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Induced sputum-retrieved matrix metalloproteinase 9 and tissue metalloproteinase inhibitor 1 in granulomatous diseases

E. FIREMAN*†, Z. KRAIEM‡, O. SADE‡, J. GREIF* & Z. FIREMAN§ **Department of Pulmonary and Allergic Diseases, †National Laboratory Service for Interstitial Lung Diseases, Tel Aviv Sourasky Medical Center, Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv, ‡Carmel Medical Center, §Department of Gastroenterology, Hillel Yaffe Medical Center, Hadera, Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel*

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SUMMARY

Matrix metalloproteinases (MMPs) capable of degrading various components of connective tissue matrices, and tissue inhibitor metalloproteinases (TIMPs) are considered important in lung parenchymal remodeling and repair processes in pulmonary diseases. Induced sputum (IS) is a reliable noninvasive method to investigate pathogenesis, pathophysiology and treatment of lung disease. This study was designed to determine whether IS-MMP9/TIMP1 levels demonstrate lung parenchymal remodeling in sarcoidosis (SA) and Crohn's disease (CRD) patients. Sputum was induced and processed conventionally in 13 SA patients, 18 CRD patients and 9 controls. Two-hundred cells were counted on Giemsa-stained cytopreps, and T lymphocytes subsets (CD4 = T helper and CD8 = T suppressor cytotoxic cells) were analysed by FACS using monoclonal antibodies.MMP-9 and TIMP-1 were measured using commercial ELISA kits. MMP-9 concentrations, but not those of TIMP-1, were significantly greater in the sputum supernatant in SA and CRD patients compared to controls ($P = 0{\cdot}018$ and $P = 0{\cdot}0019$, respectively). The molar ratio, MMP-9/TIMP-1, was significantly higher in SA and CRD patients compared to controls ($P = 0{\cdot}008$ and $P = 0{\cdot}024$, respectively). Gelatinase species having a molecular weight similar to that of MMP-9 were demonstrated by zymographic analysis. MMP-9 levels were highly correlated with the CD4/CD8 ratio and DLCO capacity in SA but less in CRD patients. MMP-9 levels in IS provide a sensitive marker for pulmonary damage.

Keywords Sarcoidosis Crohn's disease induced sputum MMP-9 TIMP1

INTRODUCTION

Granulomatous diseases are characterized by the presence of inflammatory tissue response, with granulomas appearing as an organized type of reaction. Crohn's disease (CRD) and sarcoidosis (SA) are both granulomatous diseases with different target organ involvement. The aetiologies of both diseases are unknown, but the immunological behaviour is similar, as is the medical treatment, i.e. steroids and immune suppression.

CRD is a systemic granulomatous disease that may involve any part of the alimentary tract in association with many extraintestinal manifestations, e.g. skin, eyes, liver and joints [1]. Pulmonary involvement has rarely been described [2–4], and its true incidence in CRD patients is unknown. Co-existing sarcoidosis and CRD have been reported in the literature [5]. SA is known to be a chronic granulomatous inflammatory disorder that is mainly characterized by the pronounced infiltration of CD4+ lymphocytes in the lung, but also by involvement of other extrapulmonary organs. Most SA patients experience a spontaneous remission of the disease and the histological changes are reversible and curable. Interstitial fibrosis is the final outcome only in severe cases with irreversible pulmonary structural remodeling and proteolysis of the extracellular matrix [6–8].

The matrix metalloproteinases (MMPs) are a family of zinc and calcium-dependent endopeptidases that have the combined ability to degrade the various components of connective tissue matrices [9]. They are synthesized and secreted by connective tissue and some haematopoietic cells, and are known to be important in both normal remodeling processes [10] and the accelerated destruction that occurs in many diseases [11]. Regulation of the MMPs is, occurring not only at the level of gene expression but extracellularly after secretion by the action of activators of the proenzyme forms and the specific inhibitors. The major natural

Correspondence: Dr E. Fireman, Department of Pulmonary and Allergic Diseases, Tel-Aviv Sourasky Medical Centre, 6 Weizman Street, Tel-Aviv 64239, Israel.

E-mail: fireman@tasmc.health.gov.il

inhibitor of MMPs (TIMPs) which is produced by the same cells was identified in the past decade [12].

In the present study, we measured MMP-9 and TIMP-1 levels in the induced sputum (IS) of patients with Crohn's disease and in patients with SA to determine if they can serve as a marker of the lung inflammation and fibrogenetic response. Those levels were correlated to cellular and physiological parameters.

MATERIALS AND METHODS

Study subjects

There were 18 nonsmoker patients with no lung disease and no respiratory symptoms in the CRD group. They were all diagnosed by clinical, endoscopic and imaging evaluations (small bowel passage and computerized tomography [CT]) [13]. Five patients (33·3%) were in active disease (Crohn's disease activity index CDAI > 150) [14] and 13 patients (66·6%) were in remission. The CRD duration of all the patients was 4·16 ± 4·3 years (range 1–16 years), and their mean age was 31·4 ± 18·3 years. Five patients received prednisolone (mean 20 mg/day), 10 patients received 5-ASA (5-aminosalicylic acid 2–4 g/day) and 3 patients received no medications.

There were 13 nonsmoker patients in the SA group who had been diagnosed by clinical and roentgenological evaluation. They all had a positive transbronchial biopsy which showed noncaseating granuloma. According to their chest X-ray findings, five patients were classified as being in Stage I-II, six in Stage II-III and two in Stage III-IV.

The control group was comprised of nine apparently healthy nonsmokers without any respiratory disease who were recruited from the hospital staff. All participants signed informed consent forms and the study was approved by the Institutional Review Board.

Study design and methods

Pulmonary function test. Pulmonary function tests (spirometry, lung volumes and diffusion capacity) were performed by a Masterlab (Masterlab E. Jaeger, Wurzburg, Germany). The measurement was performed using standard protocols according to American Thoracic Society guidelines [15].

Sputum induction. Sputum induction was performed with an aerosol of hypertonic saline generated by a DeVilbiss Aerosonic Ultrasonic Nebulizer 5000D/5000I (DeVilbiss-Health Care Corporation Somerset, PA, USA) with an output of 0·5 ml/min and a particle size of less than 5 microns aerodynamic mass median diameter, using a slightly modified version of the method of Pin et al. [16]. Briefly, subjects inhaled nebulized 3% saline for up to 20 min delivered by an ultrasonic nebulizer through a mouthpiece without a valve or noseclip. Ten minutes after the start of nebulization and every five minutes thereafter, the subjects were asked to rinse their nose and mouths with water to minimize contamination of the nasal secretion with saliva. They were encouraged to cough and expectorate sputum into a sterile plastic container. The nebulization was stopped after the elapse of 20 min or earlier if the sputum sample was of sufficiently good quality. The sputum was collected prior to any treatment.

Sputum examination. The method of sputum examination described by Popov *et al.* [17] was used with some modifications. Sputum was processed as soon as possible, always within 2 h. It was poured onto a Petri dish and all portions with few or non-squamous epithelial cells that were considered to originate from the lower respiratory tract were selected under an inverted microscope and placed in an Eppendorf tube, whereupon the weight was recorded. Dithiothreitol (DTT; Sputalysin, Calbiochem Corp., San Diego, CA, USA) was freshly prepared in a dilution of 1 : 10 with distilled water according to the manufacturer's instructions. The volume added was twice the recorded weight of the plugs, and it was mixed mechanically with the sputum by aspiration in and out of a pipette about 20 times to ensure an adequate blend. The sample was then placed in a shaking water bath at 37°C for 15 min to ensure complete homogenization. To stop the effect of DTT, the suspension was further diluted with phosphate-buffered solution (PBS) to a volume equal to the sputum plus DTT. The cell suspension was filtered through 52-mm nylon gauze (BNSH Thompson, Scarborough, Ontario, Canada) to remove debris and mucus, and the volume of the filtrate was recorded. The total cell count was measured by a haemocytometer (Neubauer chamber). The filtered cell suspension was centrifuged and the supernatants were store at −80°C until examination. The pellet was diluted with RPMI supplemented with 10% fetal calf serum (FCS, Biological Industries, Beit Haemek, Israel) to achieve a concentration of 10^3 cells/ml. One drop was placed in each cytocentrifuge cup already in place in a Shandon III cytocentrifuge (Shandon Southern Instruments, Sewickley, PA, USA). Separate cytospin slides were stained by Giemsa. The cell counts were performed by scanning the cytospins, starting at the top left corner and continuing in an undulating manner from top to bottom while moving across the slide using high power (× 100) magnification. Two-hundred nonsquamous cells were counted, and the results were expressed as a percentage of the total nonsquamous count.

Evaluation of phenotype of sputum cells. Flow cytometric analysis was performed on a dual FACS 440 equipped with an Ar + and Kr laser (Becton-Dickinson). Data were collected and analysed using the Consort VAX and Disp4 and Disp2D programs (Becton-Dickinson). The information was collected on a logarithmic scale. The selection of lymphocyte population was based on side scatter, and expressions of CD 45 lymphocytic subsets were identified by monoclonal antibodies as follows: CD3 = total T cells, CD4 = T helper cells, and CD8 = T suppresser-cytotoxic cells. Monoclonal antibodies were directly conjugated to either phycoerythrin (RD1) or fluorescein isothiocyanate (FITC). The cells were incubated for 10 min with Epics Coulter Q-Prep and read either immediately or 24 h later.

Measurement of MMP-9 and TIMP 1 by ELISA in sputum

Determinations of the absolute value of MMP-9 and TIMP-1 in the IS samples were performed by enzyme-linked immunoabsorbent assay (ELISA, R & D System Inc., MN, USA). MMP-9-values expressed total levels of active and pro-MMP- 9 while TIMP-1-values expressed the levels of free metabolite. Spike experiments were done with pure metabolites in order to assess the efficacy of recovery. Only 10–12% of protein was found to be equally denaturated by DTT in all samples.

Gelatin zymography

Essentially, the same method as that described by Kleiner & Stetler-Stevenson [18] was used. Samples for analysis were prepared by dilution into a buffer (4×) consisting of 0·4 M Tris (pH 6·8), 5% SDS, 20% glycerol and 0·02% bromophenol blue. The samples were applied onto an 8% polyacrylamide gel (PAGE) containing 0·5% gelatin. After 90 min of electrophoresis,

the gel was incubated for 30min at room temperature in 30 ml of 2·5% Triton X-100 in a rotary shaker. The Triton X-100 solution was decanted, replaced with 30 ml of enzyme buffer (50 mM Tris, pH 7·5, 200 mM NaCl, 5 mM CaCl 0·02% Brij 35) and incubated again for 30min at room temperature in a rotary shaker. The solution was decanted, replaced with fresh enzyme buffer and incubated overnight at 37°C. The gel was then stained with 0·5% Coomassie Blue G in methanol and 10% acetic acid for 10 min at room temperature in a rotary shaker, then washed with water until bands were visualized. Finally, the gel was incubated for 30min in 45% methanol 5% glycerol prior to drying overnight between sheets of cellophane. Areas of proteolytic activity were visualized by the absence of staining. Each sample was run twice. The bands were quantified by densitometric analysis of the zymograms using the Bio Imaging gel documentation system (Dinco & Renium, Jerusalem, Israel) and TINA software (Raytest, Staubenhardt, Germany). A positive control was used (Gelatinase zymography standards – Chemicon International, Inc., CA, USA)

Statistical analysis

Student's *t*-test was used to assess differences between groups. Spearman's rank correlation was calculated to assess correlation between data.

RESULTS

Demographics and physiological characteristics

The median ages of patients with CRD or SA and the controls were similar. Flow expiratory volume in one second (FEV1) values ranged from 65 to 120% of predicted values in the CRD patients, from 26 to 115% of predicted values in the SA patients and from 89 to 102% in the controls, $P = 0{\cdot}03$ between CRD and SA and $P = 0{\cdot}037$ between SA and the controls. Diffusion capacity values ranged from 75 to 120% of predicted values in the CRD patients and from 62 to 115% of predicted values in the SA patients ($P = 0{\cdot}0696$ between them). Total lung capacity was decreased in 1 of the 18 CRD patients and in 2 of the 13 SA patients, while it was normal for the controls (P = NS) (Table 1).

Differential cell counts in sputum cells

The differential counts are shown in Fig. 1. They showed a significantly higher percentage of lymphocytes in the CRD and SA patients compared to the controls but not between them (10·9 ± 7·1% in CRD, 16·8 ± 11·3% in SA compared to 1·2 ± 1% in the controls, $P = 0{\cdot}00018$ and $P = 0{\cdot}0037$, respectively). The percentage neutrophils was higher for the CRD and SA patients compared to the controls (neutrophils = 39·6 ± 25·4% in the CRD patients, 52·6 ± 35% in the SA patients compared to 22·5 ± 3·4% in the controls, $P = 0{\cdot}0118$ and p = 0·00979 and lower for macrophages 45·5 ± 24·5% in the CRD patients, 37·5 ± 30·9% in the SA patients compared to 76·5 ± 3·93% in the controls, $P = 0{\cdot}00528$ and $P = 0{\cdot}00183$). For the CD4/CD8 ratio, 13 of the 18 CRD patients and 10 of the 13 SA patients had abnormal values (>2·5) but the differences between them did not reach statistical significance.

MMP-9 and TIMP-1 levels in sputum

The concentrations of MMP-9 were significantly greater in the sputum supernatant of the two patient groups compared to the controls (4·20 ± 3·9 ng/ml for the SA patients, 2·7 ± 3·9 ng/ml for the CRD patients compared to 0·28 ± 0·11 ng/ml for the controls, p = 0·018 and p = 0·0019, respectively) (Fig. 2a).

The TIMP-1 levels in the sputum supernatant of the two patient groups were compared to those of the controls: they were 1 ± 1·7 ng/ml for the SA patients, 0·36 ± 0·57 ng/ml for the CRD patients, and 0·17 ± 0·23 ng/ml for the controls, $P = 0{\cdot}09$ and $P = 0{\cdot}24$, respectively) (Fig. 2b). The molar ratio MMP-9/TIMP-1 was significantly higher in all the patients compared to the controls (33 ± 32 for the SA patients, 16 ± 27 for the CRD patients, and 2·1 ± 3·1 for the controls ($P = 0{\cdot}008$ SA *versus* CO; $P = 0{\cdot}024$ CRD *versus* CO) (Fig. 3).

Correlation of MMP-9 and TIMP-1 levels with cellular and physiological parameters

In the SA group, there was a strong inverse correlation between the TIMP-1 (Fig. 4a) MMP-9 (Fig. 4b), levels and the CD4/CD8 ratio (MMP-9 *versus* CD4/CD8, r = 0·78, $P = 0{\cdot}002$; TIMP *versus*

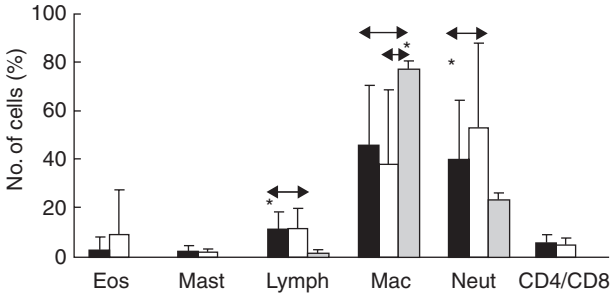

Fig. 1. Differential cell counts. Results are expressed as percentage of 200 cells counted in a Giemsa-stained cytoprep as described in Materials and methods. ■ Crohn's disease; □ Sarcoidosis; ▨ Controls. Eos, eosinophils; Mast, metachromatic cells; Lymph, lymphocytes; Mac, macrophages; neut, neutrophils.

Table 1. Demographic characteristics and pulmonary function test results of the study groups

Disease	(*n*)	Age (range)	Sex (F/M)	FEV (%)	DLCO (%)	TLC (%)
Crohns disease (CRD)	(18)	22–80	9/9	97·6 ± 18·7	94 ± 15·8	92 ± 23·8
Sarcoidosis (SA)	(13)	46–68	6/7	80·5 ± 24·2*	79·4 ± 12·9†	92·8 ± 27·8
Controls (CO)	(9)	20–60	3/6	96·2 ± 6·9	ND	99 ± 10·6

FEV1, flow expiratory volume in one second; DLCO, diffusion lung carbon monoxide; TLC, total lung capacity – percent prediction; ND, not done. *$P = 0{\cdot}03$ FEV1 CD *versus* SA and 0·037 SA *versus* C0, †$P = 0{\cdot}007$ DLCO SA *versus* CRD.

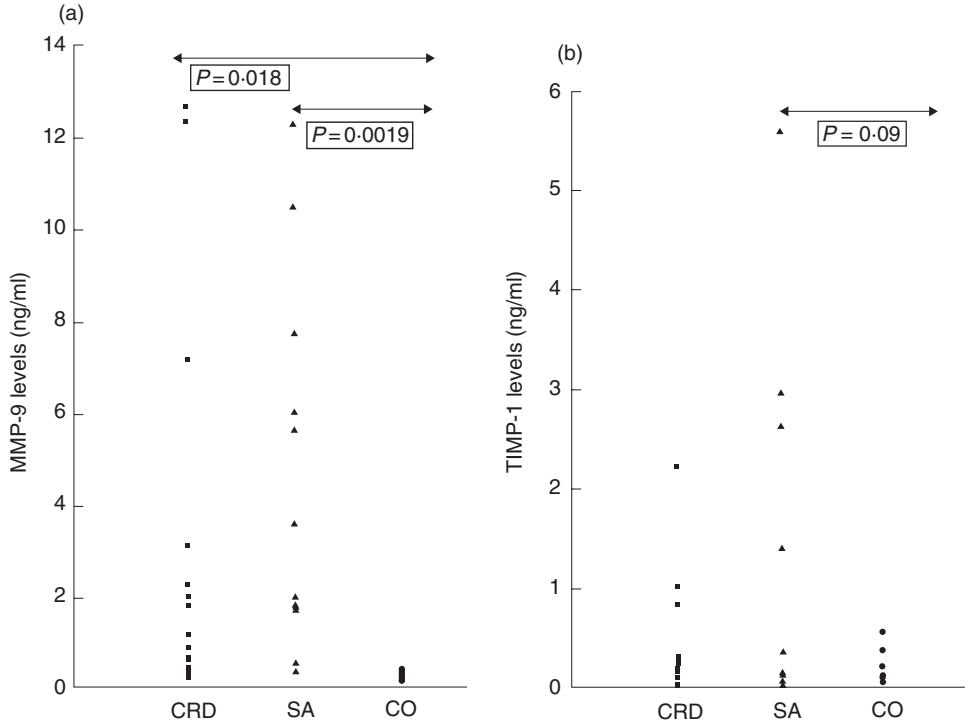

Fig. 2. MMP-9 (a) and TIMP-1 (b) concentrations in sputum samples. Supernatants were recovered and mediators expressed in ng/ml were measured as described in Materials and methods. ■ Crohn's disease; ▲ Sarcoidosis; ● Controls.

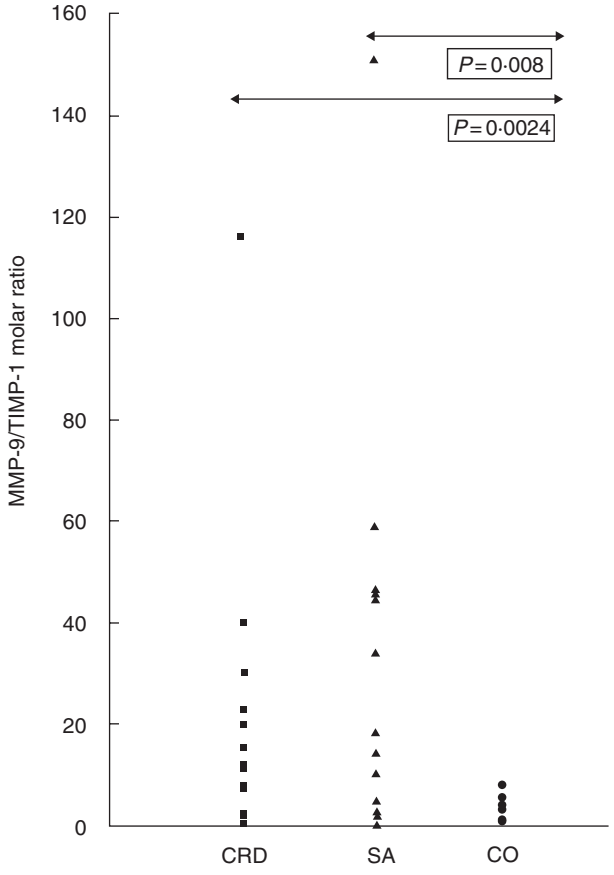

Fig. 3. MMP-9/TIMP-1 molar ratio in sputum samples. ■ Crohn's disease; ▲ Sarcoidosis; ● Controls.

CD4/CD8, r = 0·44, $P = 0·024$). The TIMP-1 (Fig. 4c) and MMP-9 (Fig. 4d) levels also correlated with diffusion lung carbon monoxide (DLCO) capacity (MMP-9 *versus* DLCO r = 0·64, $P = 0·009$; TIMP-1 *versus* DLCO, r = 0·56, $P = 0·025$).

In the CRD group, there was a weaker inverse correlation between MMP-9 *versus* DLCO ($r = 0·22$, $P = 0·002$) and *versus* CD4/CD8 ($r = 0·227$, $P = 0·022$). No other significant correlations were found in this group.

Gelatin zymography

Zymographic analysis of the sputum supernatants showed the presence of the major gelatinase species having a molecular weight similar to that of MMP-9. Representative examples of sputum samples from one patient are shown in Fig. 5. Good correlation was found between the levels of protein measured by ELISA and the OD of measured bands quantified by densitometric analysis of the zymograms ($r = 0·79$; $P = 0·006$, Figure 6.)

DISCUSSION

Pulmonary sarcoidosis (SA) is a systemic disorder of unknown aetiology characterized pathologically by the presence of non-caseating granulomas. Lungs and thoracic lymph nodes are involved in 90% of the patients who report having acute or insidious respiratory problems. Resolution of the inflammatory process occurs spontaneously in 80% of the cases, culminating in fibrosis only 10–20% of the time [8]. Crohn's disease (CRD) is also a granulomatous systemic disorder of unknown aetiology, and associated lung involvement has rarely been described [2–4]. However, the cell-mediated immune response plays somewhat similar roles in CRD and SA. Muler *et al.* [19] recently showed

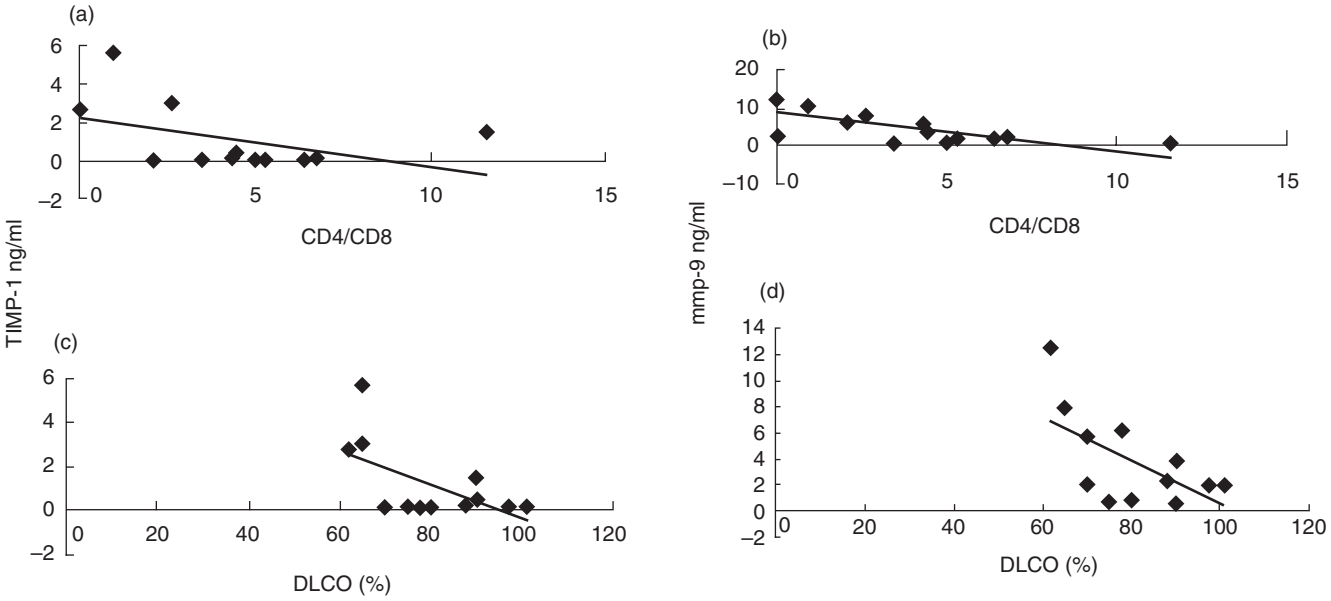

Fig. 4. Correlation between cellular and physiological parameters with MMP-9 and TIMP-1: (a) CD4/CD8 ratio and TIMP-1 (ng/ml), r = 0.44, $P = 0.024$; (b) CD4/CD8 ratio and MMP-9 (ng/ml), r = 0.78, $P = 0.002$; (c) TIMP-1 (ng/ml) and DLCO capacity, r = 0.56, $P = 0.025$; (d) MMP-9 (ng/ml) and DLCO capacity, r = 0.64, $P = 0.009$. Statistical analysis by Spearman's rank test.

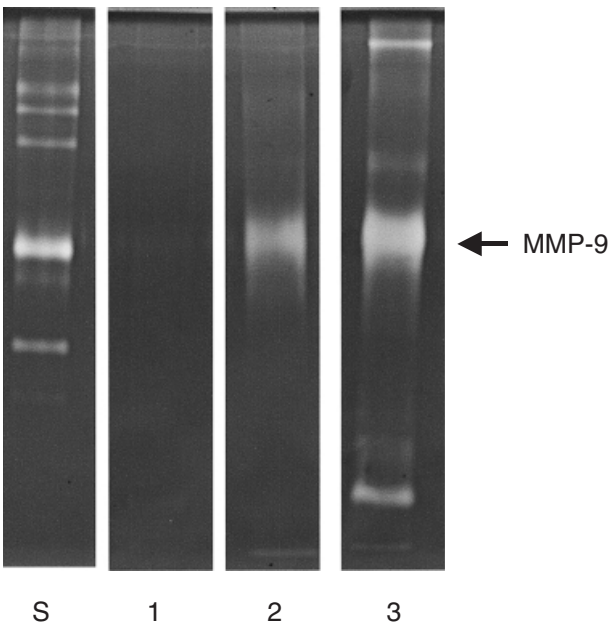

Fig. 5. Analysis of MMPs in the induced sputum by zymography. This is a representative zymographic analysis of sputum supernatant samples obtained from one control (lane 1), one patient with Crohn's disease (lane 2) and one patient with Sarcoidosis (lane 3). Gelatin zymography revealed a major band at 92 kD in all lanes. Lane 1 shows the standard.

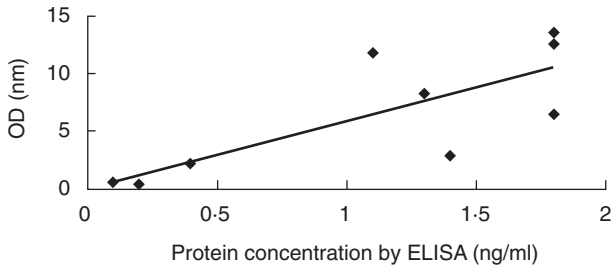

Fig. 6. Levels of concentrations (pg/ml) by ELISA were plotted against OD (nm) values of the protein performed by zymography.

that CD4 lymphocytes were vigorously activated in the intestinal mucosa of patients with active CRD.

Pulmonary involvement in both diseases may be associated with the production, deposition and proteolysis of the extracellular matrix (ECM), which may lead to irreversible pulmonary structural remodeling or to appropriate repair without fibrosis. Matrix metalloproteinases (MMPs) and the specific tissue inhibitors of metalloproteinases (TIMPs) have been recently shown to participate in the parenchymal destruction and repair processes resulting in ECM remodeling, and to be involved with different pulmonary processes associated with the destruction of the subepithelial basement membrane, e.g. diffuse alveolar damage [20], acute respiratory stress syndrome [21] and emphysema [22].

MMP-9 was reported to possess substrate specificity to type IV collagen and to degrade basement membrane structures via collagenolytic actions. MMP-9 was found in the bronchoalveolar fluid in patients with idiopathic interstitial pneumonias and hypersensitivity pneumonitis [23,24].TIMP's are the major endogenous regulators of MMP activities in the tissue microenvironment. Four homologueous TIMPs have been well characterized and each of them could noncovalently bind to the MMP with 1 : 1 stoichiometry. TIMPs have been shown to possess biological functions that are independent of MMP- inhibitory activity. We focused our study on TIMP-1 since it is more involved than other TIMPs in the pathogenesis of pulmonary fibrosis [25].

The current study shows that the concentrations of MMP-9 and the molar ratio MMP-9/TIMP-1 are significantly increased

mainly in the supernatants recovered from the IS of patients with SA compared with those recovered from apparently healthy control subjects. Moreover, in agreement with our previous reports [26,27] and with those of others [28,29], we found that the profiles of the cells recovered from induced sputum in both groups of patients showed an increased percent of lymphocytes compared with the cells in the induced sputum from the controls. We now showed that CD4/CD8 ratios are inversely correlated with the levels of MMP-9, thus showing that a high percentage of CD8-positive T lymphocytes and high levels of MMP-9 are well correlated. These results are in agreement with our previous studies and others [30–32] demonstrating that percentage CD8-cytotoxic subsets are elevated in BAL fluid from patients with IPF and pulmonary fibrosis- associated with systemic sclerosis as well as in collagen vascular disorders. Moreover, this low ratio of CD4/CD8 subtypes due to increase in CD8-cytotoxic positive cells are associated with DLCO impairment [33]. These results taken together showed that soluble MMP-9 in correlation to percentage cytotoxic CD8 T lymphocytes in sputum and diffusion capacity may be considered a marker of damage.

Correlation between cell profiles (i.e. macrophages and neutrophils) and MMP-9 had also been found in the IS of asthmatics and patients with chronic bronchitis patients [34]. Other soluble factors were largely studied in asthma COPD and chronic bronchitis [35], but this issue is still obscure for patients with interstitial lung diseases. TNF levels in sputum were shown to correlate with disease activity [29] but the present study is, to the best of our knowledge, the first report to show that the levels of metalloproteinase and its inhibitors substances strongly involved in remodeling and extracellular matrix degradation that were measured in the patients' IS showed a correlation with cellular and physiological parameters. Moreover, we also showed that their MMP-9/TIMP-1 molar ratio is higher than that of the apparently healthy controls. This may be the result of an imbalance between the expression of MMPs and TIMPs due to low secretion of the MMP-9-specific inhibitor, TIMP-1, as it had been shown in rheumatoid synovial cells [36] and experimental lung silicosis [37].

We also demonstrated herein a strong inverse correlation between the MMP-9 and TIMP-1 levels in IS supernatants with the DLCO capacity in SA patients which show pulmonary involvement and a lesser one in CRD which patients do not show clinical pulmonary manifestations. The role of MMP-9 in fibrotic diseases of the lung was already shown in many earlier studies [23–38], but we believe this to be the first study to show a correlation between MMP-9 which is mainly secreted by inflammatory cells and physiological parameters. For this purpose, DLCO proved to be a clinically useful test in the evaluation and follow-up of diseases which involve lung parenchyma [39].

In conclusion, the present study may represent a potentially useful model to measure the levels of biological factors in their IS that may identify patients with increased lung involvement and risk of lung function deterioration. Why some SA patients improve or heal and others progress to fibrosis essentially remains a mystery. Although the present proteins are not involved directly in the pathogenesis of SA and CRD, it may be that MMP-9 will emerge as being a marker for disease activity and early detection of a high-risk subgroup, insofar as the highest values for MMP-9 were recorded in the Stage IV patients with very impaired DLCO capacity. Moreover, MMPs were already identified as a possible therapeutic target: indeed, Corbel *et al.* [40] recently showed that batismat, an MMP inhibitor, is useful in preventing experimental pulmonary fibrosis induced by bleomycin. Currently small –sized inhibitors and highly specific antibodies are used to complement recombinant TIMPs as potential therapeutics [41].

Early detection of inflammatory bowel disease-related respiratory diseases is problematic [42]. The mere presence of lymphocyte in sputum that we had shown in our previous studies [27] demonstrated only lung migration of inflammatory cells, since none of our CRD patients had clinical evidence of lung disease. In the current study, we add a new perspective in terms of a marker being associated with the remodeling of the ECM, and one that is recovered by a noninvasive technique that may be clinically helpful and one that may identify a subgroup of patients early in a progressive systemic disease.

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