Serum Interleukin 9 Levels Are Increased in Patients with Systemic Sclerosis: Association with Lower Frequency and Severity of Pulmonary Fibrosis

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ABSTRACT. ***Objective.*** To determine serum interleukin 9 (IL-9) levels and their clinical associations in patients with systemic sclerosis (SSc).

Methods. Serum IL-9 levels were examined by ELISA in 71 patients with SSc, 15 with systemic lupus erythematosus (SLE), 15 with dermatomyositis (DM), 39 with atopic dermatitis, and 28 healthy individuals.

Results. Serum IL-9 levels were significantly elevated in SSc patients (84.6 ± 76.0 pg/ml) compared with healthy individuals (40.4 ± 41.7 pg/ml; $p < 0.001$), and patients with SLE (50.7 ± 52.0 pg/ml; $p < 0.05$) or DM (50.6 ± 55.8 pg/ml; $p < 0.05$) or atopic dermatitis (41.8 ± 38.8 pg/ml; $p < 0.001$). Among SSc patients, there were no differences in serum IL-9 levels between those with limited cutaneous SSc and those with diffuse cutaneous SSc. Patients with SSc and raised IL-9 levels less often had pulmonary fibrosis and decreased percentage vital capacity than those with normal IL-9 levels. IL-9 levels were positively correlated with percentage vital capacity in patients with SSc.

Conclusion. Serum IL-9 level was increased in patients with SSc, and was associated with lower frequency and severity of pulmonary fibrosis in SSc. IL-9 could be a protective factor against the development of pulmonary fibrosis in this disease, and as such would be a possible therapeutic target. (First Release Aug 1 2011; J Rheumatol 2011;38:2193–7; doi:10.3899/jrheum.110268)

Key Indexing Terms:
CYTOKINE ELISA INTERLEUKIN 9
PULMONARY FIBROSIS SYSTEMIC SCLEROSIS

Systemic sclerosis (SSc) is a generalized connective tissue disorder characterized by sclerotic and vascular changes in the skin and various internal organs. It is generally regarded as an autoimmune disorder because of the presence of antinuclear antibodies. Although the pathogenesis of SSc remains unclear, many studies have suggested that cytokines or growth factors regulate its induction by stimulating the synthesis of extracellular matrix components, which may injure endothelial cells and modulate the function of leukocytes[1,2]. These cytokines or growth factors are produced in part by inflammatory cells infiltrating the affected tissues, such as skin or lungs, of patients with SSc[1,2,3].

An imbalance between Th (T helper) 1 and Th2 immune responses is considered to play an important role in auto-immune diseases. Although the Th1/Th2 imbalance in SSc appears to be complicated, SSc is mainly thought of as a Th2-dominant autoimmune disease. Serum levels of interleukin 4 (IL-4) and IL-13, both representative Th2 cytokines, are significantly elevated in patients with SSc compared with healthy individuals[4,5,6], with IL-4 levels gradually decreasing as skin sclerosis improves[7]. By contrast, serum levels of IL-12, a Th1-inducing cytokine, are lower in the early phase of SSc than in healthy individuals, but increase in later phases[7]. Lymphocytes from patients with clinically active SSc produce lower amounts of interferon-γ, a representative Th1 cytokine, than those from patients with clinically stable disease[8]. Further, it has recently been reported that serum IL-17 levels are significantly increased in patients with SSc[9], while IL-17 is also overproduced by T cells in the skin and lungs of SSc patients[10].

IL-9 is a T cell-derived pleiotropic cytokine that was initially identified as a Th2 cytokine[11]. A number of CD4-positive T cell subsets, named Th9, have recently been shown to share the capacity to secrete IL-9[12,13]. IL-9 targets cells of the lymphoid, myeloid, and mast cell lineages, as well as lung epithelial cells, and is likely to contribute to the development of allergic and autoimmune diseases such as asthma, silica-induced lung fibrosis, and experimental autoimmune encephalomyelitis[14,15,16,17]. However, it is unclear whether IL-9 exerts mainly proinflammatory or antiinflammatory activities. We suggest that it plays a role in the pathogenesis

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of SSc; in this study, we examined serum IL-9 levels in patients with SSc, and evaluated the results with respect to clinical features.

MATERIALS AND METHODS

Patients. Serum samples were obtained from 71 Japanese patients with SSc (58 women, 13 men) with a mean age of 51 years (range 14–77 yrs). All patients fulfilled criteria for SSc proposed by the American College of Rheumatology (ACR)[18]. Patients were classified[19] into limited cutaneous SSc (lcSSc; n = 29) and diffuse cutaneous SSc (dcSSc; n = 42) groups. The mean disease duration was 4.8 ± 6.8 years (range 0.2–32 yrs). Duration was calculated from the time of onset of the first clinical event (other than Raynaud's phenomenon) that was a clear manifestation of SSc.

Fifteen patients with systemic lupus erythematosus (SLE) who fulfilled the ACR criteria[20], 15 with dermatomyositis (DM) who fulfilled the criteria of Bohan and Peter[21], and 39 patients with atopic dermatitis (AD) who fulfilled the proposed AD criteria[22] acted as disease controls. Twenty-eight age- and sex-matched healthy Japanese individuals served as healthy controls.

Antinuclear antibodies were identified by indirect immunofluorescence using HEp-2 cells as the substrate, and autoantibody specificities were further assessed by ELISA and immunoprecipitation.

At the first visit, 5 patients had been treated with low-dose steroids (prednisolone 5–20 mg/day) and 4 with low-dose D-penicillamine (100–500 mg/day). None of the patients had received immunosuppressive treatment.

Clinical assessment. Complete medical histories, physical examinations, and laboratory tests were conducted for all patients at their first visit. Organ system involvement was defined as described[23,24]. Lung involvement (bibasilar fibrosis) was observed using high-resolution computed tomography (HRCT); esophagus hypomotility was determined by barium radiography; heart involvement was indicated as pericarditis, congestive heart failure, or arrhythmias requiring treatment; kidney involvement was indicated by malignant hypertension and rapidly progressive renal failure with no other explanation; and proximal muscle weakness and elevated serum creatine kinase indicated muscle involvement. Pulmonary fibrosis was defined as bibasilar interstitial fibrosis on chest HRCT. In addition, a pulmonary function test (PFT), including vital capacity (VC) and DLCO, was evaluated to examine the severity of pulmonary fibrosis. When DLCO and VC were < 75% and < 80%, respectively, of the predicted normal values, they were considered abnormal. Patients with SSc who were smokers or had other respiratory disorders that could have affected %DLCO or %VC were excluded from our study. Sixty-nine of 71 patients with SSc had pulmonary function test data available. Pulmonary artery pressure was estimated by Doppler echocardiogram. The modified Rodnan total skin thickness score (TSS) was measured by summing skin thickness measurements, and determined by palpation on a 0–3 scale in 17 body areas[25].

The study protocol was approved by the Nagasaki University Graduate School of Biomedical Sciences and Nagasaki University Hospital, and informed consent was obtained from all patients.

ELISA. Fresh venous blood samples were drawn into pyogen-free blood collection tubes without additives, immediately immersed in melting ice, and allowed to clot 1 h before centrifugation. Only sera were separated. All serum samples were stored at –70°C before use. All these procedures were standardized. Serum IL-9 levels were measured with specific ELISA kits (BioLegend, San Diego, CA, USA), according to the manufacturer's protocol. Each sample was tested in duplicate. The detection limit of this assay was 0.5 pg/ml. Serum levels of Krebs von den Lungen (KL)-6 and surfactant protein-D (SP-D), both serological markers of pulmonary fibrosis, were investigated using specific ELISA kits (Eitest KL-6, Eisai, Tokyo, Japan, and SP-D kit, Yamasa, Chiba, Japan, respectively), according to the manufacturer's protocol.

Statistical analysis. The Mann-Whitney U test was used to compare IL-9 levels, the Fisher's exact probability test to compare frequencies, and Bonferroni's test for multiple comparisons. Spearman's rank correlation coefficient was used to examine the relationship between 2 continuous variables. Nonlinear regression was used to examine the relationship between independent variables and their associated observed dependent variables. A probability value of $p < 0.05$ was considered significant.

RESULTS

Antinuclear antibodies. Antitopoisomerase I antibodies were positive in 25 patients, anticentromere antibodies in 26, anti-RNA polymerase I and III antibodies in 6, anti-U1RNP antibodies in 4, anti-U3RNP antibodies in one, anti-Th/To antibodies in one, and 8 patients had negative results for these antibodies.

Serum IL-9 levels are elevated in patients with SSc. Serum IL-9 levels at the first visit were significantly higher in SSc patients (84.6 ± 76.0 pg/ml) than in healthy individuals (40.4 ± 41.7 pg/ml; $p < 0.001$; Figure 1), SLE patients (50.7 ± 52.0 pg/ml; $p < 0.05$), DM patients (50.6 ± 55.8 pg/ml; $p < 0.05$), and AD patients (41.8 ± 38.8 pg/ml; $p < 0.001$). Concerning SSc subgroups, IL-9 levels in patients with dcSSc (76.9 ± 67.5 pg/ml) and lcSSc (95.8 ± 86.8 pg/ml) were increased compared with healthy individuals ($p < 0.01$ and $p < 0.01$, respectively). There was no significant difference in serum IL-9 levels between dcSSc patients and lcSSc patients.

Increased IL-9 levels are associated with a lower frequency and severity of pulmonary fibrosis. Clinical and laboratory variables obtained at the first evaluation were compared between SSc patients with elevated IL-9 levels and those with normal IL-9 levels. Values higher than the mean + 2 SD (123.9 pg/ml) of control serum samples were considered to be elevated in this study. Elevated IL-9 levels were observed in 21% (15/71) of all SSc patients, 17% (7/42) of dcSSc patients, and 28% (8/29) of lcSSc patients. As shown in Table 1, the frequency of pulmonary fibrosis and decreased %VC in SSc patients with elevated IL-9 levels was significantly lower than in those with normal IL-9 levels (27% vs 57%; $p < 0.05$; and 0% vs 33%; $p < 0.01$, respectively). Serum IL-9 levels did not correlate with disease duration and the presence of antitopoisomerase I antibody and anticentromere antibody. Further, there was no significant difference in serum IL-9 levels between patients with disease duration < 2 years and those with disease duration > 2 years (data not shown).

Serum IL-9 levels were positively correlated with %VC in patients with SSc ($r^2 = 0.32$, $p < 0.01$; Figure 2). Further, we investigated correlations of serum IL-9 levels with serum KL-6, which is a mucin-like high molecular weight glycoprotein[26] and a marker of pulmonary fibrosis in patients with SSc[27], and SP-D, which is produced and secreted by alveolar type II pneumocytes in alveoli and Clara cells and is also a marker of pulmonary fibrosis in patients with SSc[28]. Serum IL-9 levels correlated inversely with serum KL-6 levels ($r = 0.51$, $p < 0.01$) and SP-D levels ($r = –0.42$, $p < 0.05$) determined by ELISA. Consistent with these findings, IL-9 levels were significantly lower in SSc patients with pulmonary fibrosis observed using HRCT and decreased %VC (Figure 3). Further, SSc patients who had both pulmonary fibrosis

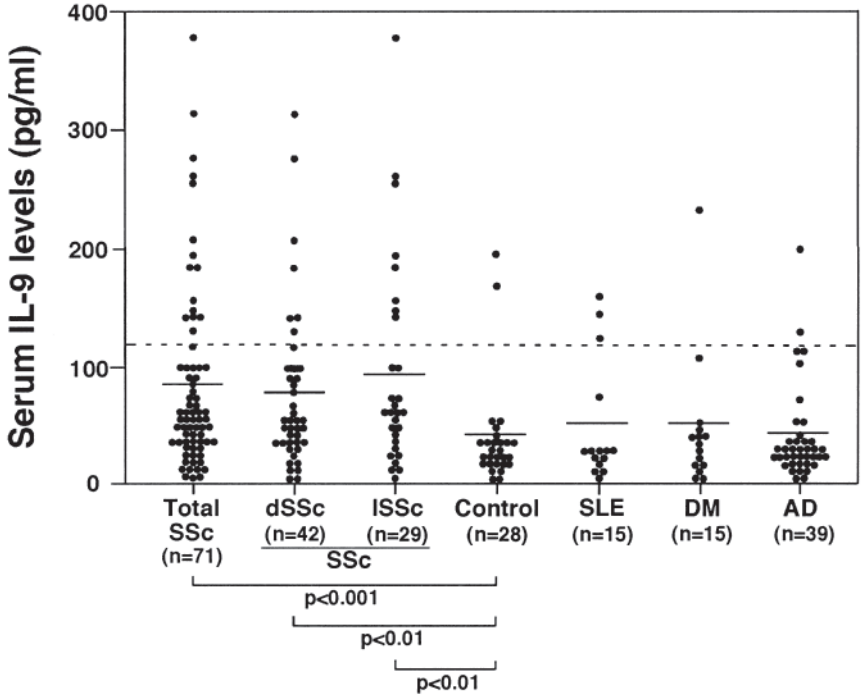

Figure 1. Serum interleukin (IL)-9 levels in patients with systemic sclerosis (SSc), diffuse cutaneous SSc (dcSSc), limited cutaneous SSc (lcSSc), systemic lupus erythematosus (SLE), dermatomyositis (DM), atopic dermatitis (AD), and healthy individuals (Control). Serum IL-9 levels were determined by a specific ELISA. Bars indicate mean values in each group. Broken line indicates the cutoff value (mean + 2 SD of control values).

using HRCT and abnormal PFT (decreased %VC and/or decreased %DLCO) had significantly lower IL-9 levels. Thus, raised IL-9 levels were associated with a lower frequency and severity of pulmonary fibrosis.

DISCUSSION

This is the first report of elevated serum IL-9 levels in patients with SSc (Figure 1). IL-9 levels were raised not only in patients with dcSSc, but also in those with lcSSc, and were associated with a lower prevalence of pulmonary involvement and better pulmonary function (Table 1, Figures 2 and 3). Thus, elevated IL-9 levels may be protective against the development of pulmonary fibrosis in SSc.

IL-4 production is enhanced by silica administration, which also induces Th2 polarization[17]. As serum IL-4 levels are raised in SSc patients[4,5,6] and contribute to the differentiation of naive CD4-positive T cells into Th9 cells together with transforming growth factor-ß (TGF-ß)[12,13], a Th2-polarized condition is likely to drive IL-9 production. Moreover, IL-9 overexpression reduces the severity of silica-induced lung fibrosis in mice[17]. Our data suggest that IL-9 may play a protective role in the development of pulmonary fibrosis. It has also been demonstrated that IL-9 in combination with TGF-ß increases the production of Th17 cells[16]. Although serum IL-17 levels are increased in patients with SSc, elevated IL-17 levels tend to correlate with a lower frequency and severity of lung fibrosis, suggesting that IL-17 production in SSc serves as a protective factor[9]. Interestingly, IL-9 also enhances the suppressive function of regulatory T cells[16]. Thus increased IL-9 may induce IL-17 production and regulatory T cell activation, thereby inhibiting the development of pulmonary fibrosis in SSc.

It has been shown that overexpression of IL-9 enhances the recruitment of B cells in the lung in murine silica-induced pulmonary fibrosis[17]. Further, B cell deficiency abolishes the protective effect of IL-9[29]. Therefore, IL-9 is likely to have a protective role in the development of lung fibrosis through the recruitment of B cells. Moreover, the numbers of B cells are elevated in the lungs of SSc patients with interstitial lung disease[30] and recent studies show that B cells and specific B cell subsets can negatively regulate immune responses, validating the existence of regulatory B cells[31,32]. These findings suggest that IL-9 may play a key role in the regulation of lung involvement in SSc by enhancing regulatory B cell recruitment in the lungs. Further studies examining the contribution of IL-9 to the regulation of interstitial lung disease and other organ involvement in SSc are required. Moreover, it is essential to examine the longitudinal changes of serum IL-9 levels in patients with SSc and to assess the association with disease activity. Nonetheless, the results of our study suggest that the administration of IL-9 might be a possible treatment in patients with SSc who have severe interstitial lung disease.

Table 1. Clinical and laboratory findings among systemic sclerosis (SSc) patients with elevated serum IL-9 levels. Unless noted otherwise, values are percentages.

	Elevated IL-9, n = 15	Normal IL-9, n = 56
Age at onset, yrs, mean ± SD	44 ± 9	47 ± 14
Male:female	3:12	10:46
Disease duration, yrs, mean ± SD	4.5 ± 7.1	4.8 ± 6.8
TSS, points, mean ± SD	10.9 ± 7.7	14.2 ± 9.6
Clinical features		
dcSSc	47	63
lcSSc	53	37
Pitting scars/ulcers	33	41
Contracture of phalanges	45	33
Diffuse pigmentation	53	50
Telangiectasia	47	39
Organ involvement		
Pulmonary fibrosis	27*	57
Decreased % VC	0**	33
Decreased % DLCO	40	63
Pulmonary hypertension	20	13
Esophagus	60	48
Heart	0	5
Kidney	0	2
Joint	13	14
Muscle	7	5
Laboratory findings		
Antitopoisomerase I antibody	33	38
Anticentromere antibody	47	36
Increased IgG	40	29
Elevated ESR	47	42
Elevated CRP	13	13

* $p < 0.05$, ** $p < 0.01$ vs SSc patients with normal serum IL-9 levels. ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; TSS: Rodnan total skin thickness score; dcSSc: diffuse cutaneous SSc; lcSSc: limited cutaneous SSc; VC: vital capacity; IL; interleukin.

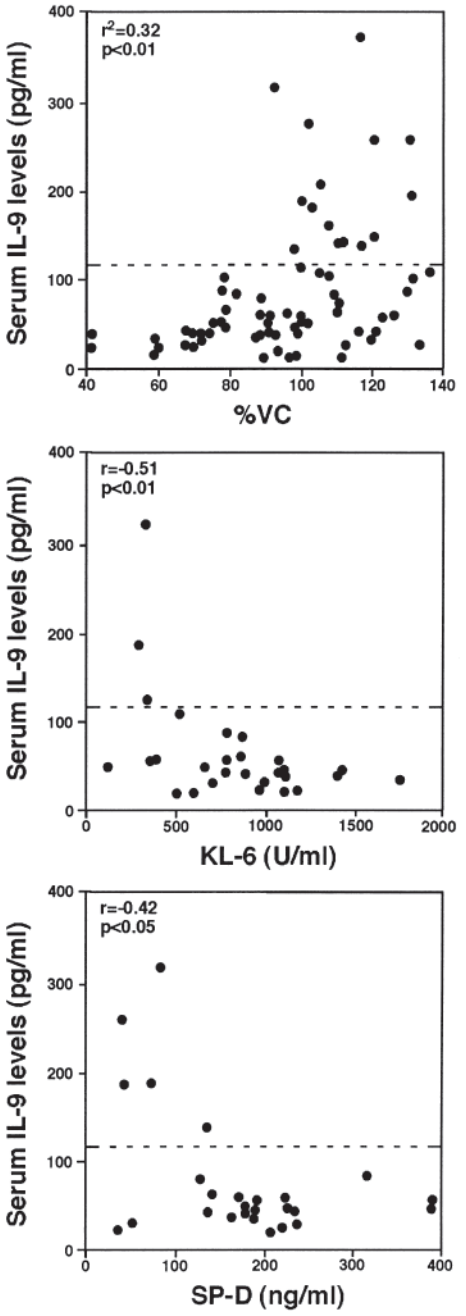

Figure 2. Correlation of serum interleukin (IL)-9 levels with percent vital capacity (VC), serum Krebs von den Lungen (KL)-6 levels, and surfactant protein-D (SP-D) levels in patients with systemic sclerosis. Serum IL-9, KL-6, and SP-D levels were determined by a specific ELISA. Broken line indicates the cutoff value of IL-9.

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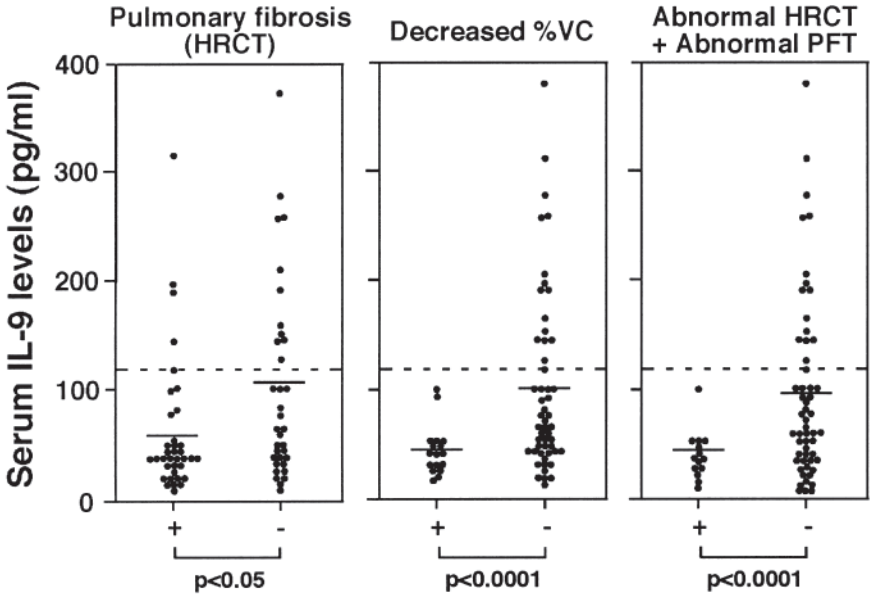

Figure 3. Serum interleukin (IL)-9 levels at first visit in the presence and absence of pulmonary fibrosis observed using high-resolution computed tomography (HRCT), decrease in percent vital capacity (VC), and both pulmonary fibrosis using HRCT and abnormal pulmonary function test (PFT; decreased %VC and/or DLCO). Bars indicate mean values in each group. Broken line indicates the cutoff value.

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