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Markers of fibrosis and inflammation in exhaled breath condensate (EBC) and bronchoalveolar lavage fluid (BALF) of patients with pulmonary sarcoidosis: a pilot study

Abstract

Introduction: Sarcoidosis is a disease of unknown aetiology. Little is known of the predictive factors of fibrosis. It has been suggested that PAI-1, uPA, TGF-β1, VEGF, IL-8, TNF-α influence this process.
The aim of the study was to assess airway inflammatory and fibrosis markers in EBC in sarcoidosis and the effects of fibreoptic bronchoscopy (FOB), bronchoalveolar lavage fluid (BALF), transbronchial lung biopsy (TBLB) and bronchial mucosa membrane biopsy on their production in the airways.
Material and methods: The study group consisted of 11 patients (five women, six men, mean age 40 $\pm$ 9 yrs, mean $\pm$ SD) with sarcoidosis stage I–III. PAI-1 (ng/ml), uPA (ng/ml), TGF-β1 (pg/ml), VEGF (pg/ml), IL-8 (pg/ml), TNF-α (pg/ml) levels were measured in BALF and EBC collected before, and 48 hours after, FOB.
Results: No significant changes in EBC levels of VEGF, PAI-1, TGF-β1, TNF-α (respectively: 8.02 $\pm$ 4.97 pg/ml; 1.1 $\pm$ 1.2 ng/ml; 2909.7 $\pm$ 206.6 pg/ml; 10.7 $\pm$ 19.9 pg/ml) after FOB were observed when compared to baseline. In contrast, IL-8 concentration in EBC (pg/ml) decreased after FOB (0.073 $\pm$ 0.13 *v.* 0.061 $\pm$ 0.1, $p = 0.006$) and was significantly lower than in BALF (BALF 0.95 $\pm$ 0.62, $p < 0.05$). Also, the mean level of VEGF was higher in BALF than in EBC both pre- and post-FOB (BALF 66.38 $\pm$ 36.95, EBC pre-FOB 6.75 $\pm$ 3.67 and EBC post-FOB 8.02 $\pm$ 4.97). A significant relationship between TNF-α in post-FOB EBC and BALF was also shown ($\beta = 0.63$, $p = 0.04$).
Conclusions: FOB does not significantly affect levels of airway inflammation and fibrosis markers present in EBC before and after FOB; they were also comparable to the concentrations marked by BALF. The lack of correlation between marker levels in EBC and BALF indicates that these methods are not equivalent. Due to the possibility of repetition, and the less invasive, simpler method of the EBC test, it would seem reasonable to continue this research on a larger number of patients.

Key words: sarcoidosis, exhaled breath condensate, fibreoptic bronchoscopy, PAI-1, uPA, TGF-β1, VEGF, IL-8, TNF-α
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Introduction

Sarcoidosis is a multi-organ disease of unknown aetiology multisystem characterized by inflammatory granulomas from lymphocytes, epithelial cells and giant cells. The disease most commonly involves the lungs and/or the mediastinal and hilar lymph nodes [1].

In about 75% of cases, the disease is self-limiting and undergoes a spontaneous or drug-induced regression, while in the remaining cases it becomes chronic. Unfavourable outcomes in the form of progressive pulmonary fibrosis leading to respiratory failure and a considerable deterioration in quality of life are seen in about 5–10% of cases.

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Unfortunately, the prognostic factors for this process are unknown. Recent multicentre studies investigating the pathomechanism of fibrosis have suggested that an important role is played by certain cytokines and growth factors, such as transforming growth factor β1 (TGF-β1), vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), tumour necrosis factor α (TNF-α); and by the following blood coagulation system factors: plasminogen activator inhibitor-1 (PAI-1) and urokinase-type plasminogen activator (uPA) [2–4].

In addition to clinical examination, the course of sarcoidosis is evaluated and monitored using radiological imaging (chest X-ray, high-resolution computed tomography [HRCT]), function testing (spirometry, respiratory mechanics, 6-minute walk test [6MWT]) and assessment of the immune profile of cells from bronchoalveolar lavage fluid (BALF) during fibreoptic bronchoscopy (FOB) [5].

The principal aim of our study was to establish whether it is possible to determine selected potential markers of inflammation and fibrosis in samples collected using a non-invasive method, specifically in exhaled breath condensate (EBC). We also assessed the effect of invasive diagnostic procedures in the airways, namely fibreoptic bronchoscopy, BAL, transbronchial lung biopsy (TBLB) and bronchial mucosal biopsy, on concentrations of these markers in EBC.

Material and methods

We enrolled male and female patients with various stages of pulmonary sarcoidosis hospitalised at the Second Department of Lung Diseases, The Institute of Tuberculosis and Lung Diseases, Warsaw, Poland. Inclusion criteria were: histopathologically confirmed sarcoidosis and no pharmacological treatment of the disease (with glucocorticosteroids or immunosuppressant agents) or on non-steroid anti-inflammatory drugs treatment during the preceding three months. None of the enrolled patients was a current or ex-smoker.

Sarcoidosis was confirmed by histopathological examination of samples collected from a bronchial mucosal biopsy (one subject), transbronchial lung biopsy (seven subjects) or mediastinal lymph nodes collected during mediastinoscopy (three subjects).

Exhaled breath condensate was collected over 10–15 minutes of quiet breathing using a condenser EcoScreen (Jaeger, Germany), according to standard protocol using a nasal clip [6]. The material was transported to the analytical laboratory in tightly closed and cooled containers and was then stored at –70°C until analysis/further exemination.

In accordance with the study protocol, EBC was collected in the morning of the day in question, and again 48 hours after, via bronchoscopy. Bronchoalveolar lavage was performed routinely as part of hospital evaluation, or for therapeutic purposes before other diagnostic procedures were performed. The bronchoalveolar lavage fluid was transported to the laboratory at 4°C in tightly closed containers. The material was filtered through a sterile ganze and centrifuged (4°C, 400 g, 10 min). The supernatant was frozen at –70°C for further analysis.

Concentration of PAI-1, uPA, TGF-β1, VEGF, IL-8, TNF-α in BALF and EBC were performed quantitative by enzyme immunoassay technique (ELISA) using commercially kits (American Diagnostica, USA, for PAI-1 and uPA, BIOSOURCE, USA, for TGF-β1, VEGF, IL-8 and TNF-α). Optical density was measured using a spectrophotometeric reader Infinite M200 (Tecan, Australia). The markers concentration were expressed in pg/ml (PAI-1, uPA) and ng/ml (TGF-β1, VEGF, IL-8, TNF-α).

All patients gave written consent for participation in the study, which had been approved by the local Ethics Committee.

Statistical analysis

Statistical analysis of the data was performed using Statistica 6.0. Comparisons of changes of the concentration of PAI-1, TGF-β1, VEGF, IL-8, TNF-α in BALF and EBC were performed using non-parametric tests for matched variables (the Wilcoxon signed-rank test) and unmatched variables (the U Mann-Whitney test). A p value of <0.05 was considered statistically significant.

Results

Eleven patients with sarcoidosis were enrolled in the study: five women (45.45%) and six men (54.55%). The mean age of the subjects was 40 ± 9 years and the mean duration of the disease was 37 ± 6 months. The mean serum angiotensin-converting enzyme (ACE) level was 84.5 ± 49.1 IU/ml and the CD4/CD8 ratio in BALF was 3.34 ± 1.89.

Based on the chest X-ray, patients were classified according to their disease stage: stage I (one patient), stage II (nine patients) and stage III (one patient).

Table 1 summarises the results of the measurements of inflammation and fibrosis markers in EBC before and after FOB with BAL and in BALF.

Table 1. The influence of invasive diagnostic procedures on inflammatory and fibrosis markers concentration in EBC and BALF

Variable		EBC before FOB	EBC after FOB	BALF
IL8 [pg/ml]	Mean	0.073 ± 0.13	0.061 ± 0.1	0.95 ± 0.62*
	Mediana	0.03 (0.022–0.05)	0.032 (0.008–0.038)	0.726 (0.577–1.432)
VEGF [pg/ml]	Mean	6.75 ± 3.67	8.02 ± 4.97	66.38 ± 36.95*
	Mediana	6.824 (3.643–9.544)	7.654 (4.288–12.772)	57.316 (42.704–97.38)
PAI-1 [ng/ml]	Mean	0.9 ± 0.6	1.1 ± 1.2	1.5 ± 3.7
	Mediana	0.857 (0.411–1.259)	0.772 (0.357–1.087)	0 (0–0.788)
TGF-β1 [pg/ml]	Mean	2871 ± 217.7	2909.7 ± 206.6	2820.1 ± 282
	Mediana	2960.48 (2628.24–3051.68)	2938.72 (2795.04–3016.56)	2875.08 (2608.56–2989.28)
TNF-α [pg/ml]	Mean	3.74 ± 5.39	5.07 ± 6.45	10.5 ± 14.01
	Mediana	2.144 (0–6.092)	2.595 (0–10.041)	6.487 (1.918–13.488)
uPA [ng/ml]	Mean	Not detected	Not detected	0.23 ± 0.1
	Mediana			0.209 (0.148–0.354)

*p < 0.05 (BALF *v.* EBC)

Table 2. Relationship between parameters analysed in all examined materials

Variable	EBC before FOB and BALF		EBC after FOB and BALF		EBC before FOB and EBC after FOB	
	R*	p	R*	p	R*	p
IL-8 [pg/ml]	–0.042	0.907	0.321	0.365	0.763	0.006
VEGF [pg/ml]	–0.272	0.445	0.018	0.960	0.163	0.630
PAI-1 [ng/ml]	0.007	0.983	0.037	0.918	–0.218	0.519
TGF-β1 [pg/ml]	0.406	0.244	0.272	0.445	0.318	0.340
TNF-α [pg/ml]	0.093	0.810	0.832	0.004	0.242	0.529

*Spearman

Table 2 summarises the statistical correlations, and Figures 1 to 5 illustrate the graphical results for individual markers.

In the EBC before and after FOB, uPA was undetectable. The mean concentration of uPA in BALF was 0.23 ± 0.1 ng/ml with the median concentration of 0.209 (0.148–0.354). The concentration of IL-8 in EBC before FOB was higher than that after FOB (0.073 ± 0.13 *v.* 0.061 ± 0.1, p = 0.006) and significantly higher in BALF than in EBC (BALF: 0.95 ± 0.62, p < 0.05) (Fig. 4). The concentration of VEGF was significantly lower in EBC than in BALF (6.75 ± 3.67 before and 8.02 ± 4.97 after FOB *v.* 66.38 ± 36.95, p < 0.05) (Fig. 5).

We showed a correlation between the concentration of TNF-α in EBC after FOB and the concentration of TNF-α measured in BALF (R = 0.832, p = 0.004) (Fig. 6). We found no correlation between the other markers we investigated in BALF and EBC.

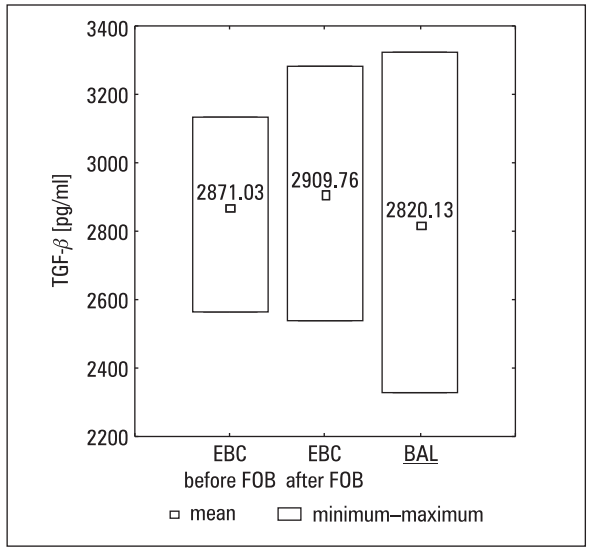

Figure 1. TGF-β concentration in all examined materials

Discussion

It is common knowledge that bronchoscopy alone and the related diagnostic procedures may contribute to inflammation in the airways. There are reports of complications of FOB unrelated to anaesthesia or premedication, including damage to the respiratory mucosa caused during biopsies, and

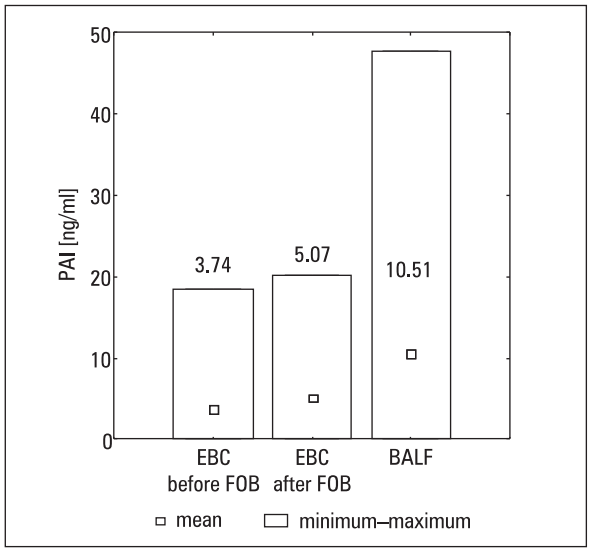

Figure 2. TNF-α concentration in all examined materials

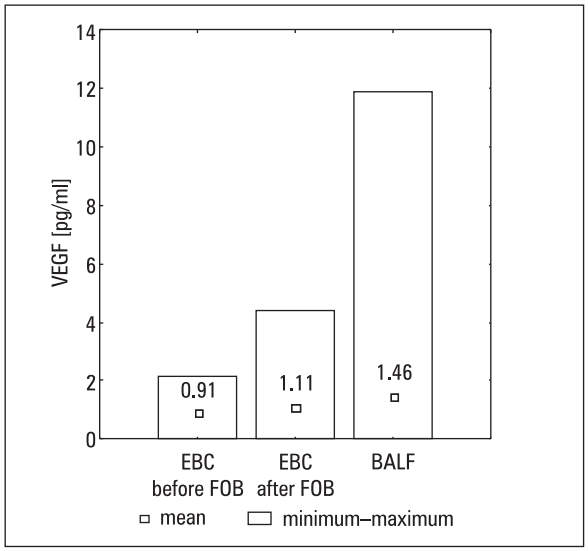

Figure 3. PAI concentration in all examined materials

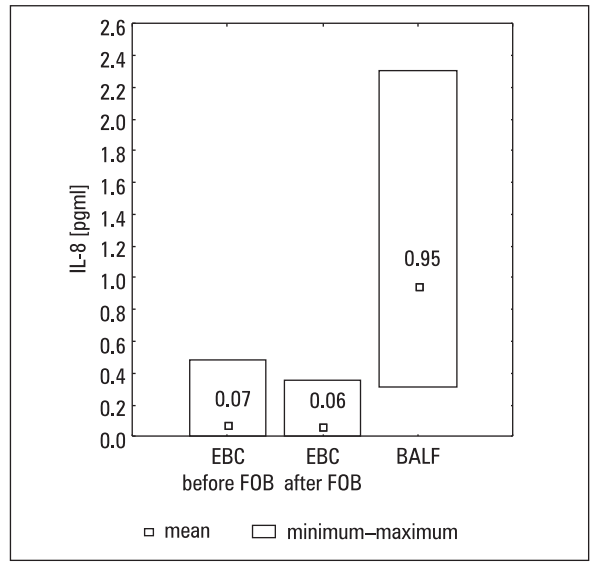

Figure 4. Concentration IL-8 in all examined materials;*p < 0.05 (BALF *v.* EBC)

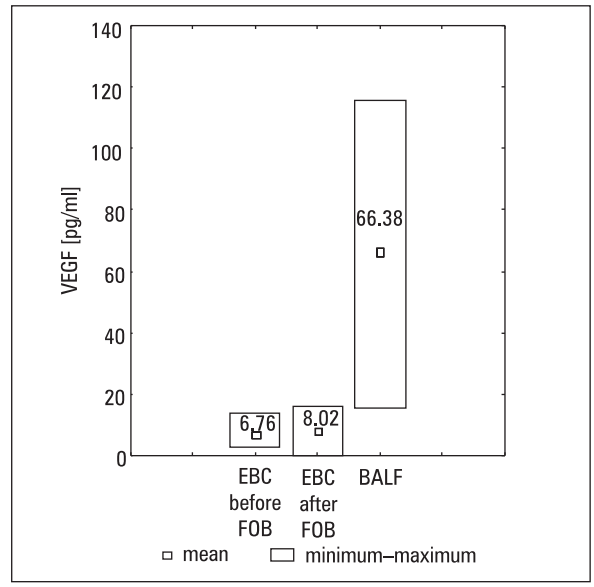

Figure 5. VEGF concentration in all examined materials; *p < 0.05 (BALF *v.* EBC)

fever (in 1.2% of patients undergoing FOB alone and in 10–30% in patients undergoing FOB and BALF) [7, 8].

In our study, FOB and the diagnostic procedures performed during FOB (BALF, TBLB or bronchial mucosa biopsy) were uncomplicated and the preliminary results we have presented seem to suggest that they have little effect on marker levels in BALF.

In previous studies, EBC levels of the following substances have been determined in patients with sarcoidosis: IL-1b, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, VEGF, PDGF-AA, EGF, eicosanoids, leukotrienes, nitric oxide (NO), H_2O_2, TGF-β1, TNF-α, PAI, Ca^{2+}, Mg^{2+} [9–12]. We found no reports of attempts to assess uPA in EBC. The only study analysing the effect of FOB and the diagnostic procedures performed during FOB to assess the presence of biologically active molecules in EBC concerned the effect of FOB on NO levels in the exhaled air. Similarly to our study, the authors failed to observe a significant effect of FOB and related instrumentations on NO levels in EBC [13].

The difficulties in determining uPA levels in EBC that we came across in our study may have been the result of the unique nature of condensate acquisition. It is well known that only molecules whose molecular weight exceeds 100 kDa enter the

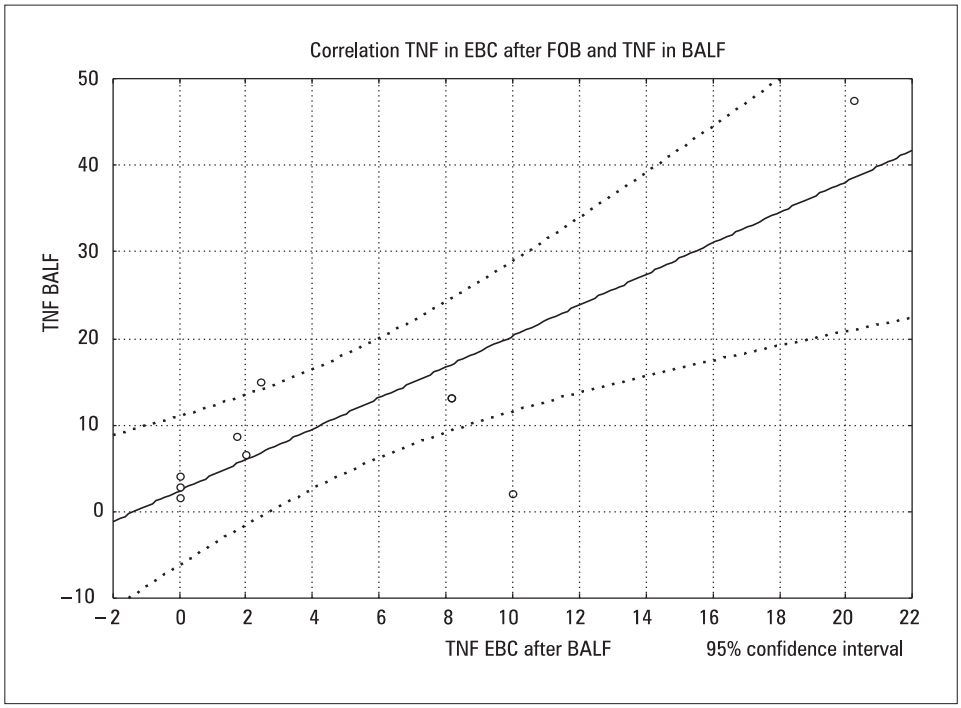

Figure 6. Significant correlation of TNF-α concentration in BALF and post-FOB EBC (R = 0.832, p = 0.004)

gaseous fraction, i.e. the exhaled air [14]. The urokinase plasminogen activator is found in an inactive form, as pro-urokinase plasminogen activator (pro-uPA), whose molecular weight is 120 kDa, or in the form of complexes, such as a complex with the protein receptor (uPAR) or PAI-1. The molecular weight of the uPA monomer is about 52–55 kDa. The relatively small uPA monomer therefore readily binds with other molecules to form complexes that are too large to diffuse to the gaseous fraction [15].

Our pilot study investigating the levels of PAI-1, uPA, TGF-β1, VEGF, IL-8, TNF-α in samples obtained during BALF and in EBC does not justify the replacement of the analysis of activity of fibrosis and inflammation markers in BALF by the assessment of these markers in EBC. However, the size of our pilot study group (11 patients) is too small to allow final conclusions. It is, however, clear that due to the confirmation of fibrosis and inflammation markers (with the exception of uPA) in the exhaled air and the possibility of repetition, low invasiveness, simple methodology and lower costs of EBC analysis, continuing the study with a larger group of patients would be fully justified.

Conclusions

1. It is possible to determine PAI-1, TGF-β1, VEGF, IL-8, TNF-α in exhaled breath condensate, and uPA did not diffuse to the gaseous fraction.
2. The concentrations of fibrosis and inflammation markers determined in EBC before and after FOB with BALF were comparable.
3. We did not observe any effects of FOB or related diagnostic procedures on the concentrations of fibrosis or inflammation markers in the samples we investigated.

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