ORIGINAL RESEARCH

Early Systemic Sclerosis: Serum Profiling of Factors Involved in Endothelial, T-cell, and Fibroblast Interplay is Marked by Elevated Interleukin-33 Levels

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Abstract

Purpose To assess the serum profile of factors involved in endothelial, T-cell, and fibroblast interplay in patients with Raynaud's phenomenon (RP) associated with nailfold vodeocapillaroscopy (NVC) scleroderma findings and/or systemic sclerosis (SSc) marker autoantibodies, recently labeled as early SSc patients.

Methods Serum levels of soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular adhesion molecule-1 (sVCAM-1), CCL2, CXCL8, IL-13, IL-33, and transforming growth factor-β (TGF-β) were measured in 24 early SSc patients, 48 definite SSc patients, and 24 osteoarthritis/fibromyalgia controls by multiplex suspension immunoassay. All SSc patients were investigated for the presence/absence of preclinical and clinical organ involvement, SSc marker autoantibodies, and NVC abnormalities.

Results Serum sICAM-1, CCL2, CXCL8, and IL-13 were increased in all SSc patients as compared to controls, and paralleled the severity of the disease subset (early SSc < limited cutaneous SSc < diffuse cutaneous SSc; $p < 0.0001$). Surprisingly, IL-33 was significantly higher in early SSc patients as compared to both controls ($p < 0.01$) and definite SSc patients ($p < 0.05$). In early SSc there were no differences in the investigated markers according to the functional and serological features assessed.

Conclusions Our study suggests that an endothelial, T-cell and fibroblast activation can be present in patients with early SSc and it is associated with a distinct profile of circulating factors involved in the cross-talk of these cells. The marked increase of IL-33 in early SSc patients suggests new routes of investigation of cell-cell dynamics in target tissues predating overt disease manifestations, thus opening to new therapeutic approaches.

Introduction

Systemic sclerosis (SSc) is a chronic systemic autoimmune disorder characterized by microvascular abnormalities, interstitial fibrosis of the skin and internal organs and oligoclonal expansion of T lymphocytes in the skin of patients with early diffuse cutaneous disease (dcSSc) [1].

Etiology and pathogenesis of SSc are unknown. It is commonly thought that microvascular abnormalities and immune system activation precede fibroblast activation and interstitial fibrosis [1]. This pathophysiologic assumption relies on old histopathology data [2] and it has been reaffirmed in recent reviews on the topic [1, 3].

We previously showed that T lymphocytes from the skin of patients with early dcSSc recognize autologous fibroblast antigens and induce fibroblast apoptosis in vitro [4]. This suggests that the primary aberrant activation of fibroblasts, that trigger the autoimmune response, might be the earliest event in SSc pathogenesis.

Recently, a condition characterized by Raynaud's phenomenon (RP) with a scleroderma pattern at nailfold videocapillaroscopy (NVC), circulating SSc marker autoantibodies or both, without any other sign/symptom of SSc except puffy fingers and/or arthritis [5, 6], has been identified. This

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condition, named early SSc, does not meet the 1980 American College of Rheumatology (ACR) and the 2013ACR/ European League Against Rheumatism (EULAR) classification criteria for SSc [7, 8], and offers an unique opportunity to investigate the pattern of cellular activation in the early stages of the disease.

In a first study we showed that 42 % of these early SSc patients can have a preclinical involvement of esophagus and/ or lung and/or heart [5]. Later, we showed that early SSc patients have increased serum levels of factors related to endothelial (soluble E-selectin) and/or T-cell (soluble IL-2 receptor alpha) and/or fibroblast activation (carboxyterminal telopeptide of type I collagen) [6].

In this scenario, we undertook the present study to investigate a wider panel of serum markers reflecting the interplay between these key cell types in early SSc patients and in patients with a definite disease for comparison, in order to shed light on the initial steps of the disease, possibly improving the non-invasive characterization of early patients lacking signs and symptoms of overt disease.

Patients and Methods

Study Population

Patients consecutively admitted to our clinic for the evaluation of RP from November 1st 2000 to June 30th 2012, who fulfilled criteria for early SSc [5, 6], did not meet classification criteria [7, 8] for SSc or any other connective tissue disease, and agreed to donate serum, were enrolled in this study. Early SSc patients were matched for sex and age with corresponding osteoarthritis and/or fibromyalgia controls, and corresponding patients with limited cutaneous (lc) and diffuse cutaneous (dc) [9] SSc. No SSc patient was affected by an overlap syndrome, including Mixed Connective Tissue Disease [10]. All patients were free from immunosuppressive therapy at the time of enrolment. All patients and controls gave their informed consent and the protocol was approved by the local Ethics Committee.

Patient Assessment

Each SSc patient underwent a complete assessment, including the evaluation of antinuclear autoantibodies (ANA) to search for SSc marker autoantibodies, NVC to search for a capillaroscopy scleroderma pattern, functional examinations to detect the preclinical involvement of esophagus (basal lower esophageal sphincter - LES- pressure <15 mmHg at manometry), heart (early- E/late –A- ventricular filling velocity ratio <1 at echocardiography), and lung (lung diffusion capacity for carbon oxyde –DLCO - <80 % of the predicted value at single breath measurement). These procedures were carried out as previously described [5, 6].

Serum Marker Selection

Among the large number of circulating markers previously investigated in sera from SSc patients meeting the ACR 1980 classification criteria [7], we chose parameters showing consistent evidence of being implicated in the pathogenesis of SSc as mediators of the cross-talk between endothelial cells, T-cells, and fibroblasts, and whose abnormal expression in SSc sera has been reported by at least two research groups. We selected soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular adhesion molecule-1 (sVCAM-1), CCL2 and CXCL8 chemokines, IL-13, IL-33, and transforming growth factor-β (TGF-β) [1, 3, 11–15].

Serum Marker Quantification

Peripheral blood was obtained at enrolment by venipuncture. Serum was separated by centrifugation at 1,500 g for 10 min, aliquoted and stored at −20 °C. All analytes were measured by single or multiple suspension fluorescence-based immunoassay (Merk Millipore, Billerica, MA, USA) using a Luminex 200 instrument (Luminex Corporation, Austin, TX, USA), as previously described [6]. Concentrations of CCL2, CXCL8, IL-33, and IL-13 were expressed as pg/ml; those of sICAM-1 and sVCAM-1 as ng/ml.

Statistical Analysis

GraphPad Prism software version 6.0 (GraphPad Software Inc, San Diego, CA, USA) was used for statistical analysis. Continuous data were expressed as mean ± standard deviation (SD) or median with range, and compared by the unpaired student's t test or the Mann–Whitney U test as appropriate for two group analysis. Three or more groups were compared by one-way ANOVA or the Kruskal-Wallis test, as appropriate. The D'Agostino-Pearson test was used to assess the normal distribution of data. Categorical data were analyzed by the Fisher's exact test and the Chi-Square test for two and three sets of data, respectively. P values less than 0.05 were considered statistically significant.

Results

Early SSc Patients are Similar to lcSSc Patients in the Autoantibody and Capillaroscopy Pattern, but Differ in the Prevalence of Preclinical Organ Involvement

Table I shows the features investigated in early SSc patients compared to the two subsets of definite SSc patients (lcSSc and dcSSc). In general, early SSc were similar to lcSSc patients in the autoantibody and capillaroscopy pattern.

Table I Features of the 24 early SSc patients as compared to definite SSc patients

Feature	early SSc (n=24)	lcSSc (n=24)	dcSSc (n=24)	p
Sex: F/M ratio	22/2	22/2	21/3	ns
Age; years (mean±SD)	42±14	49±13	45±11	ns
RP duration; years (median; range)	2 (0.5–18)[ab]	7.5 (0–39)[abc]	3 (0.5–18)[ac]	0.003[a],0.005[b],0.02[c]
Anti-Scl-70 abs	6/24 (25)	12/24 (50)	11/24 (45.8)	ns
ACA	17/24 (70.8)[ab]	11/24 (45.8)[ac]	3/24 (12.5)[abc]	0.03[a, b], <0.0001,[c]0.02
Anti-RNA polymerase III abs	1/24 (4.2)	1/24 (4.2)	3/24 (12.5)	ns
Megacapillaries	14/24 (58.3)	11/24 (45.8)	15/24 (62.5)	ns
Avascular areas	3/24 (12.5)[ab]	6/24 (25)[a]	12/24 (50)[ab]	[a]0.02,[b]0.01
DLCO <80 % of predicted value	7/24 (29.2)[abc]	13/21 (61.9)[ab]	20/24 (83.3)[ac]	[ac] <0.001,[b]0.04
E/A ratio <1	3/22 (13.6)[ab]	10/20 (50)[abc]	3/20 (15)[ac]	[a]0.01,[b]0.02,[c]0.04
Basal LES pressure <15 mmHg	6/23 (26)[ab]	11/24 (45.8)[a]	15/24 (62.5)[ab]	[a]0.009,[b]0.02

All values are expressed as numbers and percentages (in brackets) except where otherwise specified. Only features that can be found in both early and definite SSc patients were analyzed

[a] Chi-square test was used for three group comparison

[bc] Fisher's exact test was used for two group comparison

SSc systemic sclerosis; *lcSSc* limited cutaneous *SSc; dcSSc* diffuse cutaneous *SSc; F/M* female/male; *SD* standard deviation; *RP* Raynaud's phenomenon; *abs* antibodies; *ACA* anticentromere antibodies; *DLCO* diffusing lung capacity for carbon oxide; *E/A* early/atrial (late) ventricular filling velocity; *LES* lower esophageal sphincter

However, as expected, they had a lower prevalence of preclinical organ involvement. In details, a DLCO <80 % was found in 29,2 % of early cases, 61.9 % of lcSSc and 83.3 % of dcSSc ($p<0.001$). An E/A ratio <1 was found in 13.6 % early SSc, 15 % dcSSc and 50 % lcSSc patients ($p=0.02$). A basal LES pressure <15 mmHg was found in 26 % early SSc, 45.8 % lcSSc and 62.5 % dcSSc patients ($p=0.009$). Of note, lcSSc patients had a significantly longer disease duration as compared to early and dcSSc patients ($p=0.003$).

Markers Reflecting Endothelial, T-cell and Fibroblast Interplay are Elevated in Early SSc Patients with a Marked Increase of IL-33

All investigated factors were detectable in sera from SSc patients and controls. sICAM-1, CCL2, CXCL8, and IL-13 were significantly higher in early SSc patients than in controls (respectively: 2.22 ng/ml [1.23–3.07] versus 1.58 ng/ml [0.29–5.03], $p<0.05$; 464.2 pg/ml [171.4–1163] versus 128.7 pg/ml [43.01–465.7]; $p<0.0001$; 3.66 pg/ml [0.73–28.18] versus 1.89 pg/ml [0.21–4.64]; $p<0.0001$; 0.56 pg/ml [0.09–0.99] versus 0.21 pg/ml [0.04–0.78]; $p<0.01$), but lower than in SSc patients with a definite disease (Fig. 1a–d). Intriguingly, serum IL-33 had the highest levels in the early subset and the lowest in lcSSc (15.76 pg/ml [10–68.1] versus 11.08 pg/ml [5.42–38.2] in controls, $p<0.01$; versus 9.768 pg/ml [4.71–66.38] in lcSSc, $p<0.05$; versus 12.02 pg/ml [2.69–57.19] in dcSSc, $p<0.05$) (Fig. 1e). sVCAM-1 and TGF-β were significantly elevated in patients with lcSSc and dcSSc,

but not in patients with early SSc with respect to controls (Fig. 2a–b).

Disease duration, as evaluated from RP onset, SSc marker autoantibodies, NVC findings, and preclinical organ dysfunction did not affect the serum profile of the investigated soluble factors in early SSc patients.

Discussion

To our knowledge, this is the first study investigating a wide panel of serum factors implicated in the pathogenesis of the disease in early SSc patients (i.e. patients presenting with RP and NVC scleroderma findings and/or SSc marker autoantibodies not meeting 2013 ACR/EULAR criteria for SSc) [5, 6]. This newly identified early SSc population represents an ideal setting to investigate endothelial-lymphocyte-fibroblast interplay in the early stages of the disease.

In early SSc, we found that levels of sVCAM-1 and TGF-β were similar to those measured in controls and lower than those measured in lcSSc and dcSSc patients. Levels of sICAM-1, CCL2, CXCL8 and IL-13 were higher than those measured in controls, but lower than those measured in lcSSc and dcSSc patients. Lastly, levels of IL-33 were higher than those measured in controls and patients with definite disease.

sVCAM-1 is a marker of endothelial activation [1]. Serum sVCAM-1 has been found to be increased in SSc with systemic manifestations [16]. Moreover, an association between circulating sVCAM-1 and disease severity has been reported [17]. By definition, early SSc patients do not show any clinical
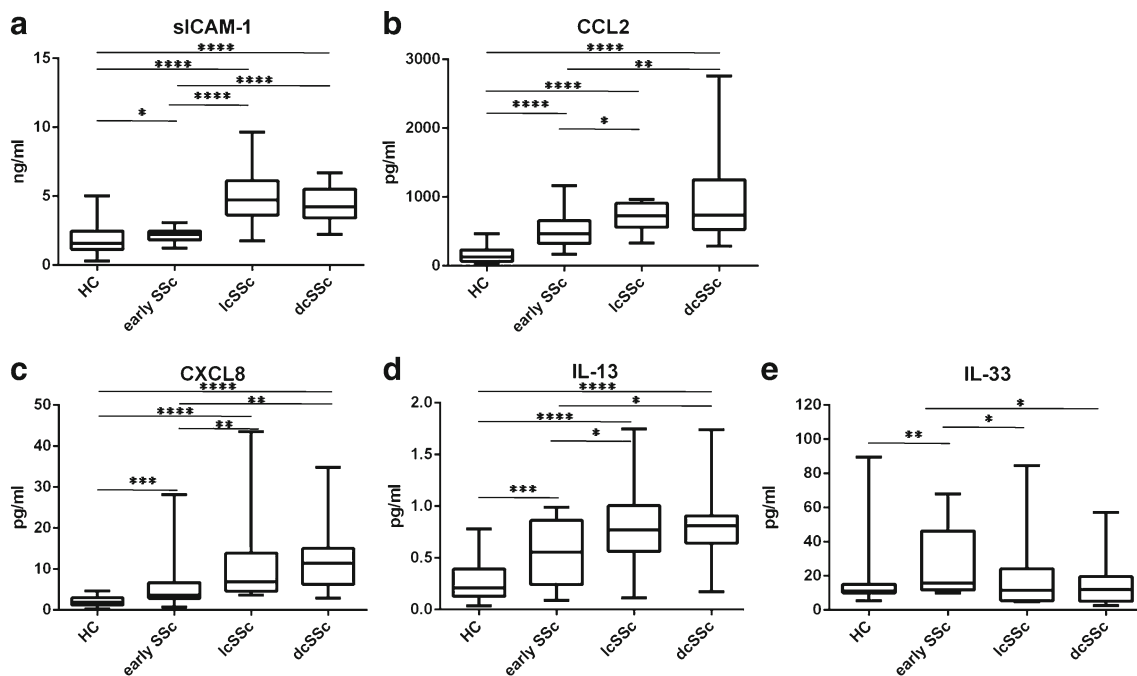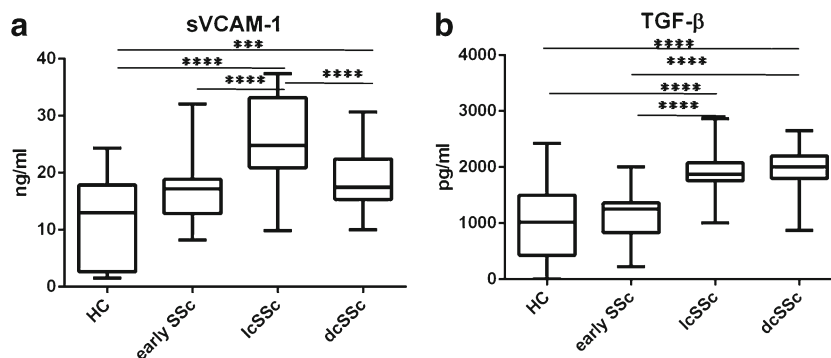
Fig. 1 Serum levels of markers reflecting endothelial, T-cell, and fibroblast interplay measured in SSc patients and osteoarthritis/fibromyalgia controls (24 patients each group) that were significantly elevated in early SSc patients. Early SSc patients were defined as patients with Raynaud's phenomenon and either SSc marker autoantibodies or scleroderma pattern at nailfold videocapillaroscopy or both [5, 6]. Definite SSc patients, lcSSc and dcSSc, were defined according to the new 2013 ACR/EULAR classification criteria [8]. In each analysis at least one data set was not-normally distributed, therefore boxes and whiskers plot was chosen. The Kruskall-Wallis test was applied for all column comparison. The Mann–Whitney test or the student's *t* test with Welch's post-test correction were applied for two column comparison. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. *SSc* systemic sclerosis; *HC* controls; *lcSSc* limited cutaneous SSc; *dcSSc* diffuse cutaneous SSc; *sICAM-1* soluble intercellular adhesion molecule-1; *CCL2* CC- chemokine 2; *CXCL8* CXC-chemokine 8; *IL-33* interleukin-33; *IL-13* interleukin-13

organ manifestation nor they can show a severe disease. Therefore, our results are in line with previous reports, since serum sVCAM-1 was similar in early SSc patients and controls but increased in lcSSc and dcSSc individuals (Fig. 2a). In details, the highest serum levels of sVCAM-1 were measured in lcSSc patients. However, the role, if any, of sVCAM-1 as a marker of this SSc subset awaits to be clarified.

Serum TGF-β has been investigated in SSc patients with a definite disease with controversial results [18, 19]. It has been found to be increased in up to 15–50 % of cases. Our data fall in this line. Indeed, we detected higher TGF-β levels in patients with a definite disease (Fig. 2b). It is possible that the slight increase of serum TGF-β measured in early SSc patients with respect to controls, not statistically significant, is

Fig. 2 Serum levels of markers reflecting endothelial, T-cell, and fibroblast interplay measured in SSc patients and osteoarthritis/fibromyalgia controls (24 patients each group), that were significantly elevated only in definite SSc patients. Early SSc patients were defined as patients with Raynaud's phenomenon and either SSc marker autoantibodies or scleroderma pattern at nailfold videocapillaroscopy or both [5, 6]. Definite SSc patients, lcSSc and dcSSc, were defined according to the new 2013 ACR/EULAR classification criteria [8]. In each analysis at least one data set was not-normally distributed, therefore boxes and whiskers plot was chosen. The Kruskall-Wallis test was applied for all column comparison. The Mann–Whitney test or the student's *t* test with Welch's post-test correction were applied for two column comparison. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. *SSc* systemic sclerosis; *HC* controls; *lcSSc* limited cutaneous SSc; *dcSSc* diffuse cutaneous SSc; *sVCAM-1* soluble vascular cellular adhesion molecule-1; *TGF-β* transforming growth factor-β

due to a general immune system activation, as observed in other autoimmune diseases, and not to an aberrant activation of the TGF-β pathway at the tissue level, similar to what we see in the definite disease [1].

sICAM-1, CCL2, CXCL8 and IL-13 levels were all increased in our early SSc patients with respect to controls, but lower than those detected in lcSSc and dcSSc patients, the highest levels being detected in patients with a larger extent of skin disease (dcSSc) (Fig. 1a–d).

sICAM-1 is another marker of endothelial activation [1]. It triggers a pro-inflammatory cascade in endothelial cells that induces the overexpression of cytokines and cell-adhesion molecules, including sVCAM-1 [20]. Therefore, the observed increase of circulating sICAM-1 (Fig. 1a) not yet associated to sVCAM-1 changes in early SSc patients (Fig. 2a) is not surprising.

CCL2 and CXCL8 are two chemokines strongly implicated in the pathogenesis of SSc. In fact, along with their chemotactic activity towards dendritic cells/monocytes/T-cells and neutrophils, respectively, both chemokines display proangiogenic [21] and pro-fibrotic properties [22], They are regarded as early mediators of inflammatory processes and can be produced by a variety of cell types, including endothelial cells and fibroblasts [1]. However, the increase of serum CCL2 and CXCL8 in parallel with the progression of skin involvement in the three SSc subsets here investigated (early SSc, with no skin involvement < lcSSc < dcSSc) (Fig. 1b–c) might indicate that these chemokines play different roles in SSc during the natural course of the disease.

IL-13 is a prototypical Th2 cytokine and one of the most potent effectors of fibrosis in SSc [1, 3, 13]. Increased serum levels of IL-13 have been shown in patients with definite disease, regardless the subset [13]. Here we show that this cytokine is already increased in early SSc when no fibrotic organ involvement is clinically detectable, and progressively increases in the lcSSc and the dcSSc subset (Fig. 1d).

The main finding of our study, however, is the peculiar increase of IL-33 in early SSc patients with respect to both controls and patients with a definite disease (Fig. 1e). IL-33 is a cytokine belonging to IL-1β superfamily, enriched in epithelial and endothelial cells [23]. IL-33 is regarded as an early inflammatory mediator targeting Th2 cells and inducing IL-13 production [14, 23]. In details, it has been clarified that IL-33 binds a heteromeric receptor consisting of two subunits: the orphan IL-1 receptor ST2, highly expressed on Th2 polarized cells and mast cells, and IL-1R accessory protein (IL-1RAcP) [24, 25]. The engagement of this complex by IL-33 induces the activation of NF-kappaB and MAP-kinases, leading to IL-4, IL-5 and IL-13 production in vitro [24]. These results were confirmed in vivo in C57BL/6 mice treated with intraperitoneal injection of IL-33 [24], and were associated with the development of pathological changes in the lungs and the gastrointestinal tract of the animals. These pathological changes were characterized by abnormalities in medium and small muscular arteries consisting of medial hypertrophy, presence of myeloid cells within the vascular lumen, and infiltrates of eosinophils and/or mononuclear cells beneath the endothelium, within the vessel wall, and adjacent to the vessels [24]. Recently, high IL-33 serum levels have been associated with peripheral vascular involvement [26, 27] and recent disease onset [26] in patients with definite SSc. The ST2 receptor has been found to be overexpressed in different organs (skin, lung, kidney, heart, esophagus, stomach and placenta) from patients with definite SSc [28]. Most interestingly, however, the overexpression of ST2 was associated with a striking lack of IL-33 in local endothelial cells in the skin and other tissues of patients with definite disease of recent onset [28]. This findings strongly support the hypothesis that IL-33 might mediate the very early pathogenic events of SSc, being crucially increased in the early subset. At the same time, these data also suggest that IL-33 gives way to Th2-type pro-fibrotic cytokines, like IL-13, in the fibrotic stages of the disease. Regarding the possible source of IL-33, we must remember that the production of this cytokine is not limited to endothelial and epithelial cells. In fact, it can be also produced by innate lymphoid cells, T cells, and fibroblasts [23, 28]. Noteworthy, both endothelial cells and fibroblasts are likely to be activated in early SSc patients, as shown by increased circulating adhesion molecules and collagen metabolites [6], respectively. Therefore, we can affirm that IL-33 is likely to be involved in the initial cascade of events in SSc pathogenesis, but we may not infer any definite conclusion on the cell type that is making it in SSc.

We underline that we are detecting here circulating levels of cytokine and soluble factors, not supplying data generated analyzing affected tissues. Nevertheless, our data clearly indicate that endothelial cells, T cells, and fibroblasts are all already activated in this early, pro-fibrotic stage of the disease, and suggest an inner loop of reciprocal stimulation going on between these cells with IL-33 playing a central role. We propose here new potential tools to improve the non-invasive characterization of early SSc patients to be confirmed on larger series. If this will be the case, serum molecules showing the attitude to increase in SSc in parallel with the onset and progression of skin sclerosis, like sICAM-1, CCL2, CXCL8 and IL-13, could be used as prognostic markers to monitor the evolution of the disease, while IL-33 might be a peculiar marker of the early subset.

Ongoing studies on tissue expression profile of the hereby investigated markers in early SSc will better help the understanding of their dynamics and potentially support the role of the molecules here investigated as novel biomarkers in SSc. Finally, finding the peculiar increase of IL-33 may possibly open the door to new therapeutic approaches to target SSc at the beginning stage to prevent overt fibrotic disease.

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