Increased Serum Interleukin 23 in Patients with Systemic Sclerosis

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ABSTRACT. ***Objective.*** The relationship between systemic sclerosis (SSc) and interleukin 23 (IL-23), a cytokine associated with the differentiation of T lymphocytes, is unknown. We investigated serum IL-23 levels and their clinical association in patients with SSc.

Methods. Serum IL-23 levels were examined by ELISA in 63 patients with SSc, 15 patients with systemic lupus erythematosus (SLE), and 31 healthy individuals. SSc patients comprised 25 with limited cutaneous SSc and 38 with diffuse cutaneous SSc.

Results. Serum IL-23 levels were significantly elevated in SSc patients compared to patients with SLE ($p < 0.05$) and controls ($p < 0.005$). Elevated serum IL-23 levels were associated with the disease duration ($p < 0.05$) and the prevalence of pulmonary fibrosis ($p < 0.05$), although they were not associated with other clinical features, including the extent of skin sclerosis or the severity of pulmonary fibrosis.

Conclusion. The results suggest that IL-23 is associated with induction of SSc and that blockade of IL-23 can be a potential therapeutic strategy in early SSc. (First Release Dec 15 2007; J Rheumatol 2008;35:120–5)

Key Indexing Terms:
SCLERODERMA INTERLEUKINS INTERLEUKIN 23 PULMONARY FIBROSIS

Systemic sclerosis (SSc) is a connective tissue disease with autoimmune background, characterized by excessive extracellular matrix deposition in the skin and other visceral organs[1,2]. Studies have attempted to elucidate the relationship between the features of fibrosis and immunological abnormalities in SSc. It has been suggested that some cytokines or growth factors, including transforming growth factor-ß (TGF-ß), regulate the induction and development of fibrosis by stimulating the synthesis of extracellular matrix components[1,2]. These cytokines and growth factors are also considered to play a role in the induction of T lymphocyte activation[1,3]. Activated T cells have been indicated to differentiate into memory/effector T cells that are classified into T helper 1 (Th1) and Th2 subsets based on their cytokine production profiles[4]. Th1 cells secrete mainly interferon-γ (IFN-γ) and interleukin 2 (IL-2), whereas Th2 cells predominantly release IL-4, IL-5, IL-6, IL-10, and IL-13[4]. Th1 cells were highly proinflammatory and were linked to the induction and progression of autoimmune diseases, including SSc[5,6]. However, unexpectedly, despite loss of IFN-γ signaling, mice deficient in IFN-γ or the IFN-γ receptor are not resistant to experimental allergic encephalomyelitis (EAE) but more susceptible to autoimmunity[7-9]. In other words, the development of autoimmune diseases was in part independent of Th1 cytokines IFN-γ. These observations suggest existence of an additional T cell subset, distinct from IFN-γ-producing Th1 cells, that is capable of inducing tissue inflammation and autoimmunity. This has led to the identification of IL-23- and IL-17-producing (Th17) cells[10].

IL-12-dependent Th1 cells were considered likely to play an essential role in the induction of autoimmunity, based on the observations in use of neutralizing p40 antibodies or p40-deficient mice. However, after the discovery of IL-23, which shares a p40 subunit with IL-12, several observations suggested the involvement of IL-23 and Th17 in autoimmunity[11]. Stimulation of activated and memory T cells in the presence of IL-23 induces Th17 cells specifically[12,13], thus IL-23 contributes to the development of autoimmune inflammation, including EAE, collagen-induced arthritis, and inflammatory bowel disease[13-15]. Further, recent reports have shown that TGF-ß and IL-6 are required for the differentiation of naive T cells into IL-23 receptor-positive IL-17-producing cells[16,17]. Since circulating levels of TGF-ß and IL-6 are elevated in patients with SSc[18,19], IL-23 is likely to play a role on the induction and development of SSc. Thus, we investigated serum IL-23 levels in SSc and relations to clinical features in SSc.

MATERIALS AND METHODS

Serum samples. Serum samples were obtained from 63 Japanese patients with

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SSc (54 women, 9 men). They had all been referred to the Department of Dermatology, Kanazawa University, between 1999 and 2005. All patients fulfilled the criteria for SSc proposed by the American College of Rheumatology (ACR)[20]. Patients were between 2 and 76 years old (mean age 55 yrs). They were grouped according to the classification system proposed by LeRoy, *et al*[21]: 25 patients (all women) had limited cutaneous SSc (lSSc) and 38 (29 women, 9 men) diffuse cutaneous SSc (dSSc). The disease duration of patients with lSSc and dSSc, defined as the time from the first symptom related to Raynaud's phenomenon, was 5.3 ± 4.2 and 5.9 ± 4.3 years, respectively. Four patients had been treated with low-dose corticosteroids (prednisolone 5–20 mg/day) and 3 patients with low-dose D-penicillamine (100–500 mg/day) at the first visit. No patient had received other immunosuppressive therapy, or had a recent history of infection or other inflammatory disease. To evaluate the relationship between steroid treatment and IL-23 concentrations, results were analyzed similarly in SSc patients without steroid treatment. As a disease control, we also examined serum samples from 15 patients with SLE that fulfilled the ACR criteria[22]. SLE patients with more than 10 points on a disease activity index were included. Thirty-one healthy Japanese (25 women, 6 men; ages 7–73 yrs, mean 55 yrs) were used as controls. Peripheral venous blood samples were drawn into pyogen-free collection tubes without additives, immediately immersed in melting ice, and allowed to clot 1 h before centrifugation (1500 g at 4°C for 10 min). All samples were stored at –70°C prior to use.

Clinical assessment. Complete medical histories, physical examinations, and laboratory tests were conducted for all patients at the first visit. Skin score was measured using the technique of the modified Rodnan total skin thickness score (TSS)[23]. Organ system involvement was defined as follows: pulmonary fibrosis: bibasilar fibrosis on chest radiography and high resolution computed tomography; esophagus: hypomotility shown by barium radiography; joint: inflammatory polyarthralgias or arthritis; heart: pericarditis, congestive heart failure, or arrhythmias requiring treatment; kidney: malignant hypertension and rapidly progressive renal failure with no other explanation; and muscle: proximal muscle weakness and elevated serum creatine kinase. Pulmonary hypertension was defined as clinical evidence of pulmonary hypertension and increased systolic pulmonary arterial pressure (> 35 mm Hg) by Doppler echocardiography. Pulmonary function tests including vital capacity (VC) and diffusion capacity for carbon monoxide (DLCO) were carried out. When DLCO and VC were < 75% and < 80%, respectively, of predicted normal values, they were considered to be abnormal. The protocol was approved by Kanazawa University School of Medicine, Kanazawa University Hospital, and by the Nagasaki University Graduate School of Biomedical Sciences.

ELISA. Specific ELISA kits were used for measuring serum IL-23 levels (BioSource, Camarillo, CA, USA) according to the manufacturer's protocol. Each sample was tested in duplicate. The detection limit of this assay in our institution is 0.1 pg/ml. Multiple tests indicated quantitative accuracy ranging from 0.1 to 15 pg/ml, although the detection limit according to the manufacturer was less than 15 pg/ml.

Statistical analysis. Statistical analysis was performed by Mann-Whitney U test for comparison of IL-23 levels, Fisher's exact probability test for comparison of frequencies, and Bonferroni test for multiple comparisons. A p value less than 0.05 was considered statistically significant. All data are shown as means ± SD.

RESULTS

Serum IL-23 levels in SSc patients. Serum IL-23 levels were significantly higher in patients with SSc than in healthy con-

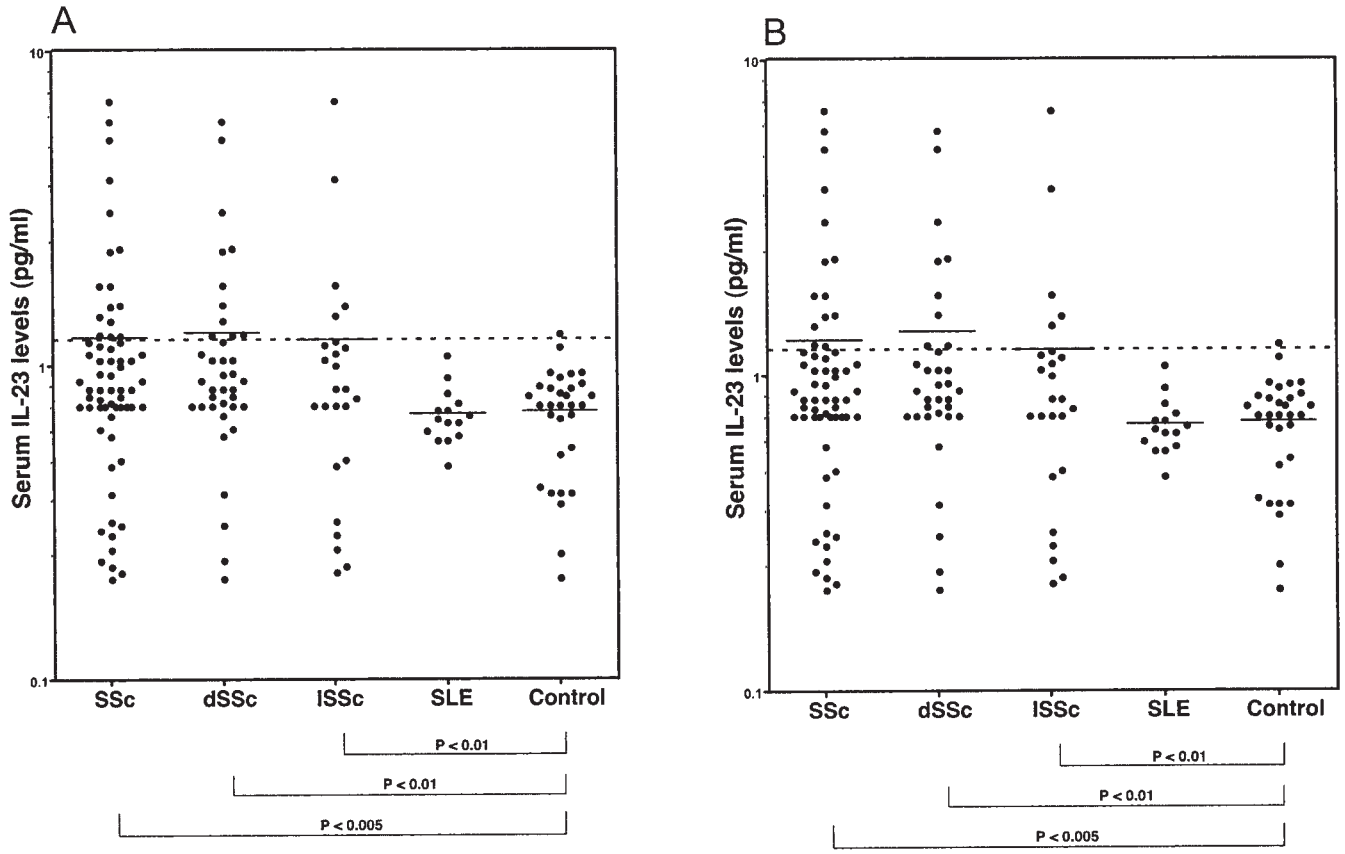

Figure 1. A. Serum levels of IL-23 in patients with limited cutaneous SSc (lSSc), diffuse cutaneous SSc (dSSc), systemic lupus erythematosus (SLE), and healthy controls. Serum IL-23 levels were determined by a specific ELISA. Short bars indicate mean values in each group. Broken line indicates the cutoff value (mean + 2 SD of control samples). B. Results from patients with no steroid therapy.

trols (1.23 ± 1.27 vs 0.71 ± 0.25 pg/ml, respectively; $p < 0.005$; Figure 1A) and patients with SLE ($p < 0.05$). As for subgroups of SSc, IL-23 levels in both lSSc (1.21 ± 1.42 pg/ml) and dSSc (1.27 ± 1.19 pg/ml) patients were significantly higher than in healthy controls ($p < 0.01$ and $p < 0.01$, respectively). However, IL-23 levels were similar between patients with dSSc and those with lSSc. Since steroid therapy may affect serum IL-23 concentrations, IL-23 levels were also analyzed within the patients without steroid therapy. The results were similar to the results from the whole patient group. Serum IL-23 levels were significantly higher in patients with SSc than in healthy controls ($p < 0.005$; Figure 1B). While abnormalities of Th17-related cytokine profiles have been reported to be associated with autoimmune diseases, including multiple sclerosis[24], rheumatoid arthritis[25], psoriasis[26], and Vogt-Koyanagi-Harada disease[27], no SSc patient in this study had these autoimmune diseases.

Elevated IL-23 and prevalence of pulmonary fibrosis in SSc. The patient sample was divided into 2 groups, according to IL-23 level of 1.20 pg/ml (mean + 2 SD of control serum samples) as the cutoff value; 27% (17/63) of SSc patients were thus classified as having elevated IL-23 levels. The frequency of pulmonary fibrosis in patients with elevated IL-23 levels was significantly higher than in those with normal IL-23 levels (71% vs 32%; $p < 0.05$; Table 1). Further, IL-23 levels were also correlated with decreased %VC, although IL-23 levels did not correlate with decreased %DLCO in patients with SSc. This is possible, since some patients with SSc show decreased %DLCO when they do not have pulmonary fibrosis or abnormality in %VC. IL-23 levels did not correlate with the extent of skin involvement, the presence of pulmonary hypertension, involvement of esophagus, heart, kidney, joint and muscle, serum levels of anti-topoisomerase I antibodies, anticentromere antibodies, IgG, IgA, IgM, CH50, C3, C4, or C-reactive protein or erythrocyte sedimentation rate (Table 1 and data not shown). The results from patients without steroid therapy were similar to the results from the whole patient group (Table 1).

Elevated IL-23 and disease duration in SSc. Patients with elevated IL-23 levels exhibited shorter disease duration ($p < 0.01$; Table 1). Moreover, dSSc patients with disease duration < 2 years had significantly elevated IL-23 levels compared to

Table 1. Clinical and laboratory features of patients with SSc showing elevated serum IL-23 levels. Unless noted otherwise, values are percentages. Values in parentheses indicate results from patients with no steroid therapy.

	Elevated IL-23, n = 17 (n = 15)	Normal IL-23, n = 46 (n = 44)	p
Age at onset, yrs, mean ± SD	46.5 ± 19.1 (46.6 ± 19.7)	43.9 ± 16.4 (44.0 ± 16.6)	0.61 (0.61)
Male:female	2:15 (1:14)	7:39 (7:37)	> 0.99 (0.67)
Duration, yrs, mean ± SD	2.25 ± 1.5 (2.14 ± 1.5)	6.91 ± 4.3 (6.97 ± 4.5)	< 0.01 (< 0.01)
Clinical features			
dSSc	65 (67)	56 (55)	0.56 (0.55)
lSSc	35 (33)	43 (45)	0.56 (0.55)
TSS, mean ± SD	15.1 ± 9.1 (14.1 ± 8.5)	13.3 ± 10.7 (13.1 ± 11.0)	0.56 (0.75)
Pitting scars	41 (39)	41 (39)	0.77 (> 0.99)
Contracture of phalanges	35 (40)	52 (48)	0.40 (0.77)
Diffuse pigmentation	58 (60)	52 (48)	0.56 (0.55)
Organ involvement			
Pulmonary hypertension	0 (0)	15 (16)	0.24 (0.21)
Pulmonary fibrosis	71 (73)	32 (27)	0.004 (0.002)
Decreased %VC	59 (53)	21 (21)	0.03 (0.02)
Decreased %DLCO	88 (87)	56 (59)	0.06 (0.06)
Esophagus	76 (73)	74 (75)	0.47 (0.58)
Heart	18 (13)	17 (11)	0.80 (0.84)
Kidney	6 (7)	2 (2)	0.45 (0.45)
Joint	12 (13)	26 (23)	0.53 (0.44)
Muscle	6 (7)	24 (20)	0.31 (0.22)
Laboratory findings			
Anti-topoisomerase I antibody	65 (67)	41 (36)	0.08 (0.07)
Anticentromere antibody	24 (27)	39 (39)	0.38 (0.54)
Increased IgG	53 (47)	28 (25)	0.24 (0.19)
Elevated ESR	35 (33)	28 (20)	0.76 (0.52)
Elevated CRP	24 (13)	15 (11)	0.58 (0.22)

TSS: modified Rodnan total skin thickness score.

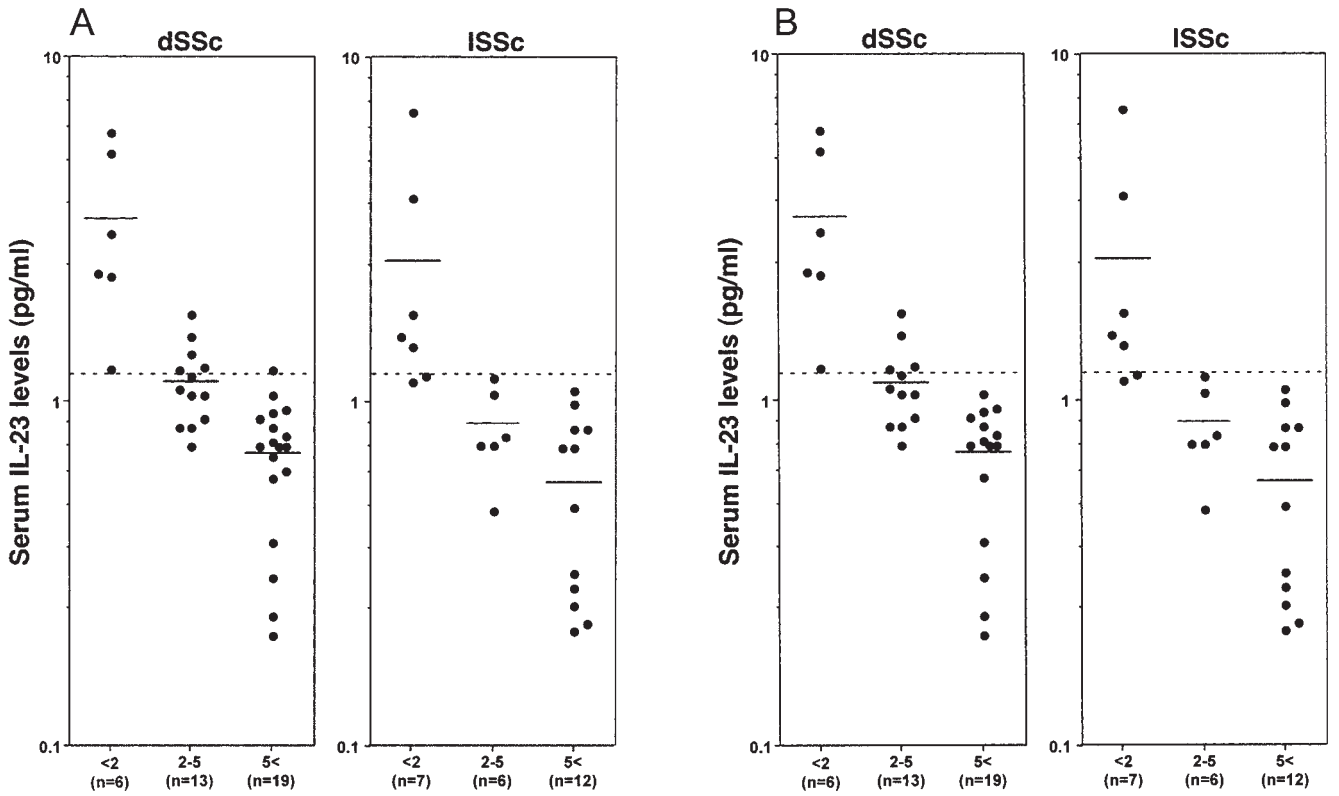

Figure 2. A. Correlation of disease duration with serum IL-23 level in patients with dSSc and lSSc. Serum IL-23 levels were determined by a specific ELISA. Broken line indicates the cutoff value. B. Results from patients with no steroid therapy.

those with duration of 2–5 years ($p < 0.05$) and those with duration > 5 years ($p < 0.01$; Figure 2A). lSSc patients with duration < 2 years also had significantly elevated IL-23 levels compared to those with duration of 2–5 years ($p < 0.05$) and those with duration > 5 years ($p < 0.01$; Figure 2B). Thus, serum IL-23 levels were elevated in early-phase SSc. These results were independent of steroid therapy, since similar results were obtained from patients using no steroids (Figure 2B).

DISCUSSION

This is the first study showing that serum IL-23 levels were elevated in patients with SSc. We found that IL-23 elevation was related to early-stage SSc and the presence of pulmonary fibrosis, but not to other clinical features of SSc. IL-23 has been suggested to be involved in the induction of autoimmunity, including EAE, collagen-induced arthritis, and inflammatory bowel disease[13-15]. In SSc, chronic T cell activation characterized by increased T cell-associated cytokines is thought to contribute to tissue inflammation[1]. Since recent studies suggest that IL-23 can differentiate activated T cells into effector T cells, IL-23 may be associated with the process of T cell activation in SSc[10,11,16].

Dysregulated balance of Th1/Th2 has been suggested in various immune diseases[4]. In SSc, it has been reported that serum concentrations of Th2 cytokines such as IL-4, IL-6, IL-10, and IL-13 were increased[28-30]. Th2 cytokine production by stimulated peripheral blood lymphocytes was also elevated in SSc. Further, SSc patients exhibited Th2 cytokine production by cultured CD4+ T cells isolated from skin lesions. These findings have suggested Th2 dominancy in SSc. However, activation of Th1 responses has been reported in SSc, as well. Serum levels and spontaneous production of IL-12, a potent inducer of Th1 cells, by circulating lymphocytes were elevated in SSc[6]. Moreover, there was a report that both Th1 and Th2 responses were activated in SSc[31]. Therefore, it remains unclear which T cell response, Th1, Th2, or other type of response, is predominant in SSc. Th17, a newly discovered subset of CD4+ T cells, is induced by IL-23 in the presence of IL-6 and TGF-ß[16,17]. Th17 produces high amounts of IL-17[32]. This IL-17 has been observed to cause inflammatory responses in autoimmune diseases[32,33]. Kurasawa, *et al* have described elevated circulating IL-17 levels in SSc, suggesting the relationship between Th17 and SSc[34]. In their study, IL-17 was overproduced by T cells from the peripheral blood and fibrotic lesions in patients with SSc. Th17 cells may cause vascular injury and fibrosis as well as autoimmunity in SSc, since IL-17 receptor is expressed on fibroblasts and endothelial cells[34]. Consistently, IL-17 can cause fibroblast proliferation, and upregulation of cell adhesion molecules on endothelial cells[34]. Thus, it is possible that IL-23-induced Th17 cells secrete IL-17, which causes autoimmunity, vascular injury, and fibrosis in patients with SSc. Since IL-17 is significantly

related to the early stage of SSc but not to other clinical features of SSc, IL-17 is suggested to play an important role in the induction of SSc[34]. Similarly, in our study, shorter duration from disease onset and the prevalence of pulmonary fibrosis were related to the elevation of IL-23, although IL-23 was not associated with the extent of skin sclerosis. Thus, our study and previous investigations suggest that Th17 is related to the induction of SSc, but not to progression or maintenance of the disease.

There are some limitations of this study. A larger study as well as a longitudinal study will provide more meaningful information about the relationship between IL-23 and SSc. Recently, several immune suppressive drugs, including cyclophosphamide, have been implicated in certain effects, by mechanisms unknown, on inflammation and fibrosis in SSc[35]. In this regard, the serial changes of IL-23 levels during treatment are a very important focus of the issue. Our investigation, a cross-sectional study with only 4 steroid-treated patients, did not determine differences in IL-23 levels before and after excluding patients using steroid therapy. Thus, more studies are necessary to clarify the association between IL-23 and SSc. Further, the behavior of Th17 cells in autoimmune diseases is not fully understood. For example, there is technical difficulty in identifying or evaluating the activation of Th17 cells in human subjects. It remains unclear how Th17 induces autoimmune disorders, including SSc. The relationship between Th17 and other T cell subsets, including Th1/Th2 and regulatory T cells, remains unknown. It is also unknown how TGF-ß and IL-6 relate with Th17 in patients with SSc. Since IL-23 causes inflammation through IL-17-dependent and IL-17-independent pathways[36], the relationship between IL-23 and IL-17 in SSc should also be clarified. However, since there are few established basic therapies for skin sclerosis and lung fibrosis in SSc, new therapeutic agents have been investigated. The regulation of Th17 cells by IL-23, a specific upstream cytokine of Th17, might provide new insight for the therapy of patients with SSc.

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