CXCL11 in bronchoalveolar lavage fluid and pulmonary function decline in systemic sclerosis

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Received on September 29, 2010; accepted in revised form on April 16, 2012.

Clin Exp Rheumatol 2012; 30 (Suppl. 71): S71-S75.

Key words: bronchoalveolar lavage fluid, chemokine CXCL11, interstitial lung diseases, systemic sclerosis

Funding: this work was supported by the Ministero dell'Istruzione dell'Università e della Ricerca, PRIN 2005, prot. n. 2005 064413.

Competing interests: none declared.

ABSTRACT

Objective. *Several studies have focused on the antifibrotic potential of the Th1 cytokine IFN-γ-1b through suppression of Th2 fibrogenic functions. It has been reported that IFN-γ induces the production of CXCL11 in the lung and plasma of patients with lung-fibrosis. The aim of the present study was to determine whether the levels of CXCL11 in the bronchoalveolar lavage fluid (BALF) of SSc patients might be a predictor of clinically significant fibrotic lung involvement.*

Methods. *In a retrospective longitudinal study we analysed BALF samples from 16 SSc patients with interstitial lung disease (ILD) and 16 matched control patient without ILD. Patients were eligible if they did not have evidence of ILD at the time of BAL as shown by HRCT. A standard morphological and immunological analysis of BALF cellular components was performed. CXCL11 was measured in BALF by specific ELISA assay.*

Results. *BALF CXCL11 concentrations were significantly elevated in the samples taken from patients who did not developed ILD as compared to those who developed ILD ($p<0.001$). Stepwise logistic regression analysis revealed that BALF CXCL11 levels predicted clinically significant ILD ($p<0.001$).*

Conclusion. *The presence of elevated BALF concentrations of CXCL11 in SSc patients who do not developed lung fibrosis suggest that determination of CXCL11 in BALF could serve as a prognostic factor for pulmonary function decline.*

Introduction

Systemic sclerosis (SSc) is a generalised connective tissue disease clinically characterised by fibrotic changes in the skin and internal organs. Respiratory system involvement occurs more frequently in SSc than in other connective tissue diseases representing a significant cause of morbidity and mortality (1). The most common pulmonary manifestation is interstitial lung diseases (ILD), which occurs in up to 80% of patients with SSc. However, only 30% of cases will develop a clinically significant respiratory involvement and, unlike idiopathic pulmonary fibrosis (IPF), progression of disease is not severe in many patients (2). The most frequent histopathologic character is a non specific interstitial pneumonia (NSIP) rather than the usual interstitial pneumonia (UIP) and ILD is less extensive in SSc than in IPF (3, 4). Nevertheless, pulmonary fibrosis has become the most common cause of death in SSc (5, 6). The diagnosis is easy in advanced stages of the disease, when the lesions are clinically and radiologically evident, but in the early phases symptoms and signs are often mild and insidious.

Detection of lung involvement as early as possible would give the chance to use immunosuppressive drugs, thus possibly altering the course of the disease (7-10). Furthermore a better understanding of the markers predictive for pulmonary complications would minimise the risk of invasive over-diagnosis and over-treatment. Unfortunately, validated markers for predicting progression and poor outcome are missing.

Several studies have focused on the antifibrotic potential of the Th1 cytokine IFN-γ-1b through suppression of Th2 fibrogenic functions (11). IFN-γ is known to induce the production of CXCL11 in the lung and plasma of patients with lung-fibrosis (UIP pattern) (12). Furthermore, data in animal models indicate that CXCL11 attenuates bleomycin-induced pulmonary fibrosis (13). The objective of the present study was to verify if bronchoalveolar lavage fluid (BALF) levels of CXCL11 represent a predictor of clinically significant lung involvement in SSc.

Materials and methods

Patients

In a retrospective study we identified SSc patients who had undergone bronchoalveolar lavage (BAL) and high-resolution computed tomography (HRCT) at the Rheumatology Unit of the University of Padova between 1985 and 2006. All patients fulfilled the criteria for SSc proposed by the American College of Rheumatology (14). Patients were eligible if they did not have evidence of ILD at the time of BAL as shown by HRCT scans performed at the time of BAL. We identified 16 subjects who subsequently developed ILD. Control patients were selected from among those meeting the same eligibility criteria but who did not develop ILD. Records were then reviewed in detail to identify one suitably matched control patient for each case based on the following criteria: sex, age, disease duration, autoantibody profile and cutaneous subset (15). The baseline characteristics of the patients are reported in Table I.

None of the patients had ever received systemic immunosuppressants or steroids. Patients with respiratory infections and/or isolated pulmonary hypertension were excluded. ILD was diagnosed by an independent investigator (CF) based on characteristic features at HRCT of the lungs: ground glass opacification, reticular, mixed and honeycomb pattern (16). In addition, lung function was assessed by forced vital capacity (FVC) and diffusing lung capacity for CO (DLCO) at rest (Master Screen spirometer; Erich Jaeger, Hoechberg, Germany).

All research methods were approved by the Ethics Committee of the Padova University Hospital, and participating subjects gave their written informed consent to all procedures.

Bronchoalveolar lavage (BAL)

BAL was performed according to the technical recommendations and guidelines for the standardisation of BAL procedures (17). Briefly, a total of 200 ml of sterile saline (0.9% sodium chloride) was injected in 25-ml aliquots via fiberoptic bronchoscopy, with immediate gentle vacuum aspiration after each aliquot. Immediately after the BAL, the fluid was filtered through sterile gauze and the volume measured. A volume of 100–200 ml of BAL recovery and a sample of 50% of the instilled volume with a minimum of 50 ml was considered acceptable. The percentage of BAL recovery was 54.9%±4.2. The volume of the recovered fluid was pooled and centrifuged at 800g for 10 minutes at 4°C. Supernatants were immediately frozen at -80°C for further analyses. The recovered cells were resuspended in phosphate buffered saline. A standard morphological and immunologic analysis of BAL cellular components was performed and included cell recovery, differential count of macrophages, lymphocytes, neutrophils, and eosinophils, and flow cytometry analysis of the lymphocytes subsets, including CD4/CD8 BAL T-cell ratio.

Measurement of CXCL11

CXCL11 concentrations in cell-free supernatants from BAL were measured by specific ELISA for CXCL11 (Quantikine DCX110) purchased from R&D Systems. Detection limit was 3.4 pg/ml.

Statistical analysis

The results are presented as median, SE and range or mean ± SD according to data distribution. Differences between cases and controls were compared with Mann-Whitney U-test. Spearman's rank correlation coefficient was used to study correlations between CXCL11 concentrations and both BAL cellular populations and pulmonary function tests obtained at the time of BAL sampling. Correlations between CXCL11 and follow-up pulmonary function tests were based on the differences in paired observations between pulmonary function tests follow-up and baseline values. Stepwise logistic regression analysis was used to identify variables independently associated with significant fibrotic lung involvement. Statistical significance was defined as $p<0.05$. The analysis was performed using SPSS 12.0 (SPSS, Chicago, IL-USA).

Table I. Baseline characteristics of the study subjects.

	Total patient population (n=32)	patients with ILD (n=16)	patients without ILD (n=16)
Age, years	55.1±9.2 (45.6–67.4)	57.3±9.0 (45.9–66.7)	53.8±9.6 (45.6–67.4)
Female	32	16	16
Diffuse SSc	17	8	9
Limited SSc	15	8	7
Anti-Scl70	15	8	7
Anti-centromere	17	8	9
SSc duration at BALF sampling, years	2.5±0.8 (1.2–3.8)	2.2±0.9 (1.2–3.7)	2.9±0.7 (1.9–3.8)
Time to ILD onset#	–	2.1±0.8 (1.1–2.9)	–
Time to last follow-up#	5.6±1.7 (3.1–8.2)	5.7±1.9 (3.4–8.2)	5.5±1.6 (3.1–6.9)
Treatment (n. patients)^			
Plasmapheresis	8	4	4
Penicillamine	8	3	5
Calcium channel blockers	31	15	16
PPIs / H2 antagonists	30	15	15

Data are presented as mean±standard deviation (range), unless otherwise stated. # from bronchoalveolar lavage, years; ^ type of treatment received between time of BALF sampling and ILD diagnosis or last follow-up (for patients without ILD); ILD: interstitial lung disease; SSc: systemic sclerosis; BALF: bronchoalveolar lavage fluid; PPIs: Proton pump inhibitors.

Results

Thirty-two SSc caucasian patients were selected for analysis. Cases and controls were well matched for most variables considered in this study (Table I). Sex, age, disease duration, autoantibody profile and cutaneous subset were not statistically different between the groups ($p>0.05$ for all).

Total and differential cell counts in BALF were within the normal range in all patients (Table II). No difference between total and differential cell counts was detected in both groups.

HRCT and PFT (Table III) at the time of BALF sampling showed no evidence of ILD in all patients. Sixteen patients

Table II. Total and differential cell counts in bronchoalveolar lavage fluid of patients who developed or not interstitial lung disease.

	patients with ILD (n=16)	patients without ILD (n=16)	*p*-value[#]
Total cell counts (x10^5/ml)	1.2 (0.2–4.8)	1.5 (0.3–4.6)	0.742
Macrophages (%)	91.0 (68.0–98.0)	91.0 (71.0–97.0)	0.624
Lymphocytes (%)	4.0 (1.0–18.0)	8.5 (1.0–19.0)	0.202
Neutrophils (%)	2.0 (0.0–3.0)	1.0 (0.0–3.0)	0.329
Eosinophils (%)	0.5 (0.0–2.0)	0.0 (0.0–2.0)	0.231
Lymphocyte subsets			
CD3 (%)	70.0 (58.0–85.0)	76.5 (64.0–95.0)	0.101
CD4 (%)	36.0 (30.0–63.0)	41.5 (6.0–73.0)	1.000
CD8 (%)	23.0 (16.0–45.0)	42.5 (10.0–77.0)	0.320
CD4/CD8	1.4 (0.8–3.9)	0.9 (0.1–7.3)	0.424
CD19 (%)	1.0 (1.0–12.0)	1.0 (1.0–7.0)	0.883
CD16 (%)	1.2 (0.2–4.8)	2.5 (1.0–10.0)	0.547
HLA-DR$^+$ (%)	10.0 (1.0–17.0)	8.0 (1.0–50.0)	0.934

Data are presented as median (range), unless otherwise stated. [#] using Mann-Whitney U-test; ILD: interstitial lung disease.

Table III: Pulmonary Function test in the studied SSc patients

	Baseline[#]		Follow-up^		Last follow-up	
Test	patients with ILD median (SE) range	patients without ILD median (SE) range	patients with ILD median (SE) range	patients without ILD median (SE) range	patients with ILD median (SE) range	patients without ILD median (SE) range
FVC (% of predicted value)	94.0 (2.6) 84–103	98.0 (3.1) 86–110	65.5 (5.4) 40–72	97.0 (3.9) 80–107	49.0 (2.6) 45–59	105.0 (4.3) 80–115
DLCO (% of predicted value)	99.0 (3.9) 87–110	105.0 (3.1) 89–109	49.0 (4.2) 30–60	98.0 (2.8) 83–104	43.0 (4.0) 28–52	90.0 (6.0) 76–125

[#] at the time of BALF sampling, no patient had evidence of ILD; ^ at the time of ILD diagnosis; matched time for controls; ILD: interstitial lung disease; FVC: forced vital capacity; DLCO: diffusing lung capacity for carbon oxide.

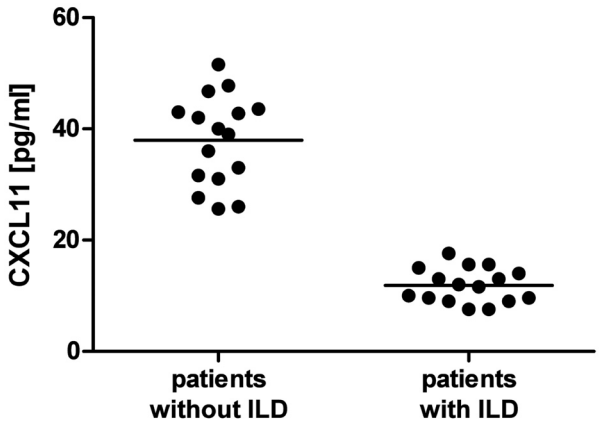

Fig. 1. CXCL11 concentrations in bronchoalveolar lavage fluid of patients who developed or not interstitial lung disease. Mann-Whitney U-test: $p<0.001$.

developed HRCT abnormalities suggestive of ILD during the follow-up (10 had evidence of reticular pattern, 4 showed mixed ground glass opacification and reticular pattern and two developed subpleural honeycombing).

BALF CXCL11 concentrations were significantly elevated in the samples taken from patients who did not developed ILD (median 43.2 pg/ml, SE 3.5, range 25.6–51.6) as compared to those who developed ILD (median 10.6 pg/ml, SE 1.4, range 7.6–17.6, Mann-Whitney U-test: $p<0.001$) (Fig. 1).

There was no significant correlation between CXCL11 concentrations and BAL cellular data within the whole study population. No significant correlations were found also comparing the concentrations of CXCL11 and cellular data.

As shown in Table IV, there was no correlation between BALF CXCL11 concentrations and pulmonary function tests at the time of BALF sampling. Conversely, the levels of CXCL11 in the BALF correlated significantly with subsequent changes, at the time of ILD diagnosis and at the last follow-up, in FVC (r=0.85, $p<0.001$ and r=0.96, $p<0.001$, respectively) and DLCO (r=0.56, $p<0.04$ and r=0.75, $p<0.002$, respectively).

A stepwise conditional logistic regression analysis revealed that only CXCL11 BALF levels (β=-0.891, SE=0.004) predicted clinically significant ILD ($p<0.001$). No other variable was found to have sufficient predictive value to be included in the model.

Discussion

Our data suggest that SSc patients with low lung levels of CXCL11, measured within a 4-years period from disease onset, have a greater likelihood of decreasing significantly their pulmonary function with respect to patients with high CXCL11 BALF levels. ILD is the most common cause of death in SSc but its pathogenesis remains unclear. There is increasing evidence that local immune dysregulation leads to an overproduction of cytokines that could lead to progressive fibrosis. In the present study baseline patient age, DLCO, FVC, cutaneous subset and specific autoantibodies were not found to significantly correlate with pulmonary function loss and were not able to predict clinically significant fibrotic lung development. All these parameters have been proposed as adverse prognostic markers for lung function deterioration, however their ability to predict function loss in patients with scleroderma is still controversial (18, 2).

Cellular differentiation of BAL cells is often used to define alveolitis. In addition, neutrophilic alveolitis has been suggested to predict the progression of

Table IV. Correlation coefficients between CXCL11 concentrations and pulmonary function tests.

	Baseline	Follow-up†	Last follow-up†
pulmonary function tests			
FVC	0.19	0.85#	0.96#
DLCO	-0.09	0.56^	0.75#

FVC: forced vital capacity; DLCO: diffusing lung capacity for carbon oxide. Correlation coefficient and *p*-value by non-parametric Spearman rank correlation. #$p<0.01$; ^$p<0.05$
† Calculations are based on the differences in paired observations between follow-up values and baseline.

fibrosing alveolitis (19). In a recent multicentre study including 141 patients, BAL neutrophilia was associated with early and overall mortality, but the effect on overall mortality was lost when disease severity was taken into account. The authors concluded that BAL findings add only limited prognostic information in SSc-related interstitial lung disease in addition to HR-CT scans and lung-function parameters (20).

Our results show that baseline CXCL11 BALF levels were able to predict the progressive deterioration of pulmonary function. In fact patients with low BALF levels of CXCL11 at initial assessment were at risk to develop a significant impairment of pulmonary function tests. The finding that BALF CXCL11 levels relate to severity of lung involvement suggests that this chemokine may influence ILD progression.

Inflammation and excessive fibrosis of the lungs are key features of ILD, although the exact mechanisms involved in the development of this condition are still poorly understood. Accumulating data support the concept that the specific cytokine phenotype may provide a key mechanism for the development of the fibrotic process (21). Schimdt *et al*. identified several abnormalities in the cytokine and chemokine patterns in BALF of SSc patients, suggesting an important role of these mediators in the pathogenesis of ILD. Cytokines/chemokines produced by lymphocytes (*e.g*. IL-4, IL-2) and monocytes/macrophages (CCL2, CCL4, TNF-α, IL-8, IL-6), as well as other cell types, were shown to be increased, indicating activation of different cell types in SSc (22).

Under the influence of type-2 cytokines, fibroblasts become activated, proliferate, and deposit extracellular matrix (23), indicating the importance of immune deviation to a Th2 cytokine profile in the fibroproliferative response. CXCL11 acts primarily on activated T and NK cells, attracting Th1 cells and blocking the migration of Th2 cells. Our data suggest that a high CXCL11 BALF level, reflecting a local inflammatory process characterised by a type-1 cytokines response, might contribute to limit the development of tissue fibrosis.

Our study has several limitations that are necessary to point out. One major limitation is that this is a case-control study on a small number of patients. A larger study may reveal more conclusive data.

Secondly, although case-control studies are powerful tools for highlighting the differences, their cross-sectional nature do not allow definition of causal relations. Another limitation would be that the retrospective search of the cases might have not included patients with very poor outcome, due to their short follow-up.

In conclusion the presence of elevated BALF concentrations of CXCL11 in SSc patients who do not developed lung fibrosis suggest that determination of CXCL11 in BALF could serve as a prognostic factor for pulmonary function decline. Additional and larger scale studies are needed for the confirmation of these findings.

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