# Longitudinal Analysis of Serum Cytokine Concentrations in Systemic Sclerosis: Association of Interleukin 12 Elevation with Spontaneous Regression of Skin Sclerosis

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***ABSTRACT.*** ***Objective.*** Skin sclerosis that progresses in the earlier disease phase in systemic sclerosis (SSc) spontaneously regresses thereafter. We investigated the relationship between changes of the serum cytokine profile and changes in skin fibrosis in patients with SSc.

***Methods.*** Serum cytokine levels were examined by ELISA using 180 sera samples from 26 patients with early diffuse cutaneous SSc (dcSSc) with mean disease duration of 2.1 years. The mean followup period was 4.9 years (range 2–8). Cytokine mRNA expression in the affected skin was quantified by real-time reverse transcription-polymerase chain reaction.

***Results.*** Modified Rodnan total skin thickness score decreased after 2, 4, and 6 years compared to that at first visit. Serum levels of the Th2 cytokines interleukin 6 (IL-6) and IL-10 and monocyte chemotactic protein-1 (MCP-1) were higher at first evaluation compared to healthy controls, while IL-4 levels were normal. Levels of all Th2 cytokines generally decreased as skin sclerosis regressed. Conversely, levels of serum IL-12, a Th1-inducing cytokine, were lower at first visit relative to controls, but increased by roughly 15-fold after 6 years to significantly higher levels than controls. Surviving dcSSc patients exhibited elevated IL-12 levels compared to deceased patients. Serum levels of transforming growth factor-$ß_1$ (TGF-$ß_1$), a fibrogenic cytokine, increased throughout followup, with slightly decreased levels at later timepoints. IL-12 mRNA expression was upregulated in affected skin from patients with late-stage dcSSc, while TGF-$ß_1$ and MCP-1 expression was downregulated.

***Conclusion.*** These results suggest that a shift from Th2 to Th1 response correlates with improvement in skin fibrosis in SSc, and that IL-12 level is a serologically useful marker for disease activity and prognosis. (J Rheumatol 2006;33:275–84)

*Key Indexing Terms:*
SYSTEMIC SCLEROSIS Th1 Th2 INTERLEUKIN 12

Systemic sclerosis (SSc) is a multisystem disorder of connective tissue characterized by excessive fibrosis in the skin and various internal organs, with an autoimmune background. Although the pathogenesis of SSc remains unknown, immunologic abnormalities have been suggested to play an important role. Most of the infiltrating cells in the skin of patients with SSc are activated T lymphocytes with a predominant CD4+ phenotype[1]. Hyperactivity of circulating CD4+ T cells has also been detected in patients with SSc, since they show augmented CD25 expression[2]. Cytokines play a major role in regulating extracellular matrix deposition by fibroblasts[3]. Stimulated naive T cells 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]. It has been suggested that Th1 cytokines generally decrease extracellular matrix deposition, whereas Th2 cytokines increase it[3]. Thus, cytokines produced by activated T cells may regulate fibrosis associated with SSc.

It has been reported that serum concentrations of Th2 cytokines such as IL-4, IL-6, IL-10, and IL-13 are increased in SSc[5-8]. Th2 cytokine production by stimulated peripheral blood lymphocytes is also augmented in SSc[6,9]. In addition, SSc patients exhibit Th2 cytokine production by cultured CD4+ T cells isolated from affected skin. Serum levels of soluble CD30, which reflects Th2 cell activation, are also

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*Accepted for publication September 20, 2005.*

increased in SSc, and expression of IL-4 mRNA and CD30 by mononuclear cells that infiltrate the affected skin is elevated[10,11]. These findings suggest that Th2 responses are predominant in SSc. However, activation of Th1 response in SSc has also been reported: serum levels and spontaneous production of IL-12, a potent inducer of Th1 cells, by circulating lymphocytes are elevated in SSc patients[12]. Moreover, studies have suggested mixed activation of Th1 and Th2 responses in SSc: a study using intracellular cytokine analysis at the single-cell level has revealed Th1 polarization of γ/δ T cells in addition to Th2 predominance of α/ß T cells[13]; another showed increased numbers of IFN-γ-producing CD8+ T cells in SSc[14]. Further, cells expressing Th1 cytokine mRNA (IFN-γ and IL-2) as well as those expressing Th2 cytokine mRNA (IL-4 and IL-5) were found to be increased in the lung interstitium of SSc patients with fibrosing alveolitis, while the ratios of Th1 to Th2 cytokines were similar to healthy controls[15]. Yet it remains unknown which T cell response, Th1 or Th2, is predominant in SSc.

It was previously thought that the risk of internal organ involvement in patients with diffuse cutaneous SSc (dcSSc) increased in parallel with the disease duration[16]. In 1986, however, spontaneous improvement in skin sclerosis was first reported as "regressive scleroderma"[17]. Clinical trials have consistently revealed that skin involvement spontaneously improves even in untreated patients[18]. A recent study described that severe organ damage occurs within the first 3 years of disease onset in most patients with dcSSc, and progression to severe skin thickening seldom occurs after 5 or 6 years[16]. Collectively, these findings indicate that SSc is not progressive, but has a distinct early phase of higher disease activity. Although the reasons for the discrepancy among previous studies regarding the Th1/Th2 imbalance in SSc are not clear, it may be due to qualitative differences in the patient populations, especially with regard to disease activity. Almost all previous studies analyzed cytokine levels at only one timepoint during the disease course in SSc. Therefore, to clarify the role of the cytokine imbalance in the development of SSc, it is necessary to assess cytokine profiles according to disease duration and activity. We performed a retrospective longitudinal study of serum cytokine levels in patients with early dcSSc. Our results indicate that a shift from a Th2 response to a Th1 response is associated with the improvement of cutaneous involvement in dcSSc.

## MATERIALS AND METHODS

*Patients*. Serum samples were obtained from 26 Japanese patients with SSc (22 women, 4 men). All patients fulfilled the criteria for SSc proposed by the American College of Rheumatology[19] and were classifed as having dcSSc according to the system proposed by LeRoy, *et al*[20]. Antitopoisomerase I antibodies were positive for 20 patients, anticentromere antibodies for 2, and anti-RNA polymerases I and III antibodies for 4. Their average age (mean ± SD) was 48 ± 20 years and the mean disease duration was 2.1 ± 1.0 years (range 0.2–3). Disease duration was calculated from the time of the first clinical event (other than Raynaud's phenomenon) that was a clear manifestation of SSc. Fifteen age and sex matched healthy Japanese individuals (12 women, 3 men; age 43 ± 12 yrs) were recruited as healthy controls.

In a retrospective longitudinal study, we analyzed 180 serum samples from an identical patient population of 26 patients with early dcSSc. The followup period was 4.9 ± 1.9 years (range 2–8) with 7 ± 2 (range 5–11) assessment timepoints. At the first visit, 5 patients were treated with low dose corticosteroid (prednisolone, 5–10 mg/day). Afterward, 18 additional patients received a low dose oral steroid (prednisolone, 5–20 mg/day), while the remaining 3 patients did not receive oral steroid. In addition to steroid treatment, 8 patients were treated with low dose D-penicillamine (100–200 mg/day) at the first visit. Afterward, 5 additional patients received low dose D-penicillamine (100–200 mg/day) in addition to steroid treatment. No patient received other immunosuppressive therapy. Complete medical histories, physical examinations, and laboratory tests were conducted on all patients at their first visit, with limited evaluations during followup visits. The modified Rodnan total skin thickness score (TSS) was assessed as described[21]. Fresh venous blood samples were centrifuged shortly after clot formation and all samples were stored at –70°C before use. The protocol was approved by the Kanazawa University Graduate School of Medical Science and Kanazawa University Hospital, and informed consent was obtained from all patients.

*Detection of soluble mediators*. Specific ELISA kits were used to measure serum concentrations of IL-2, IL-4, IL-6, IL-10, IL-12, monocyte chemotactic protein-1 (MCP-1; PharMingen, San Diego, CA, USA), tumor necrosis factor-α (TNF-α; R&D Systems, Minneapolis, MN, USA), and transforming growth factor-$ß_1$ (TGF-$ß_1$; GT, Minneapolis, MN, USA) according to the manufacturer's protocols. IL-12p70 consists of 2 disulfide-linked chains of 40 kDa and 35 kDa[22]. An ELISA for IL-12 measured the entire IL-12p70 molecule. Each sample was tested in duplicate. The detection limits of the assays were as follows: IL-2, 1.0 pg/ml; IL-4, 2.0 pg/ml; IL-6, 4.0 pg/ml; IL-10, 2.0 pg/ml; IL-12, 4.0 pg/ml; MCP-1, 1.0 pg/ml; TNF-α, 4.4 pg/ml; and TGF-$ß_1$, 7.0 pg/ml.

*RNA isolation and real-time polymerase chain reaction (RT-PCR)*. Skin biopsy specimens were obtained from the forearm of 9 patients with dcSSc (7 women, 2 men, aged 40 ± 15 yrs). Five of these patients were grouped into a category of early dcSSc with a disease duration < 3 years, while 4 patients were grouped as late dcSSc, with a disease duration > 6 years. No early dcSSc patient received therapy. All late dcSSc patients received a low dose oral steroid (prednisolone, 5–20 mg/day). Five age and sex matched healthy Japanese (3 women, 2 men, aged 39 ± 10 yrs) served as healthy controls. All skin samples were snap-frozen in liquid nitrogen and stored at –80°C before use. Total RNA was isolated from frozen tissue with Qiagen RNeasy spin columns (Qiagen Ltd., Crawley, UK) and then was reverse-transcribed into cDNA according to the protocol for the Reverse Transcription System (Promega, Madison, WI, USA). Expression of IL-12p35, TGF-$ß_1$, and MCP-1 mRNA was analyzed by RT-PCR quantification according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Sequence-specific primers and probes were designed by pre-developed TaqMan® assay reagents (Applied Biosystems). RT-PCR (one cycle at 50°C for 2 min, one at 95°C for 10 min; 40 cycles at 92°C for 15 s and at 60°C for 60 s) was performed on an ABI Prism 7000 sequence detector (Applied Biosystems). GAPDH was used to normalize the mRNA. To compare target gene and housekeeping GAPDH gene mRNA expression, the relative expression of PCR products was determined using the ΔΔCt method[23]. The fold induction equaled $2^{-[\Delta\Delta Ct]}$, where Ct = the threshold cycle, and ΔΔCt = [Ct gene interest (unknown sample) – Ct GAPDH (unknown sample)] – [Ct gene interest (calibrator sample) – Ct GAPDH (calibrator sample)]. One of the control samples was chosen as a calibrator sample. Each sample was examined in triplicate and the mean Ct was used in the equation.

*Statistical analysis*. The Mann-Whitney U test was used to determine the level of significance of differences between the sample means, and the Bonferroni test was used for multiple comparisons. A p value < 0.05 was considered statistically significant. All values are shown as mean ± SD.

## RESULTS

*Longitudinal change in serum Th2 cytokine levels in SSc*. To determine changes in serum cytokine levels during followup in patients with SSc, the levels were studied at the first visit and after 2, 4, and 6 years. Four SSc patients had been followed for 2 years, 10 for 4 years, and 12 for 6 years. All patients had early dcSSc with a mean disease duration of 2.1 years (range 0.2–3). First, changes in skin sclerosis during the followup period were assessed by the modified Rodnan TSS (Figure 1). In comparison with the modified Rodnan TSS at the first visit, the skin score was significantly decreased after 2 years (35% decrease; $p < 0.0005$), 4 years (48% decrease; $p < 0.0001$), and 6 years (52% decrease; $p < 0.0005$). Serum levels of IL-6 and IL-10, which belong to the Th2 cytokines, at the first visit were significantly 9 to 12-fold higher in SSc patients than levels in controls ($p < 0.05$ and $p < 0.01$, respectively; Figure 2). However, IL-6 levels decreased by 6 years after the first visit, while IL-10 levels were reduced after 4 years. In contrast to IL-6 and IL-10, serum levels of IL-4, another Th2 cytokine, in SSc patients at the first visit were similar to those in controls, whereas IL-4 levels after 4 and 6 years were significantly reduced by 60%–80% relative to controls ($p < 0.05$). Serum levels of MCP-1, a Th2 chemokine that induces Th2 cytokine expression[24], were significantly elevated by roughly 3-fold at all timepoints in SSc patients compared with controls ($p < 0.0001$). Although serum MCP-1 levels after 6 years tended to be decreased by 10% to 20% relative to those at earlier timepoints, there was no significant difference between any of the timepoints. Thus, in general, serum Th2 cytokine levels decreased as skin sclerosis improved in patients with dcSSc.

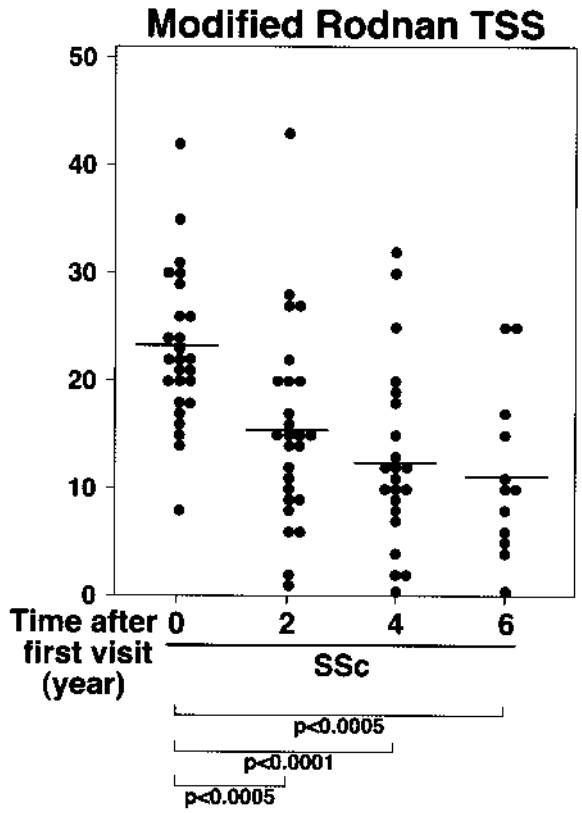


*Figure 1*. Changes in skin fibrosis during the followup period in patients with dcSSc. All patients had early dcSSc, with a mean disease duration of 2.1 years (range 0.2–3), and the mean followup period was 4.9 years (range 2–8). The extent of skin sclerosis was measured by modified Rodnan TSS at the first visit and after 2, 4, and 6 years. The 17 anatomic areas were rated as 0 (normal), 1+ (mild skin thickening), 2+ (moderate), and 3+ (severe) and the TSS was derived by summing scores from all areas. Horizontal bars indicate mean values.

*Longitudinal change in serum Th1 cytokine levels in SSc*. IL-12 polarizes undifferentiated T cells and commits them to the Th1 lineage, and reduces Th2 activity[22]. Further, IL-12 is the most potent regulator of Th1/Th2 cytokine balance *in vivo*[22]. Unlike Th2 cytokines, SSc patients exhibited significantly reduced serum IL-12 levels at the first visit (78% decrease) compared to controls ($p < 0.0005$; Figure 3). Although IL-12 levels after 2 years were increased in several patients and were 3.7-fold higher than at the first visit, IL-12 levels after 2 years in the total cohort of SSc patients remained significantly decreased by 17% relative to controls ($p < 0.05$). Serum IL-12 levels increased further after that: they were significantly 7.8-fold and 15.3-fold higher after 4 years ($p < 0.05$) and 6 years ($p < 0.0005$), respectively, than at the first visit. Further, IL-12 levels after 6 years were significantly increased by 3.4-fold relative to controls ($p < 0.05$).

The correlation between elevated serum IL-12 levels and mortality in SSc was also examined. Maximal serum IL-12 levels throughout the followup period in individual patients were selected for this analysis. Serum IL-12 levels in deceased SSc patients were significantly lower than those in surviving patients ($p < 0.05$; Figure 4).

IL-2 is a Th1 cytokine, but is also produced by undifferentiated precursor CD4+ T cells[4,25]. In contrast to IL-12, serum IL-2 levels at the first visit tended to be increased compared to those found in controls or at later timepoints in SSc patients; however, the differences were not statistically significant (Figure 3). Serum IL-2 levels decreased as the disease course progressed: they were significantly reduced after 4 years (66% decrease) and 6 years (83% decrease) relative to controls ($p < 0.05$). Serum IFN-γ levels could not be detected in any SSc patients or controls (data not shown), as previously reported[5]. Thus, serum IL-12 levels increased as cutaneous involvement regressed, while serum IL-2 levels decreased. Further, the lack of increase in IL-12 levels throughout the followup period correlated with a higher mortality in patients with SSc.

*Longitudinal change in serum levels of other cytokines related to fibrosis in SSc*. TGF-ß stimulates the synthesis and assembly of matrix proteins such as fibronectin and collagen, and is involved in fibrosis associated with SSc[26]. Serum TGF-$ß_1$ levels were significantly elevated by 30%–40% at all timepoints in SSc patients compared with

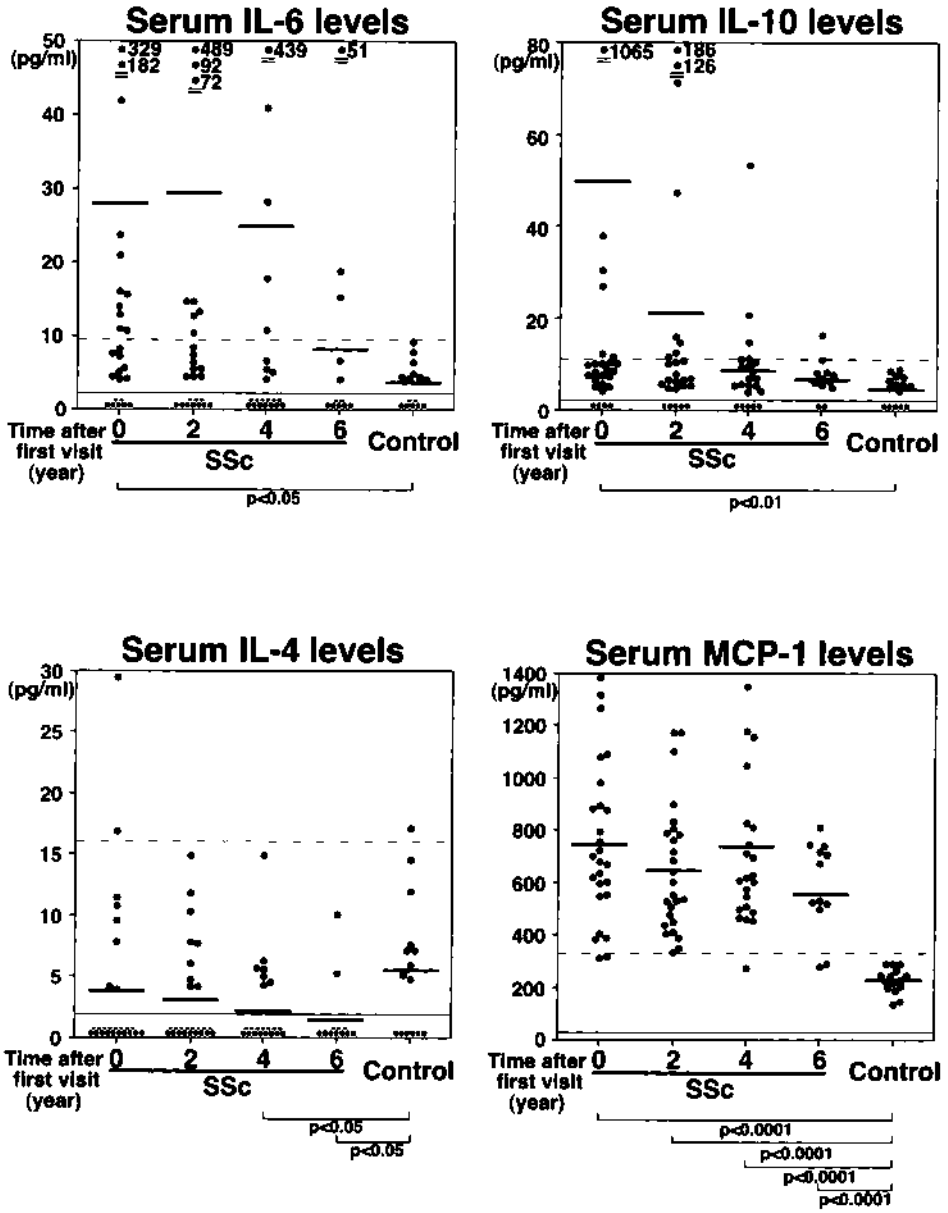


*Figure 2*. Changes in serum levels of Th2 cytokines (IL-6, IL-10, IL-4, and MCP-1) during the followup period. Cytokine levels were determined by ELISA at the first visit and after 2, 4, and 6 years. Healthy individuals served as controls. Horizontal bars indicate mean values; broken lines indicate the cutoff values (mean + 2 SD of control samples). Thin lines at the bottom of each figure indicate the detection limits.

controls ($p < 0.05$; Figure 3). Serum TGF-ß$_1$ levels after 2, 4, and 6 years tended to be decreased by roughly 10% relative to those at the first visit; however, there were no significant differences between any of the timepoints. TNF-α regulates fibrosis by inhibiting TGF-ß signaling in human fibroblasts[27]. Further, a recent study has shown that TNF-α inhibits collagen synthesis promoted by Th2 cells in SSc[28]. Although serum TNF-α levels were increased in several SSc patients, there was no significant difference between the groups. Thus, serum TGF-ß$_1$ levels were increased throughout the followup period, with slightly decreased levels at later timepoints.

*Longitudinal change in the frequency of elevated serum cytokine levels in SSc*. When serum levels higher than the mean + 2 SD of control samples were considered elevated in this study, the frequency of elevated cytokine levels was compared during the followup period (Table 1). The frequency of IL-12 elevation after 6 years was significantly increased compared to the first visit (42% vs 4%; $p < 0.01$). This was the only statistically significant difference when all frequencies of elevated cytokines were compared. Similar to the serum levels, the frequency of IL-6, IL-10, TGF-ß$_1$, and MCP-1 elevation tended to decrease as the disease progressed. The frequency of IL-2 and IL-4 elevation

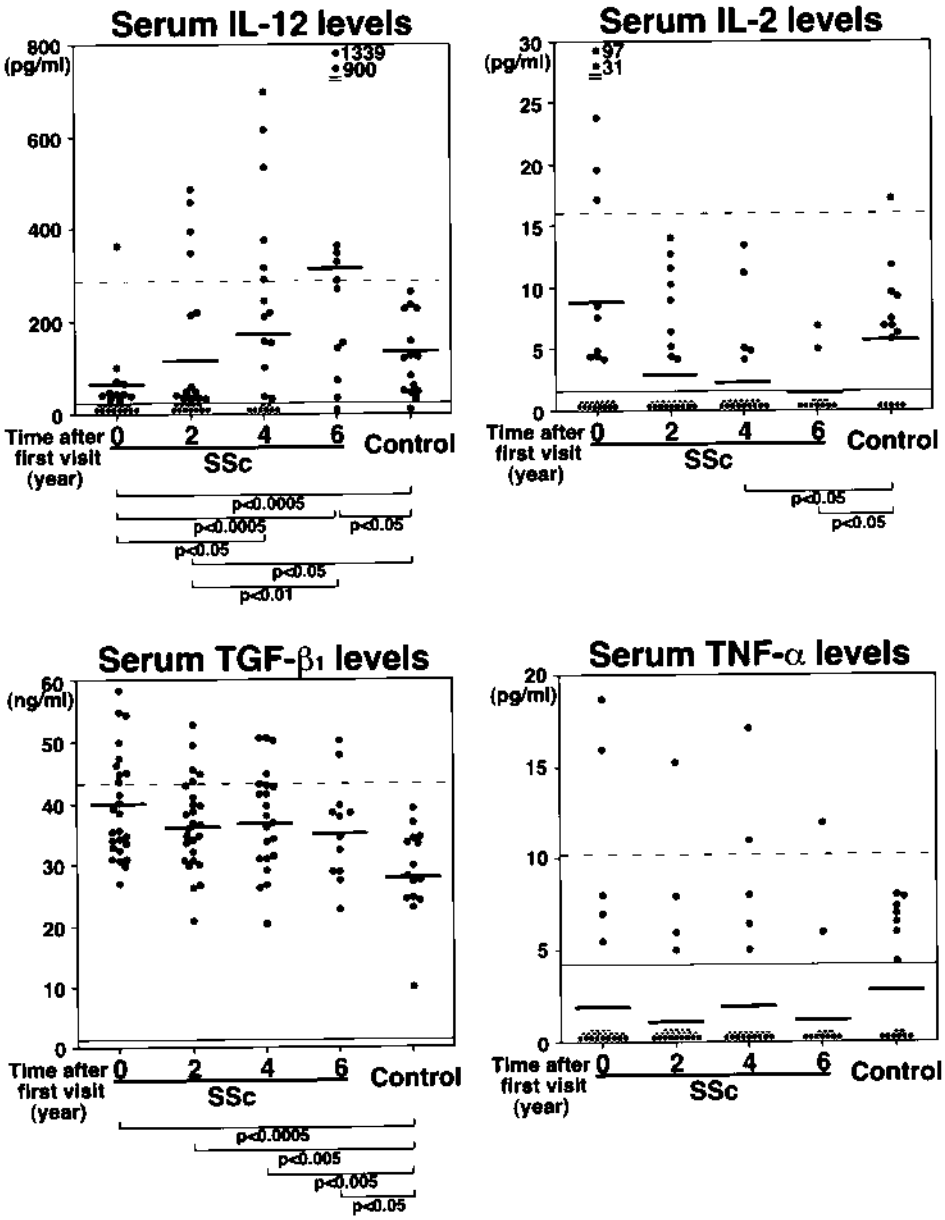


*Figure 3*. Changes in serum levels of Th1 cytokines (IL-12, IL-2) and cytokines related to fibrosis (TGF-ß$_1$, TNF-α) during the followup period. Cytokine levels were determined by ELISA at the first visit and after 2, 4, and 6 years. Healthy individuals served as controls. Horizontal bars indicate mean values; broken lines indicate cutoff values (mean + 2 SD of control samples). Thin lines indicate the detection limits.

was only detected in patients at the first visit (19% and 8%, respectively). The frequency of TNF-α elevation was similar and low throughout the followup period. Thus, the prevalence of IL-12 elevation was increased in the later stages of Ssc.

*Representative longitudinal change in serum cytokine levels in SSc*. Representative longitudinal changes of the modified Rodnan TSS and serum IL-10 and IL-12 levels are shown in Figure 5. At the first visit, a male patient (Case 1) with disease duration of 3 years had increased serum IL-10 levels, which were decreased 3–4 years later, in parallel with an improvement of skin fibrosis. In contrast, serum IL-12 was not detected at his first visit, but began to increase after 4 years, when skin sclerosis began to improve, with decreased levels of IL-10. He was treated with 10 mg/day prednisolone and 300 mg/day D-penicillamine just after his first visit. A similar pattern was observed for Case 2 (male patient, disease duration 2 yrs) and Case 3 (female patient, disease duration 1 yr). Both patients were treated with 10 mg/day prednisolone just after their first visit. In contrast, for Case 4 (a female patient, disease duration 1 month), the modified Rodnan TSS did not decrease throughout the followup period, during which slightly elevated serum IL-10 levels were

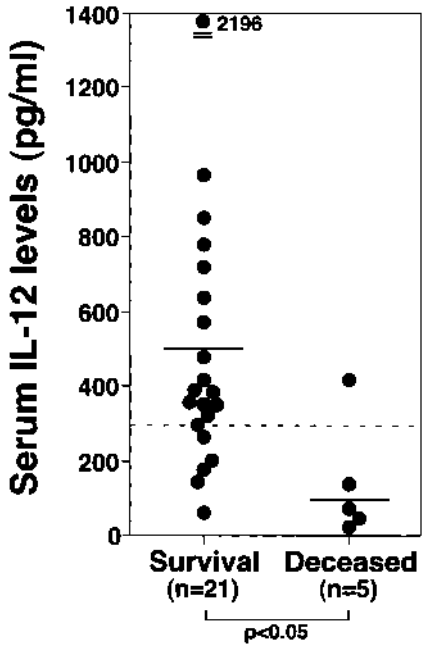


*Figure 4.* Correlation of elevated serum IL-12 levels with mortality in patients with early dcSSc. Serum IL-12 levels were determined by ELISA at all timepoints throughout the followup period. Maximal serum IL-12 levels for individual patients were selected for this analysis and were compared between surviving and deceased patients. Horizontal bars indicate mean values; broken line indicates the cutoff value (mean + 2 SD of control samples).

*Table 1.* The frequency of elevated serum cytokine levels in patients with dcSSc. Except for the modified Rodnan total skin thickness score (TSS), values are the percentages of patients with elevated serum cytokine levels.

| | Time After the First Visit, yrs | | | |
|---|---|---|---|---|
| | 0 (n = 26) | 2 (n = 26) | 4 (n = 22) | 6 (n = 12) |
| Modified Rodnan TSS | 23 ± 7 | 15 ± 9 | 13 ± 8 | 11 ± 8 |
| Cytokines | | | | |
| IL-2, pg/ml | 19 | 0 | 0 | 0 |
| IL-4, pg/ml | 8 | 0 | 0 | 0 |
| IL-6, pg/ml | 42 | 31 | 23 | 25 |
| IL-10, pg/ml | 23 | 27 | 18 | 8 |
| IL-12, pg/ml | 4 | 15 | 23 | 42* |
| TNF-α, pg/ml | 8 | 4 | 9 | 8 |
| TGF-ß$_1$, ng/ml | 35 | 23 | 27 | 17 |
| MCP-1, pg/ml | 92 | 100 | 96 | 83 |

* Frequency of elevated cytokine levels after 6 years was significantly different from that at the first visit ($p < 0.01$). IL: interleukin, TNF-α: tumor necrosis factor-α, TGF-ß$_1$: transforming growth factor-ß$_1$, MCP-1: monocyte chemoattractant protein-1.

observed, whereas serum IL-12 levels also did not increase. Treatment with 7.5 mg/day prednisolone began after her first visit. In 26 patients with dcSSc, 10 were treated with low dose steroid alone and 13 with low dose steroid and D-penicillamine. However, the serum cytokine levels did not correlate with treatment (data not shown). Thus, serum IL-12 levels were increased in parallel with the decrease in the modified Rodnan TSS and IL-10 levels.

*Cytokine mRNA expression in SSc affected skin.* To assess the correlation of disease duration with local cytokine expression in affected skin from patients with dcSSc, cytokine mRNA expression in the skin was quantified by RT-PCR. The mRNA expression of IL-2, IL-4, IL-6, IL-10, IFN-γ, and TNF-α was not detected in SSc affected or normal skin (data not shown), while IL-12p35, TGF-ß$_1$, and MCP-1 mRNA expression was detected in SSc affected skin. IL-12p35 mRNA expression was not detected in skin from patients with early SSc with a disease duration of less than 3 years or in normal skin (Figure 6A). In contrast, IL-12p35 mRNA was expressed in skin from patients with late SSc with a disease duration of more than 6 years. Affected skin from patients with early SSc showed fibrosis on histological examination, while skin from patients with late SSc exhibited modest or absent skin fibrosis (data not shown). Fibrotic skin from patients with early SSc showed expression of TGF-ß$_1$ and MCP-1 mRNA that was significantly decreased in affected skin from patients with late SSc ($p < 0.05$; Figure 6B, 6C). TGF-ß$_1$ and MCP-1 mRNA expression was not detected in the normal skin. Thus, local IL-12 expression was upregulated in the later stages of SSc, with downregulated TGF-ß$_1$ and MCP-1 expression.

## DISCUSSION

The most important finding in our study was the correlation of increased serum IL-12 concentrations with improved skin fibrosis. IL-12 was expressed only during the regression stage of SSc skin samples. Further, absence of increased IL-12 levels during the disease course correlated with a higher mortality in patients with SSc. IL-12 is produced by activated macrophages and dendritic cells and is the principal Th1-inducing cytokine[22]. Since IL-12 induces the secretion of IFN-γ, which reduces excessive collagen synthesis by SSc-derived fibroblasts[29], IL-12-induced IFN-γ production might be related to improvement in skin fibrosis. However, this is unlikely, since IFN-γ was not detected in the sera or affected skin of SSc patients (data not shown). Consistent with this, clinical trials of recombinant IFN-γ in treatment of SSc revealed that IFN-γ has only modest beneficial clinical effects[30,31]. IL-12 may inhibit skin fibrosis by suppressing Th2 differentiation, since Th2 cytokines generally enhance collagen production by fibroblasts[3]. Alternatively, IL-12 is an important negative regulator of TGF-ß production both *in vivo* and *in vitro*[32]; therefore, it may reduce skin fibrosis via the inhibition of TGF-ß production. TGF-ß expression was consistently downregulated in affected skin in the later stages of SSc. The importance of IL-12 in inhibition of skin fibrosis is also supported by a recent report that IL-12 administration to tight-skin mice, a murine model of SSc, had beneficial effects in reducing collagen accumulation in the skin[33]. Unlike IL-12, IL-2, another Th1 cytokine, decreased as skin sclerosis regressed. However, IL-2 production is not limited to Th1 cells, as it is produced by undif-

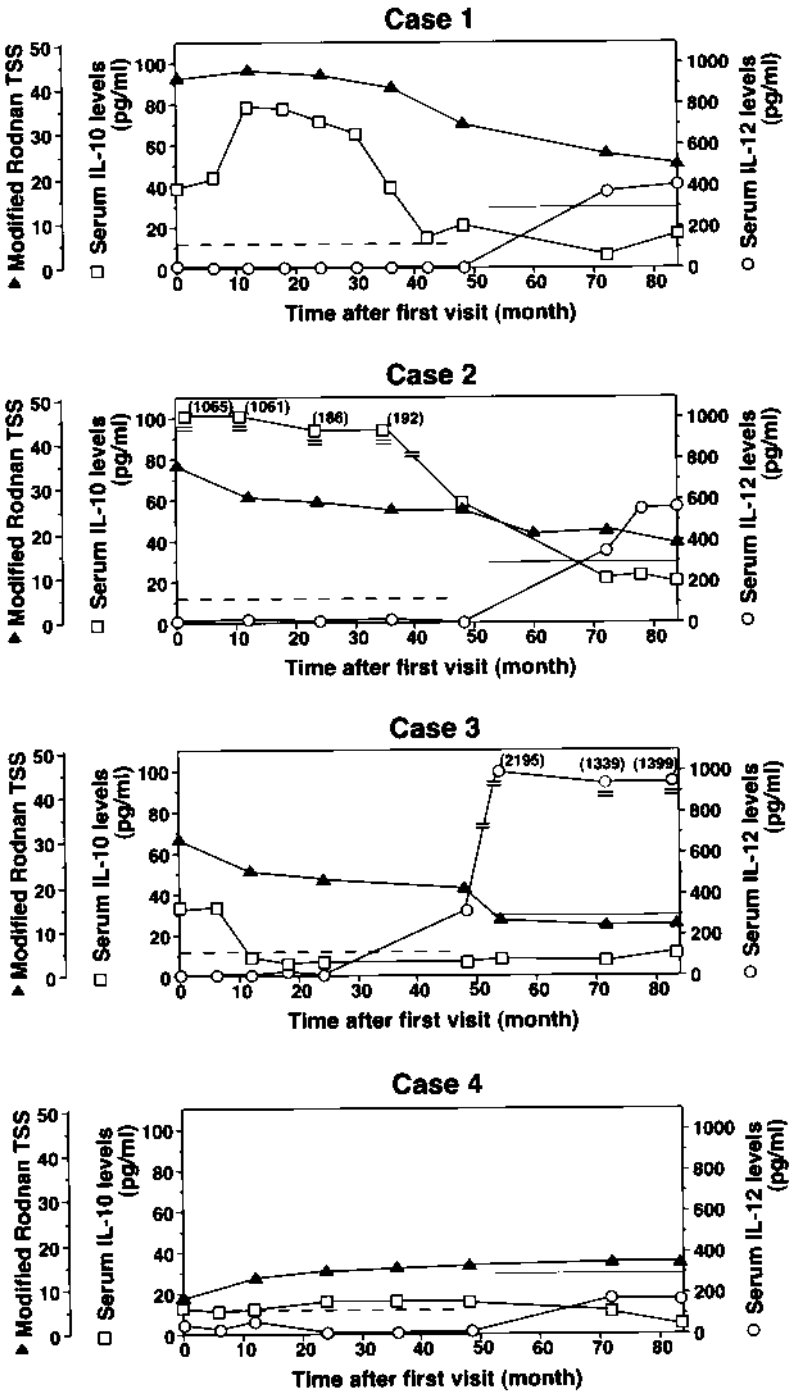


*Figure 5*. Representative longitudinal changes in skin fibrosis and serum levels of IL-10 and IL-12 in patients with dcSSc. Extent of skin sclerosis was measured by the modified Rodnan TSS (▲). Serum levels of IL-10 (pg/ml, □) and IL-12 (pg/ml, ○) were determined by ELISA. Broken lines indicate cutoff values of serum IL-10 levels; thin lines indicate cutoff values of serum IL-12 levels. Cutoff values represent the mean + 2 SD of control samples.

ferentiated precursor CD4+ T cells and enhances their proliferation[4,25]. Although the mechanisms by which IL-12 regulates fibrosis remain unknown, our results suggest that increased IL-12 production is related to the improvement of cutaneous fibrosis in SSc.

In contrast to IL-12, serum Th2 cytokine levels generally increased during the earlier stages of SSc, while they decreased as skin sclerosis improved. IL-4 stimulates collagen synthesis by fibroblasts derived from SSc[34]. Other studies report that serum IL-4 levels were elevated in SSc

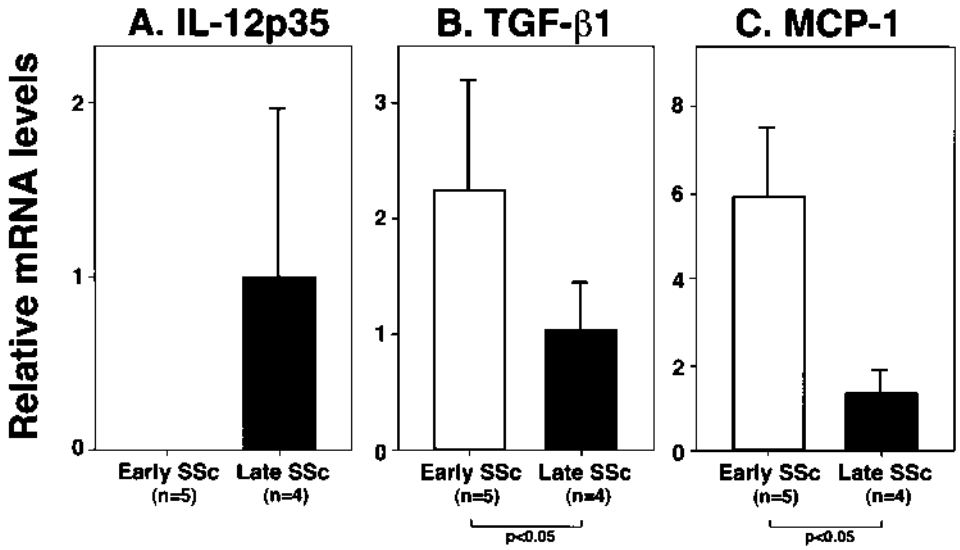


*Figure 6.* IL-12 (A), TGF-ß$_1$ (B), and MCP-1 (C) mRNA expression in dcSSc skin samples. Cytokine mRNA expression was quantified by RT-PCR in samples from 5 early dcSSc patients, with a disease duration < 3 years, and from 4 late dcSSc patients, with a disease duration > 6 years. Samples from early SSc patients showed fibrosis by histological examination, while samples from late SSc patients exhibited modest or absent skin fibrosis (data not shown).

patients[5]. However, in our study, serum IL-4 concentrations were normal at first visit, and decreased as skin sclerosis improved. This suggests that IL-4 reduction might only reflect suppressed Th2 polarization, since IL-12 suppresses IL-4 production[22]. Similarly to IL-4, IL-6 induces concentration-dependent increases in the production of collagen and glycosaminoglycans from human dermal fibroblast *in vitro*[35]. Further, IL-6 production by fibroblasts derived from affected SSc skin is augmented at protein and mRNA levels compared with normal fibroblasts[36,37]. Remarkably, blocking the IL-6 response by anti-IL-6 antibody results in a significant reduction in procollagen type I by cultured SSc fibroblasts[36]. Skin fibrosis in the tight-skin mouse is improved with a parallel decrease in IL-6 production[38]. In addition, serum IL-6 levels are elevated in patients with early-stage dcSSc[8], and correlate with the extent of skin fibrosis[39]. Unlike IL-4 and IL-6, IL-10 is suggested to downregulate type I collagen gene expression and enhance collagenase gene expression *in vitro*[40]. However, several reports have described the involvement of IL-10 in organ fibrosis *in vivo*[41,42]. Further, 2 studies have revealed that serum IL-10 levels are positively correlated with the extent of skin fibrosis in SSc[39,43]. Together, these results suggest that elevated production of Th2 cytokines in the early phase of SSc contributes to skin fibrosis. Consistent with this notion, high mRNA expression of Th2 cytokines produced by bronchoalveolar lavage cells is associated with a future decline in lung function[44,45].

MCP-1, an important chemotactic mediator of monocytes/macrophages, also functions as a Th2 chemokine because it stimulates IL-4, IL-5, and IL-10 production[24]. Our finding that serum MCP-1 levels were elevated throughout the followup period, especially during the earlier stages of SSc, suggests that MCP-1 contributes to Th2 polarization in SSc. In addition, fibrotic skin from patients with early SSc exhibited MCP-1 expression that was downregulated during the regression stage. MCP-1 is consistently expressed in inflammatory mononuclear cells, endothelial cells, keratinocytes, and fibroblasts in skin from patients with earlier onset SSc, leading to enhanced leukocyte migration into affected tissues[46,47]. Further, in a bleomycin-induced skin fibrosis model, MCP-1 mRNA expression is increased, and treatment with neutralizing anti-MCP-1 antibody inhibits cutaneous fibrosis[48]. Since treatment with recombinant MCP-1 does not augment the synthesis of type I collagen by cultured dermal fibroblasts[47], these findings suggest that MCP-1 contributes to the initiation of inflammatory infiltrates and also promotes Th2 differentiation in SSc.

TGF-ß is a major fibrogenic growth factor, since it not only stimulates matrix synthesis, but also controls virtually all fibroblast function relevant to fibrosis including proliferation, chemotaxis, and differentiation[26]. Many studies have suggested a role of TGF-ß in the development of fibrosis in SSc[26]. In our study, serum TGF-ß$_1$ levels were elevated throughout the followup period, with slightly decreased levels at later timepoints. Further, expression of TGF-ß$_1$ was downregulated in the regression stage of SSc skin. TGF-ß$_1$ is consistently most strongly expressed in the early, uninvolved skin of patients with SSc, rather than in areas of advanced disease that are already highly sclerotic[49]. In addition, studies have shown that TGF-ß drives the shift in the Th1/Th2 balance toward Th2 by way of the IL-10-mediated development of Th2 responses and the inhibition of Th1

responses[50]. Thus, our results confirm previous findings indicating that TGF-ß contributes to the development of skin fibrosis in SSc, and also suggest that TGF-ß is involved in Th2 polarization.

In multiple sclerosis, a prototype of Th1-mediated disorders, the shift from a Th1 response to a Th2 response is associated with clinical improvement[51]. Conversely, our results suggest that the shift from a Th2 response to a Th1 response correlates with improvement of skin fibrosis in SSc. This implies that the inhibition of Th2 cytokines or a forced shift toward a Th1 response may be a possible therapy for SSc. However, we cannot exclude the possibility that a shift from a Th2 to Th1 response may only be secondary to the regression of skin sclerosis. Furthermore, as almost all patients with SSc we examined received low-dose steroid and/or D-penicillamine treatment, we also cannot rule out the possibility that treatment affects Th1/Th2 balance in SSc. Nonetheless, serum IL-12 could be a serologically useful marker for disease activity and prognosis, since patients' clinical course is heterogeneous, and they should be closely monitored for the onset of new organ involvement during the early years of illness[16]. A future prospective study will be needed to confirm our results.


## ACKNOWLEDGMENT

We thank M. Matsubara and Y. Yamada for their technical assistance.


## REFERENCES


1. Roumm AD, Whiteside TL, Medsger TA Jr, Rodnan GP. Lymphocytes in the skin of patients with progressive systemic sclerosis. Quantification, subtyping, and clinical correlations. Arthritis Rheum 1984;27:645-53.
2. Fiocco U, Rosada M, Cozzi L, et al. Early phenotypic activation of circulating helper memory T cells in scleroderma: correlation with disease activity. Ann Rheum Dis 1993;52:272-7.
3. Chizzolini C. T lymphocyte and fibroblast interactions: the case of skin involvement in systemic sclerosis and other examples. Springer Semin Immunopathol 1999;21:431-50.
4. Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. Nature 1996;383:787-93.
5. Needleman BW, Wigley FM, Stair RW. Interleukin-1, interleukin-2, interleukin-4, interleukin-6, tumor necrosis factor-α, and interferon-γ levels in sera from patients with scleroderma. Arthritis Rheum 1992;35:67-72.
6. Famularo G, Procopio A, Giacomelli R, et al. Soluble interleukin-2 receptor, interleukin-2 and interleukin-4 in sera and supernatants from patients with progressive systemic sclerosis. Clin Exp Immunol 1990;81:368-72.
7. Hasegawa M, Fujimoto M, Kikuchi K, Takehara K. Elevated serum levels of interleukin 4 (IL-4), IL-10, and IL-13 in patients with systemic sclerosis. J Rheumatol 1997;24:328-32.
8. Hasegawa M, Sato S, Fujimoto M, Ihn H, Kikuchi K, Takehara K. Serum levels of interleukin 6 (IL-6), oncostatin M, soluble IL-6 receptor, and soluble gp130 in patients with systemic sclerosis. J Rheumatol 1998;25:308-13.
9. Hasegawa M, Sato S, Ihn H, Takehara K. Enhanced production of interleukin-6 (IL-6), oncostatin M and soluble IL-6 receptor by cultured peripheral blood mononuclear cells from patients with systemic sclerosis. Rheumatology Oxford 1999;38:612-7.
10. Mavalia C, Scaletti C, Romagnani P, et al. Type 2 helper T-cell predominance and high CD30 expression in systemic sclerosis. Am J Pathol 1997;151:1751-8.
11. Giacomelli R, Cipriani P, Lattanzio R, et al. Circulating levels of soluble CD30 are increased in patients with systemic sclerosis (SSc) and correlate with serological and clinical features of the disease. Clin Exp Immunol 1997;108:42-6.
12. Sato S, Hanakawa H, Hasegawa M, et al. Levels of interleukin 12, a cytokine of type 1 helper T cells, are elevated in sera from patients with systemic sclerosis. J Rheumatol 2000;27:2838-42.
13. Giacomelli R, Cipriani P, Fulminis A, et al. Circulating γ/Δ T lymphocytes from systemic sclerosis (SSc) patients display a T helper (Th) 1 polarization. Clin Exp Immunol 2001;125:310-5.
14. Fujii H, Hasegawa M, Takehara K, Mukaida N, Sato S. Abnormal expression of intracellular cytokines and chemokine receptors in peripheral blood T lymphocytes from patients with systemic sclerosis. Clin Exp Immunol 2002;130:548-56.
15. Majumdar S, Li D, Ansari T, et al. Different cytokine profiles in cryptogenic fibrosing alveolitis and fibrosing alveolitis associated with systemic sclerosis: a quantitative study of open lung biopsies. Eur Respir J 1999;14:251-7.
16. Steen VD, Medsger TA Jr. Severe organ involvement in systemic sclerosis with diffuse scleroderma. Arthritis Rheum 2000;43:2437-44.
17. Black C, Dieppe P, Huskisson T, Hart FD. Regressive systemic sclerosis. Ann Rheum Dis 1986;45:384-8.
18. Clements PJ, Hurwitz EL, Wong WK, et al. Skin thickness score as a predictor and correlate of outcome in systemic sclerosis: high-dose versus low-dose penicillamine trial. Arthritis Rheum 2000;43:2445-54.
19. Subcommittee for Scleroderma Criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Preliminary criteria for the classification of systemic sclerosis (scleroderma). Arthritis Rheum 1980;23:581-90.
20. LeRoy EC, Krieg T, Black C, et al. Scleroderma (systemic sclerosis): classification, subsets, and pathogenesis. J Rheumatol 1988;15:202-5.
21. Clements PJ, Lachenbruch PA, Seibold JR, et al. Skin thickness score in systemic sclerosis: an assessment of interobserver variability in 3 independent studies. J Rheumatol 1993;20:1892-6.
22. Trinchieri G. Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. Blood 1994;84:4008-27.
23. Meijerink J, Mandigers C, van de Locht L, Tonnissen E, Goodsaid F, Raemaekers J. A novel method to compensate for different amplification efficiencies between patient DNA samples in quantitative real-time PCR. J Mol Diag 2001;3:55-61.
24. Gu L, Tseng S, Horner RM, Tam C, Loda M, Rollins BJ. Control of TH2 polarization by the chemokine monocyte chemoattractant protein-1. Nature 2000;404:407-11.
25. Sad S, Mosmann TR. Single IL-2-secreting precursor CD4 T cell can develop into either Th1 or Th2 cytokine secretion phenotype. J Immunol 1994;153:3514-22.
26. Varga J. Scleroderma and Smads: dysfunctional Smad family dynamics culminating in fibrosis. Arthritis Rheum 2002;46:1703-13.
27. Verrecchia F, Pessah M, Atfi A, Mauviel A. Tumor necrosis factor-α inhibits transforming growth factor-ß/Smad signaling in human dermal fibroblasts via AP-1 activation. J Biol Chem 2000;275:30226-31.
28. Chizzolini C, Parel Y, De Luca C, et al. Systemic sclerosis Th2 cells inhibit collagen production by dermal fibroblasts via membrane-associated tumor necrosis factor α. Arthritis Rheum 2003;48:2593-604.
29. Rosenbloom J, Feldman G, Freundlich B, Jimenez SA. Inhibition of

excessive scleroderma fibroblast collagen production by recombinant γ-interferon. Association with a coordinate decrease in types I and III procollagen messenger RNA levels. Arthritis Rheum 1986;29:851-6.
30. Polisson RP, Gilkeson GS, Pyun EH, Pisetsky DS, Smith EA, Simon LS. A multicenter trial of recombinant human interferon γ in patients with systemic sclerosis: effects on cutaneous fibrosis and interleukin 2 receptor levels. J Rheumatol 1996;23:654-8.
31. Grassegger A, Schuler G, Hessenberger G, et al. Interferon-γ in the treatment of systemic sclerosis: a randomized controlled multicentre trial. Br J Dermatol 1998;139:639-48.
32. Marth T, Strober W, Seder RA, Kelsall BL. Regulation of transforming growth factor-ß production by interleukin-12. Eur J Immunol 1997;27:1213-20.
33. Tsuji-Yamada J, Nakazawa M, Takahashi K, et al. Effect of IL-12 encoding plasmid administration on tight-skin mouse. Biochem Biophys Res Commun 2001;280:707-12.
34. Gillery P, Fertin C, Nicolas JF, et al. Interleukin-4 stimulates collagen gene expression in human fibroblast monolayer cultures. Potential role in fibrosis. FEBS Lett 1992;302:231-4.
35. Duncan MR, Berman B. Stimulation of collagen and glycosaminoglycan production in cultured human adult dermal fibroblasts by recombinant human interleukin 6. J Invest Dermatol 1991;97:686-92.
36. Kawaguchi Y, Hara M, Wright TM. Endogenous IL-1α from systemic sclerosis fibroblasts induces IL-6 and PDGF-A. J Clin Invest 1999;103:1253-60.
37. Feghali CA, Bost KL, Boulware DW, Levy LS. Mechanisms of pathogenesis in scleroderma. I. Overproduction of interleukin 6 by fibroblasts cultured from affected skin sites of patients with scleroderma. J Rheumatol 1992;19:1207-11.
38. Saito E, Fujimoto M, Hasegawa M, et al. CD19-dependent B lymphocyte signaling thresholds influence skin fibrosis and autoimmunity in the tight-skin mouse. J Clin Invest 2002;109:1453-62.
39. Sato S, Hasegawa M, Takehara K. Serum levels of interleukin-6 and interleukin-10 correlate with total skin thickness score in patients with systemic sclerosis. J Dermatol Sci 2001;27:140-6.
40. Reitamo S, Remitz A, Tamai K, Uitto J. Interleukin-10 modulates type I collagen and matrix metalloprotease gene expression in cultured human skin fibroblasts. J Clin Invest 1994;94:2489-92.
41. Huaux F, Louahed J, Hudspith B, et al. Role of interleukin-10 in the lung response to silica in mice. Am J Respir Cell Mol Biol 1998;18:51-9.
42. Louis H, Le Moine A, Quertinmont E, et al. Repeated concanavalin A challenge in mice induces an interleukin 10-producing phenotype and liver fibrosis. Hepatology 2000;31:381-90.
43. Veale DJ, Fearon UM, Henshaw K, et al. Clinical and laboratory measures of disease activity in systemic sclerosis [abstract]. Arthritis Rheum 2001;Suppl 44:S327.
44. Atamas SP, Yurovsky VV, Wise R, et al. Production of type 2 cytokines by CD8+ lung cells is associated with greater decline in pulmonary function in patients with systemic sclerosis. Arthritis Rheum 1999;42:1168-78.
45. Spinozzi F, Agea E, Russano A, et al. Th2-type cytokines and the Fas way to pulmonary fibrosis: comment on the article by Atamas et al [letter]. Arthritis Rheum 2000;43:236-8.
46. Hasegawa M, Sato S, Takehara K. Augmented production of chemokines (MCP-1, MIP-1a, and MIP-1ß) in patients with systemic sclerosis: MCP-1 and MIP-1α may be involved in the development of pulmonary fibrosis. Clin Exp Immunol 1999;117:159-65.
47. Distler O, Pap T, Kowal-Bielecka O, et al. Overexpression of monocyte chemoattractant protein 1 in systemic sclerosis: role of platelet-derived growth factor and effects on monocyte chemotaxis and collagen synthesis. Arthritis Rheum 2001;44:2665-78.
48. Yamamoto T, Nishioka K. Role of monocyte chemoattractant protein-1 and its receptor, CCR-2, in the pathogenesis of bleomycin-induced scleroderma. J Invest Dermatol 2003;121:510-6.
49. Higley H, Persichitte K, Chu S, Waegell W, Vancheeswaran R, Black C. Immunocytochemical localization and serologic detection of transforming growth factor ß1: association with type I procollagen and inflammatory cell markers in diffuse and limited systemic sclerosis, morphea, and Raynaud's phenomenon. Arthritis Rheum 1994;37:278-88.
50. Maeda H, Shiraishi A. TGF-ß contributes to the shift toward Th2-type responses through direct and IL-10-mediated pathways in tumor-bearing mice. J Immunol 1996;156:73-8.
51. Takahashi K, Miyake S, Kondo T, et al. Natural killer type 2 bias in remission of multiple sclerosis. J Clin Invest 2001;107:R23-R9.