Concise report

Increased cysteinyl-leukotrienes and 8-isoprostane in exhaled breath condensate from systemic sclerosis patients

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Abstract

Objectives. SSc is a systemic CTD characterized by fibrosis in skin and internal organs. Interstitial lung disease is a frequent complication with fibrosis in the lung parenchyma. The fibrotic process is believed to be influenced by leukotrienes (LTs) and also by oxidative stress. The aim of this study was to investigate the amount of LTs and 8-isoprostane, a marker of oxidative stress, in exhaled breath condensate (EBC) from SSc patients.

Methods. Twenty-two SSc patients with median disease duration of 2.1 years were investigated. Fifteen patients had lcSSc, four patients had dcSSc and three patients only fulfilled criteria for limited SSc. Sixteen healthy controls were enrolled. Cysteinyl-LTs (CysLTs), LTB4 and 8-isoprostane were measured in EBC with EIA and related to the radiologic extent of pulmonary fibrosis.

Results. Compared with controls, SSc patients displayed higher median (interquartile range) CysLT [6.1 (5.3–6.8) vs 4.9 (3.7–6.3) pg/ml; \( P = 0.040 \)], 8-isoprostane [0.23 (0.20–0.46) vs 0.19 (0.12–0.20) pg/ml; \( P = 0.0020 \)], but similar levels of LTB4 [0.70 (0.50–0.83) vs 0.60 (0.42–0.70) pg/ml]. CysLT correlated to LTB4, while 8-isoprostane did not correlate to any of the LTs. None of the biomarkers measured in EBC correlated to radiologic findings.

Conclusion. Increased levels of CysLT and 8-isoprostane in EBC from patients with SSc reflect the inflammatory pattern involving LTs as well as oxidative stress. These findings may indicate a possible non-invasive assessment of pulmonary involvement in SSc with a potential value for assessment of disease progress and therapy evaluation.

Key words: Exhaled breath condensate, Fibrosis, High-resolution computer tomography, Leukotriene, Oxidative stress, Systemic sclerosis, Traction bronchiectasis, 8-Isoprostane.

Introduction

SSc is a systemic CTD characterized by fibrosis in skin and internal organs. Interstitial lung disease (ILD) is a frequent complication and is characterized by infiltration of inflammatory cells and excessive fibrosis in the lungs leading to respiratory failure [1]. High-resolution CT (HRCT) of the lungs and pulmonary function tests are currently used as standard assessment of SSc–ILD [2].

Leukotrienes (LTs) are potent pro-inflammatory and pro-fibrotic mediators. They are believed to stimulate fibroblast proliferation, chemotaxis and collagen synthesis and thereby stimulate airway remodelling. LTB4 contributes to leucocyte recruitment into tissue compartments, while the cysteinyl-LTs (CysLTs) are involved in airway smooth muscle contraction and increasing vascular permeability. Previous publications have revealed increased levels of LTB4 and LTE4 in bronchoalveolar lavage (BAL) from patients with SSc [3, 4], and a correlation with total cell number in BAL reflecting the intensity of the inflammatory process in the lungs. In addition, an increase in LTE4 has been measured in urine from SSc patients [5].
Oxidative stress is believed to contribute to the pathophysiology of ILD in SSc [6]. 8-Isoprostane is produced primarily by free radical-induced peroxidation of arachidonic acid [7] and is regarded as a biomarker of oxidative stress. It is also biologically active and has been shown to be increased in BAL, serum and urine from SSc patients [6, 8, 9].

Exhaled breath condensate (EBC) is a new non-invasive tool, having the potential of being useful in diagnosing respiratory disorders. Several inflammatory biomarkers have been measured in EBC, and are suggested to reflect lower airway inflammation [10]. It has previously shown that exhaled levels of H$_2$O$_2$ [11], vitronectin and endothelin [12] are increased in SSc patients. The aim of the present study was to investigate the amount of LTs (CysLT and LTB$_4$) and 8-isoprostane in EBC from patients with SSc compared with healthy controls. A secondary aim was to relate these EBC results to radiologic findings using HRCT.

Materials and methods

Patients

During September 2008 to May 2009, 22 consecutive patients were included at the initial assessment for SSc. Nineteen fulfilled the criteria for SSc according to the criteria of the ACR; 15 patients (12 women and 3 men) had lcSSc and 4 (2 women and 2 men) had proximal skin involvement and fulfilled the criteria for dcSSc [13]. Three patients (three women) with a median disease duration of 0.5 (range 0.5–3.4) years did not fulfil the ACR criteria for SSc, but fulfilled criteria for limited SSc (lSSc) suggested by LeRoy and Medsger [14]. Four patients were on NSAIDs, two were on inhaled corticosteroids (200–400 µg budesonide daily), three were on oral corticosteroids (2.5–10 mg daily) and none of the patients were on immunosuppressive medication. Sixteen healthy controls were enrolled. The study was approved by the Regional Ethical Review Board in Lund, and all subjects gave written informed consent in accordance with the Declaration of Helsinki.

Clinical assessment

All clinical and laboratory data were obtained within 1 week. Clinical characterization was performed as previously described [15]. Lung function tests included assessment of forced expiratory volume in 1 s (FEV$_1$), vital capacity (VC) and diffusing capacity of the lung for carbon monoxide (DLCO).

EBC collection and analysis

EBC was collected using ECoScreen (Jaeger, Wuerzburg, Germany) and to eliminate loss of biomarkers due to absorbance, all plastic surfaces were coated with 1% BSA and 0.01% Tween-20 as previously described [16]. Subjects were asked to rinse their mouth with water and breathe tidally for 15 min, wearing a nose clip. The condensates, as well as the serum samples, were stored at −70°C, until analysis. Activity of α-amylase was analysed using the EnzChek Ultra Amylase Assay Kit (E33651) from Molecular Probes (Eugene, OR, USA) to exclude saliva contamination [17].

Due to low concentrations in EBC, samples were concentrated (5–10 times depending on the kind of biomarker) by freeze-drying and resolved in the respective assay buffer. The final concentrations were calculated from the specific freeze-dried volumes. CysLT, LTB$_4$ and 8-isoprostane were analysed using EIA kit from Cayman Chemical (Ann Arbor, MI, USA) with a detection limit of 13, 6 and 2.7 pg/ml, respectively.

Radiologic analysis of HRCT images

The HRCT images were analysed by a chest radiologist blinded for EBC results and clinical data of the patients. Each patient was analysed for the extent of ground glass opacities (GGOs) and reticulations (estimated in percentage of the total lung volume, minimum step size 5%). Fibrosis was defined as the presence of traction bronchiectasis within areas of GGO and reticulation [18]. In the absence of traction bronchiectasis and reticulation, GGO was regarded as an unspecific finding not corresponding to fibrosis. The extent of traction bronchiectasis was described and grouped as 0 = non-detectable, 1 = few (1–5), 2 = intermediary (6–10) and 3 = extensive (>10). The HRCT images were also analysed for the presence of honeycombing and emphysema.

Statistical analysis

Data are shown as median [interquartile range (IQR)]. Mann–Whitney U-test was used for statistical comparison between groups and Spearman’s rho test was used for correlation analysis. $P < 0.05$ was considered to be statistically significant.

Results

Patients

Fibrosing alveolitis was present in 12 patients as defined by traction bronchiectasis. Of these patients, 10 also had reticulation and 9 had GGO (Table 1). In order to exclude saliva contamination in our EBC samples, we measured the levels of α-amylase. In EBC from all subjects, both SSc patients and healthy controls, the levels of α-amylase were low, confirming that the levels of LTs and 8-isoprostane were not due to saliva contamination [17].

SSc patients display increased CysLT but normal LTB$_4$ levels in EBC

The CysLT levels were significantly increased in SSc patients [6.1 (5.3–6.8) pg/ml] compared with healthy controls [4.9 (3.7–6.3) pg/ml] as shown in Fig. 1A ($P = 0.040$). The LTB$_4$ levels were not significantly increased in SSc patients [0.70 (0.50–0.83) pg/ml] compared with healthy controls [0.60 (0.42–0.70) pg/ml] as shown in Fig. 1B ($P = 0.29$). LTB$_4$ levels correlated with CysLT levels within the SSc-patient group ($P = 0.0015, r = 0.64$).
8-Isoprostane levels in EBC are increased in SSc

The levels of 8-isoprostane were significantly increased in SSc patients [0.23 (0.20–0.46) pg/ml] compared with healthy controls [0.19 (0.12–0.20) pg/ml] as shown in Fig. 1 C (P = 0.0020). 8-Isoprostane did not correlate with either CysLT (P = 0.14) or LTB4 within the SSc-patient group (P = 0.17).

No correlations between EBC and radiologic findings

There was no difference in LT levels between SSc patients with or without lung fibrosis on HRCT. Consequently, none of the LTs in EBC correlated to radiologic findings, either GGO, reticulation or traction bronchiectasis grading. Neither 8-isoprostane in EBC correlated to either radiologic finding.

There was no correlation between CysLT, LTB4 or 8-isoprostane levels in EBC and lung function parameters, such as FEV1, VC or DLco. There was no difference in any of the biomarkers in patients with or without anti-inflammatory medication.

8-Isoprostane levels in EBC and serum

As the amount of 8-isoprostane in EBC could be an indicator of systemic oxidative stress, the levels of 8-isoprostane were also measured in serum from SSc patients. However, we found no correlation between EBC and serum levels of 8-isoprostane (P = 0.82, data not shown).

Two populations of SSc patients could be distinguished, as seen in Fig. 1 C, and those SSc patients (n = 7) with 8-isoprostane levels >95 percentile of the controls (>0.37 pg/ml) were further investigated. This group of patients had significantly lower extent of traction bronchiectasis (P = 0.027) as well as lower extent of reticulation (P = 0.047). These patients also had significantly lower estimated pulmonary arterial systolic pressure (PASP; P = 0.005), as measured by echocardiography, and CRP.

**TABLE 1** Demographic dataa

<table>
<thead>
<tr>
<th></th>
<th>SSc (n = 22)</th>
<th>Healthy (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical data</td>
<td></td>
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<tr>
<td>Gender: female/male,</td>
<td>17/5</td>
<td>15/1</td>
</tr>
<tr>
<td>Age, years</td>
<td>58 (44–65)</td>
<td>55 (52–62)</td>
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<tr>
<td>Smokers, n</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>FEV1, %p</td>
<td>87 (78–98)</td>
<td>92 (86–106)</td>
</tr>
<tr>
<td>VC, %p</td>
<td>91 (76–104)</td>
<td>101 (96–107)</td>
</tr>
<tr>
<td>DLco, %p</td>
<td>70 (60–94)</td>
<td>ND</td>
</tr>
<tr>
<td>Duration, year</td>
<td>2.1 (0.8–4.9)</td>
<td>ND</td>
</tr>
<tr>
<td>Skin score</td>
<td>7 (2–13)</td>
<td>ND</td>
</tr>
<tr>
<td>dcSSc/lcSSc/SSc, n</td>
<td>4/15/3</td>
<td>ND</td>
</tr>
<tr>
<td>Radiologic findings</td>
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<td></td>
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<tr>
<td>GGO, n (5–20/25–40/</td>
<td>9 (5/3/1)</td>
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</tr>
<tr>
<td>45–50%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reticulation, n</td>
<td>10 (6/3/1)</td>
<td>ND</td>
</tr>
<tr>
<td>(5–20/25–40/45–50%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Traction bronchiectasis, n</td>
<td>12 (8/2/2)</td>
<td>ND</td>
</tr>
<tr>
<td>(1/2/3)</td>
<td></td>
<td></td>
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<tr>
<td>Serology</td>
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<tr>
<td>ANA/Scl-70/ACA, n</td>
<td>22/4/9</td>
<td>ND</td>
</tr>
</tbody>
</table>

aData are shown as numbers or as median (IQR). Scl-70: anti-scleroderma 70 antibodies; %p: percentage of predicted value; ND: not determined.
LTs in EBC from SSc patients

(P = 0.045). In addition, in all SSc patients, 8-isoprostanate in both EBC and serum correlated negatively to PASP (P = 0.026 and P = 0.033, respectively).

EBC findings do not differ between dcSSc and lcSSc

There were no differences in either CysLT or LTB4 in EBC from patients with dcSSc [6.0 (4.8–6.2) and 0.50 (0.40–0.75) pg/ml] compared with those with lcSSc [6.3 (5.3–7.9) and 0.7 (0.5–1.3) pg/ml]. Neither were there any differences in 8-isoprostanate in EBC between patients with dcSSc [0.33 (0.20–0.47) pg/ml] compared with patients with lcSSc [0.23 (0.20–0.47) pg/ml].

Patients with dcSSc had significantly higher levels of 8-isoprostanate in serum [45.6 (33.8–81.8) pg/ml] compared with patients with lcSSc [18.6 (12.1–50.7) pg/ml; P = 0.044]. In addition, none of the biomarkers in EBC correlated to disease duration from non-Raynaud’s (= skin) onset.

Discussion

LTs are believed to be involved in the fibrotic process of the lungs in SSc [19]. Increased levels of LTB4 and 8-isoprostanate have been reported in BAL fluid from SSc patients with fibrosing alveolitis [4, 6], and the present study shows that increased levels can also be measured non-invasively in EBC. Increased levels of CysLT were found in EBC from SSc patients compared with controls.

In addition, the level of 8-isoprostanate was elevated in EBC from SSc patients suggesting that oxidative stress within the lungs is part of the pulmonary fibrotic process.

There was no difference in LTB4 between SSc patients and healthy controls. This indicates the different roles of these two subsets of LT. However, the correlation between CysLT and LTB4 reflects an inflammatory pattern involving both types of LTs. However, the correlation between 8-isoprostanate and any of the LTs was found, showing that the LT-driven inflammatory process is separated from the oxidative stress process.

The finding of increased CysLT, but not LTB4, is in contrast with previous publications where an increase in both LTE4 and LTB4 is seen in BAL fluid from SSc patients [3, 4], and specifically in those patients with lung disease defined as pathological HRCT. An important difference between these studies and the present study is the disease duration in the patients, which is much shorter in the present study. This suggests that the onset of CysLT-driven inflammation comes early in the disease process.

An increase in 8-isoprostanate in EBC was seen in SSc patients, in accordance with previous publications on BAL, serum and urine [6, 8, 9], showing the involvement of oxidative stress in SSc. Serum levels of 8-isoprostanate were also measured to investigate whether the increased levels in EBC reflect local or systemic oxidative stress. As there was no correlation between EBC and serum levels of 8-isoprostanate, we assume that the oxidative process measured in EBC reflects the process in the lungs.

The biological role of 8-isoprostanate in SSc is not clear, but it has been shown to be a potent vasoconstrictor as well as bronchoconstrictor [7]. Interestingly, the EBC levels of 8-isoprostanate were high in some individuals who were distinguished from the rest of the SSc patients (Fig. 1C). These patients had in fact, a lower extent of traction bronchiectasis and reticulation, as well as lower PASP and CRP, compared with those with lower levels of 8-isoprostanate. This might suggest either a protective role of 8-isoprostanate, or that an early occurrence of 8-isoprostanate precedes subsequent fibrosis, but it needs to be further investigated.

Another interesting finding was that the serum levels, but not EBC levels, of 8-isoprostanate were higher in patients with dcSSc compared with lcSSc. This indicates that systemic oxidative stress is more prominent in patients with more extensive SSc, but needs to be interpreted cautiously due to the low number of patients.

In conclusion, increased levels of CysLT and 8-isoprostanate may be measured in EBC from patients with SSc indicating a possible significance in the pathogenesis of SSc. Further, increased levels of 8-isoprostanate in EBC supports the hypothesis that free radical-induced oxidative injury occurs in the lungs in SSc. Measurement of these two parameters non-invasively in EBC might provide biological markers which may be related to disease activity to follow the natural course of the disease. Since a decrease in LTB4 has been reported in BAL after cyclophosphamide therapy [4], EBC measurement of LTs could be of value for evaluation of therapy.

Rheumatology key messages

- CysLTs and 8-isoprostanate are increased in EBC from SSc patients.
- EBC may be used for assessment of inflammation and oxidative stress in the lungs of SSc patients.

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