Serum CXCL16 Concentrations Correlate with the Extent of Skin Sclerosis in Patients with Systemic Sclerosis

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ABSTRACT. ***Objective.*** To determine serum concentrations of soluble CXCL16 and its clinical associations in patients with systemic sclerosis (SSc).

Methods. Serum CXCL16 levels from 89 patients with SSc were examined by ELISA. In a retrospective longitudinal study, 68 sera from 28 patients with SSc were analyzed (followup 1.3 to 7.3 yrs).

Results. Serum CXCL16 levels were elevated in patients with SSc compared with healthy controls (n = 42). Patients with diffuse cutaneous SSc (n = 52) had higher levels of CXCL16 than those with limited cutaneous SSc (n = 37). Serum CXCL16 levels correlated positively with the extent of skin sclerosis. In the longitudinal study, CXCL16 levels generally decreased on a parallel with the improvement in skin sclerosis.

Conclusion. CXCL16 levels were increased in patients with SSc, and correlated with the extent of skin sclerosis, suggesting that CXCL16 may have a role in the development of skin fibrosis in SSc. Blockade of CXCL16 interaction might be a potential therapeutic target in patients with SSc. (First Release July 15 2009; J Rheumatol 2009;36:1917–23; doi:10.3899/jrheum.090108)

Key Indexing Terms:
SYSTEMIC SCLEROSIS FIBROSIS CHEMOKINE CXCL16

Systemic sclerosis (SSc) is a generalized connective tissue disorder characterized by sclerotic and vascular changes in the skin and various internal organs. Although the pathogenesis of SSc remains unclear, many studies have suggested that some cytokines or growth factors regulate the induction of SSc by stimulating the synthesis of extracellular matrix components, injuring the endothelial cells, and modulating the function of leukocytes[1,2]. In early skin lesions of patients with SSc, mononuclear cell infiltration is first seen around small vessels in the dermis[3,4]. Further, the degree of mononuclear cell infiltration correlates with both the degree and progression of skin thickening[5]. Thus, these cells are potent candidates for releasing cytokines or growth factors, which play a crucial part in initiating and developing fibrosis in SSc.

Recent investigations have identified many potential molecules, including chemokines, that regulate the migration and recruitment of specific leukocytes to the inflammatory regions. CXCL16 and CX3CL1 are the only transmembrane chemokines thus far known[6,7]. CXCL16 is expressed on macrophages, dendritic cells, B cells, T cells, smooth-muscle cells, endothelial cells, and keratinocytes[6-11]. The membrane-bound form of CXCL16 specifically interacts with its unique receptor, CXCR6, to effect firm adhesion of CXCR6-expressing cells such as effector/memory T cells and natural killer cells[6,7]. The membrane-bound CXCL16 can be cleaved by a disintegrin and metalloproteinase 10[12], and the cleaved soluble form of CXCL16 exhibits chemotactic activity for CXCR6-expressing cells[6].

CXCL16 plays an important role in CXCR6+ T cell accumulation and stimulation in the synovium of patients with rheumatoid arthritis[13,14]. Further, soluble CXCL16 levels are elevated in sera from patients with systemic lupus erythematosus (SLE), Crohn's disease, and ulcerative colitis and associated with disease activity[15,16]. We suggest that CXCL16 may have some role in the pathogenesis of SSc.

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MATERIALS AND METHODS

Serum samples. Serum samples were obtained from 89 Japanese patients with SSc (76 female, 13 male). All patients fulfilled criteria for SSc proposed by the American College of Rheumatology (ACR)[17]. These patients were grouped according to the classification system proposed by LeRoy, *et al*[18]: 37 patients had limited cutaneous SSc (lSSc) and 52 had diffuse cutaneous SSc (dSSc). Anti-topoisomerase I (topo I) antibodies were positive in 36 patients (30 dSSc, 6 lSSc); anticentromere antibodies (ACA) in 30 (2 dSSc, 28 lSSc); anti-RNA polymerase I and III (RNAP) antibodies in 9 (8 dSSc, 1 lSSc); anti-U1RNP antibodies in 2 (1 dSSc, 1 lSSc); anti-U3RNP antibodies in 1 (dSSc); anti-Th/To antibodies in 1 (dSSc); and 10 were negative (9 dSSc, 1 lSSc). These patients were aged 9–76 years (mean 47 yrs). The mean disease duration was 4.4 ± 5.7 (range 0.2–29) years. At the first visit, 8 patients had been treated with low-dose steroids (prednisolone 5 to 20 mg/day) and 6 with low-dose D-penicillamine (100 to 500 mg/day). None of the patients had received immunosuppressive treatment.

Thirty patients with SLE who fulfilled the ACR criteria[19] and 25 with dermatomyositis (DM) who fulfilled the criteria of Bohan and Peter[20] acted as disease controls. Forty-two age- and sex-matched healthy Japanese individuals were used as healthy controls.

In a retrospective longitudinal study, we analyzed 68 serum samples from 28 of 89 patients with SSc who had been followed longitudinally (27 women, 1 man). There was no bias in patient selection. The mean followup period was 3.9 ± 1.6 (1.3–7.3) years with 2.4 (2–3) timepoints. The mean disease duration was 4.1 ± 5.1 (0.2–13.3) years. Fifteen patients had dSSc and 13 had lSSc; anti-topo I antibodies were positive in 11 patients (all dSSc); ACA in 14 (1 dSSc, 13 lSSc); anti-RNAP antibodies in 2 (all dSSc); and 1 was negative (dSSc). These patients were aged 9–71 years (mean age 46 yrs).

Fresh venous blood samples were centrifuged shortly after clot formation. All samples were stored at –70°C before use.

Clinical assessment. Complete medical histories, examinations, and laboratory tests were conducted for all patients at their first visit, with evaluations especially for pulmonary fibrosis during followup visits. Organ system involvement was defined as described[21,22]: lung = bibasilar fibrosis on chest radiography and high-resolution computed tomography; esophagus = hypomotility shown by barium radiography; 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 fibrosis was defined as bibasilar interstitial fibrosis on chest high-resolution computed tomography. In addition, pulmonary function tests including vital capacity (VC) and diffusion capacity for carbon monoxide (DLCO) were used to examine the severity of pulmonary fibrosis. When the DLCO and VC were < 75% and < 80%, respectively, of the predicted normal values, they were considered abnormal. Patients with SSc who were smokers or who had other respiratory disorders that could have affected %DLCO or %VC were excluded from this study. The modified Rodnan total skin thickness score (TSS) was measured by summing the skin thickness measurements, and determined by palpation on a 0–3 scale in 17 body areas[23]. The study protocol was approved by the Nagasaki University Graduate School of Biomedicinal Sciences and Nagasaki University Hospital, and informed consent was obtained from all patients.

Detection of serum CXCL16. Serum CXCL16 levels were measured with specific ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's protocol. This ELISA system can detect all circulating CXCL16 isoforms. Each sample was tested in duplicate. The detection limit of this assay was 0.007 ng/ml.

Immunohistochemical staining. Skin biopsy samples were taken from the dorsal aspect of the mid-forearm of 5 female patients with dSSc and 5 healthy female volunteers. CXCL16 expression in the skin tissue was determined using goat IgG anti-human CXCL16 monoclonal antibody (R&D Systems). Frozen dermal tissues were air-dried and subsequently fixed in cold acetone. The tissue sections were incubated overnight with anti-CXCL16 monoclonal antibody at 4°C, and then treated with biotinylated rabbit anti-goat IgG antibody for 45 min at room temperature, followed by incubation with avidin-biotin-peroxidase complex for 30 min. Sections were incubated with diaminobenzidine tetrahydrochloride substrate solution for 5 min, counterstained with methyl green, and embedded in balsam. Isotype-matched control monoclonal antibody was used as a negative control.

Statistical analysis. The Mann-Whitney U-test was used to compare CXCL16 levels, 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. p values less than 0.05 were considered statistically significant.

RESULTS

Serum CXCL16 levels elevated in patients with SSc. Serum CXCL16 levels were significantly higher in patients with SSc (3.15 ± 1.1 pg/ml) than healthy controls (1.29 ± 0.95 pg/ml; $p < 0.001$; Figure 1). Similarly, serum CXCL16 levels were significantly higher in patients with SLE (2.67 ± 0.83 pg/ml; $p < 0.001$) and in patients with DM (3.02 ± 0.97 pg/ml; $p < 0.001$) than in healthy controls. Patients with SSc had the highest median serum CXCL16 levels. As for subgroups of SSc, CXCL16 levels in both dSSc (3.43 ± 1.17 pg/ml) and lSSc (2.76 ± 0.88 pg/ml) patients were significantly higher than in healthy controls ($p < 0.001$ for both). Further, serum CXCL16 levels were significantly elevated in patients with dSSc relative to those with lSSc ($p < 0.05$).

Clinical correlation of serum CXCL16 levels. Clinical and laboratory measures obtained at the first evaluation were compared between SSc patients with elevated CXCL16 levels and those with normal CXCL16 levels. Values higher than the mean + 2 standard deviations (3.19 ng/ml) of the control serum samples were considered to be elevated in our study. Elevated CXCL16 levels were observed in 38% (34/89) of total patients with SSc, 48% (25/52) with dSSc, and 24% (9/37) with lSSc. SSc patients with elevated CXCL16 levels were more frequently male ($p < 0.01$) and had more frequent presence of dSSc ($p < 0.01$) and anti-RNAP antibody ($p < 0.05$) than those with normal CXCL16 levels (Table 1). Inversely, SSc patients with elevated CXCL16 levels had less frequent presence of lSSc ($p < 0.01$) and ACA ($p < 0.05$) than those with normal CXCL16 levels. Consistent with the association of higher CXCL16 levels with dSSc, SSc patients with elevated CXCL16 levels had significantly higher modified Rodnan TSS compared with those with normal CXCL16 ($p < 0.01$). Moreover, CXCL16 levels correlated positively with modified Rodnan TSS ($p < 0.01$, $r = 0.43$; Figure 2). Thus, elevated CXCL16 levels were associated with the severity of skin sclerosis in SSc.

Immunohistochemical expression of CXCL16 in the skin of patients with SSc. Whether CXCL16 expression was augmented in the skin of patients with SSc was assessed by immunohistochemical analysis. Patients with SSc had remarkably higher expression of CXCL16 in the epidermis

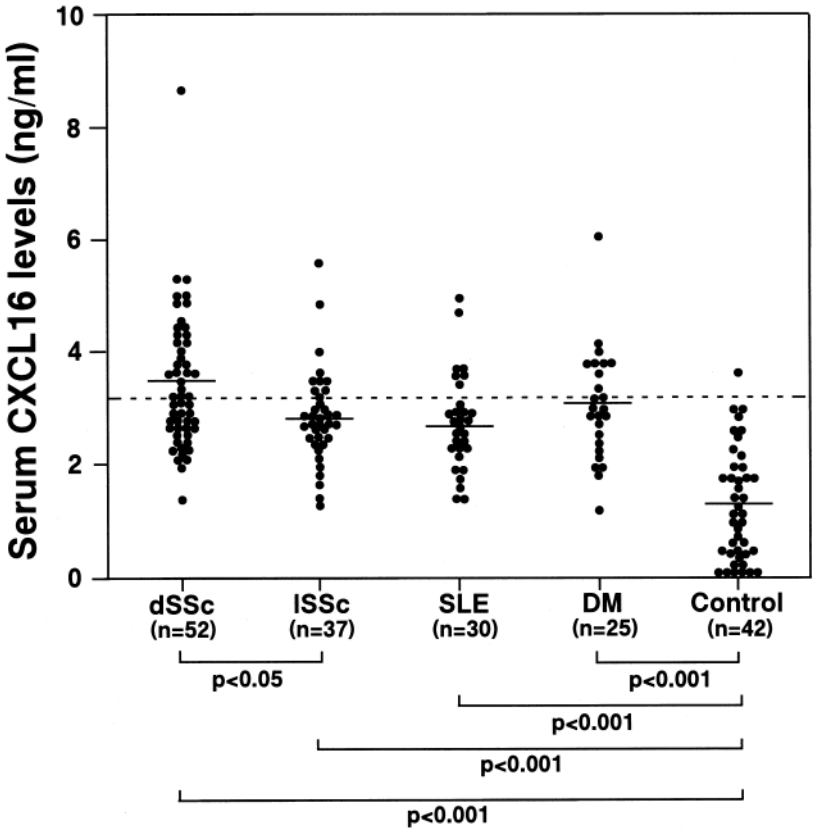

Figure 1. Serum levels of CXCL16 in patients with dSSc, lSSc, SLE, DM, and normal controls (Control). Serum CXCL16 levels were determined by a specific ELISA. Bars indicate mean value in each group. Broken line indicates cutoff value (mean + 2 standard deviations of normal control samples).

compared with healthy controls (Figure 3). Further, infiltrating dermal mononuclear cells in the skin of patients with SSc exhibited strong CXCL16 expression. Thus, augmented CXCL16 expression was found in the lesional skin from patients with SSc.

Longitudinal study of serum CXCL16 levels. To assess changes in serum CXCL16 levels over time, 68 serum samples from 28 patients with SSc (15 dSSc and 13 lSSc) were analyzed retrospectively. None of these patients had received any treatment at their first visit. Seven of 15 patients with dSSc exhibited elevated CXCL16 levels at their first visit. Serum CXCL16 levels in 4 of these 7 patients were decreasing during the followup of 2.9 ± 1.7 (1.3–5.3) years (Figure 4A). Their disease duration at the initial visit was 2.3 ± 3.2 (0.3–7.0) years. Serum CXCL16 levels at the final evaluation were within normal range in all of these patients. During the followup, 3 of the 4 patients exhibited subacute deterioration of interstitial pneumonitis. Two of the 3 patients received cyclophosphamide pulse therapy, and one received steroid pulse therapy for the pulmonary involvement. Low-dose steroids were started in the remaining patient. Skin sclerosis was improving in all the patients during followup (modified Rodnan TSS 21.0 ± 3.6 at first visit to 12.8 ± 3.9 at the final evaluation; 39% decrease; $p < 0.05$).

Serum CXCL16 levels in 3 of the 7 dSSc patients with high CXCL16 levels at first visit remained high during the followup of 4.6 ± 0.5 (4.1–5.2) years (Figure 4B). Their disease duration was 2.8 ± 3.5 (0.2–6.8) years. After the initial visit, treatment with low-dose steroids alone was started in 2 of the 3 patients and treatment with both low-dose steroids and D-penicillamine was started in the remaining patient. Skin sclerosis tended to improve (modified Rodnan TSS 13.3 ± 6.4 at first visit to 11.6 ± 5.9 at the final evaluation; 13% decrease); however, the decrease was not significant.

Serum CXCL16 levels in 8 of 15 dSSc patients with normal serum CXCL16 levels at first visit remained within normal range throughout the followup period of 4.1 ± 1.8 (2.4–7.3) years (Figure 4C). Their disease duration was 1.7 ± 1.3 (0.2–4.0) years. After the initial visit, treatment with low-dose steroids alone was started in 4 of the 8 patients, while treatment with both low-dose steroids and D-penicillamine was started in 3 patients. In addition, the remaining patient received cyclophosphamide pulse therapy for sub-

Table 1. Clinical and laboratory characteristics in patients with SSc showing elevated serum CXCL16 levels.

Characteristic	Elevated CXCL16, n = 34	Normal CXCL16, n = 55
Age at onset, yrs, mean ± SD	49 ± 13	45 ± 17
Male:female	10:24**	3:52
Disease duration, yrs, mean ± SD	3.3 ± 5.0	3.5 ± 6.6
TSS, mean ± SD	16.6 ± 10.6**	10.6 ± 7.2
Clinical features, %		
dSSc	73**	48
lSSc	26**	52
Pitting scars/ulcers	29	35
Contracture of phalanges	44	39
Diffuse pigmentation	50	44
Telangiectasia	30	44
Calcinosis	6	8
Organ involvement, %		
Pulmonary fibrosis	41	52
Decreased % VC	25	19
Decreased % DLCO	66	58
Pulmonary hypertension	10	13
Esophagus	58	72
Heart	6	13
Kidney	2	0
Joint	12	22
Muscle	9	22
Laboratory findings, %		
Anti-topo I antibody	47	37
Anticentromere antibody	24*	41
Anti-RNAP antibody	18*	6
Increased IgG	27	31
Elevated ESR	47	45
Elevated CRP	6	14

* $p < 0.05$, ** $p < 0.01$ versus SSc patients with normal serum CXCL16 levels. TSS: total skin thickness score; VC: vital capacity; DLCO: diffusion capacity for carbon monoxide; RNAP: RNA polymerase; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein.

acute deterioration of interstitial pneumonitis. Skin sclerosis was improving in all the patients during followup (modified Rodnan TSS 16.5 ± 5.7 at first visit to 12.4 ± 4.7 at the final evaluation; 25% decrease; $p < 0.05$).

Finally, 9 of 13 patients with lSSc examined in this study exhibited normal CXCL16 levels at the first visit that stayed within normal range throughout the followup period of 4.0 ± 1.7 (1.8–5.5) years, although only 1 exhibited transiently elevated CXCL16 levels during the followup (Figure 4D). Their disease duration was 7.0 ± 7.4 (1.0–23) years. Serum CXCL16 levels in 4 of the 13 patients with lSSc showed elevated CXCL16 levels at their first visit, although their CXCL16 levels were lower than 3.5 ng/ml. Serum CXCL16 levels in 2 of the 4 patients with lSSc were decreasing during the followup of 3.0 ± 0.1 (2.9–3.0) years. The remaining 2 patients with increased CXCL16 levels at their first visit remained elevated during the followup of 4.3 ± 2.6 (2.5–6.2) years. None of these patients with lSSc had received D-penicillamine or steroids during the followup period. Thus, CXCL16 levels generally decreased on a parallel with the improvement in skin sclerosis, while steroid or immunosuppressive therapy might affect the change.

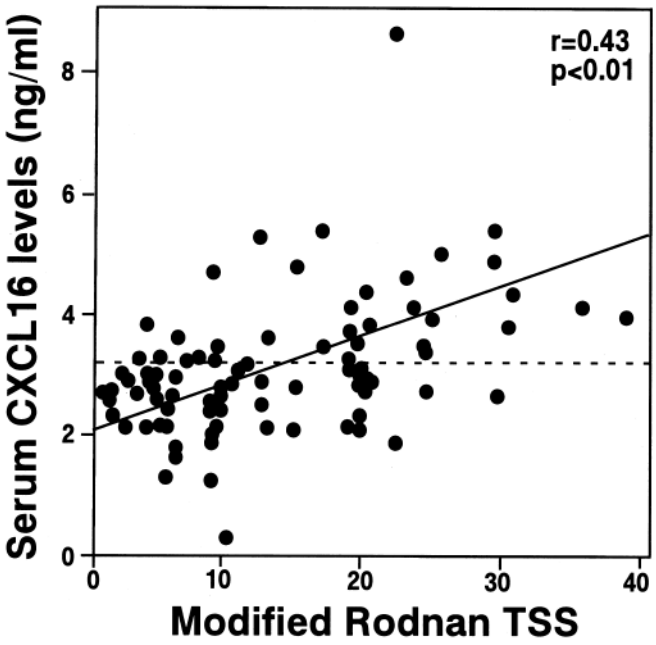

Figure 2. Correlation of serum CXCL16 levels with modified Rodnan TSS in patients with SSc. Serum CXCL16 levels were determined by a specific ELISA. Broken lines indicate cutoff value.

DISCUSSION

Ours is the first report of elevated serum CXCL16 levels in patients with SSc. Although the elevation of serum CXCL16 levels was also observed in patients with SLE and DM, mean CXCL16 levels in patients with SSc were higher than those found in SLE and DM patients (Figure 1). Remarkably, the elevation of serum CXCL16 levels in patients with SSc was associated with greater extent of skin fibrosis (Figure 2 and Table 1). In the longitudinal study, CXCL16 levels generally decreased on a parallel with the improvement in skin sclerosis (Figure 4). Taken together, these results suggest that CXCL16 may play an important role in the development of skin sclerosis in patients with SSc.

CXCL16 expression in epidermal keratinocytes and infiltrating dermal mononuclear cells was substantially augmented in patients with SSc compared with healthy controls (Figure 3). Stimulation with tumor necrosis factor-α (TNF-α) and interleukin 1α (IL-1α) enhances CXCL16 expression in keratinocytes[11,24]. Further, serum TNF-α levels are significantly elevated in patients with SSc compared with healthy control subjects[25], while dermal fibroblasts from patients with SSc exhibit increased IL-1α production[26]. This suggests the contribution of TNF-α and IL-1α to the elevated CXCL16 levels in patients with SSc.

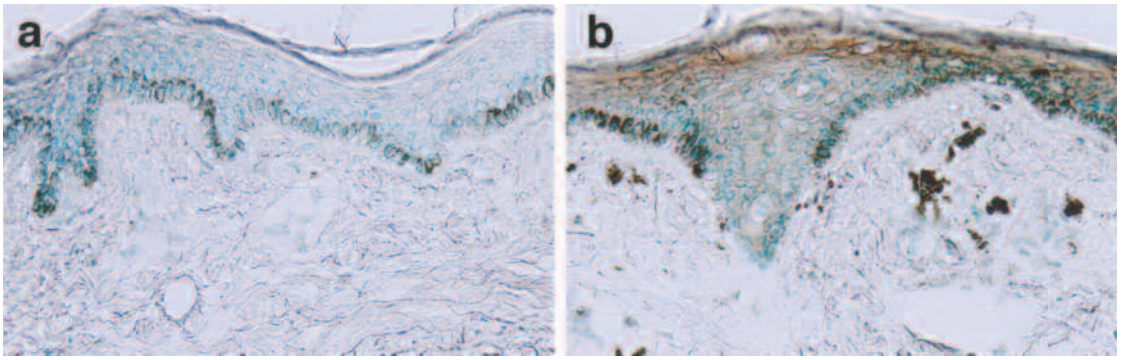

Figure 3. Representative CXCL16 expression in normal skin tissues (a) and lesional skin tissues from patients with SSc (b). Original magnification ×250.

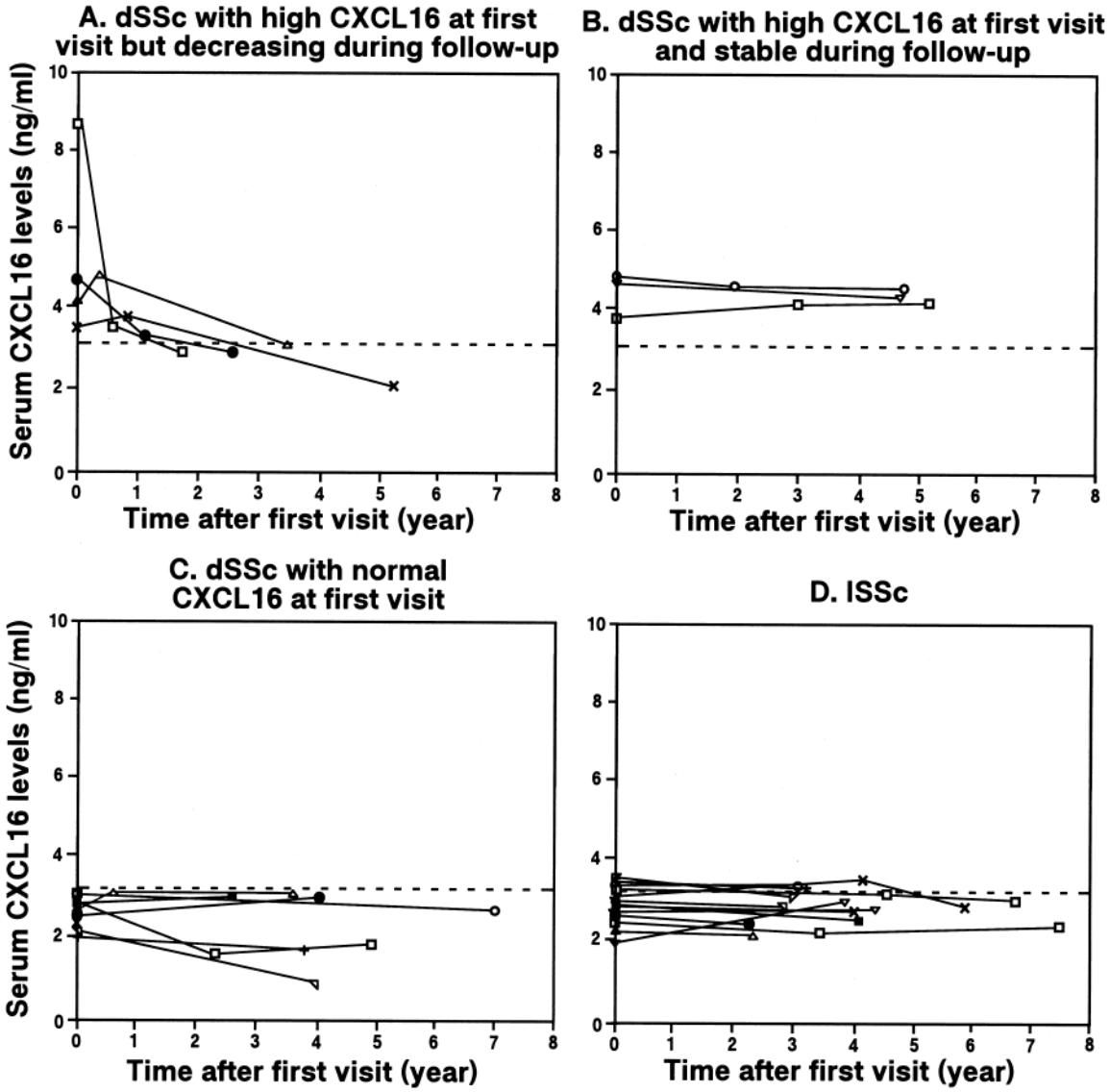

Figure 4. Serial changes of serum CXCL16 levels during followup period in dSSc patients with elevated CXCL16 levels at their first visit (a and b), dSSc patients with normal CXCL16 levels at their first visit (c), and lSSc patients (d). Serum CXCL16 levels were determined by a specific ELISA. Broken lines indicate cutoff value.

In addition, CXCL16 production from keratinocytes is enhanced by Toll-like receptor (TLR) 2 and TLR3 stimulation[24]. Interestingly, serum levels of hyaluronan, which activates TLR2 and TLR4, are elevated in patients with SSc are and associated with the extent of skin sclerosis[27]. Moreover, hyaluronan expression in the dermis was enhanced in a mouse model for SSc[28]. Therefore, the elevation of serum CXCL16 levels may also be attributable to TLR stimulation by hyaluronan. The infiltrated dermal mononuclear cells observed in patients with early-stage SSc mostly consist of activated T cells[5]. Moreover, CXCR6 is expressed on activated T cells in the papillary dermis[11,29]. Thus, CXCL16 production from keratinocytes and infiltrating mononuclear cells may be enhanced by TNF-α, IL-1α, and TLR stimulation, thereby recruiting activated T cells to the skin, leading to skin sclerosis.

In our study, serum CXCL16 levels were associated with the extent of skin sclerosis, but not with the severity of pulmonary fibrosis in SSc. This suggests that serum CXCL16 is not useful as a clinical marker of pulmonary fibrosis in SSc. However, this does not imply that CXCL16 does not play a role in the pathogenesis of pulmonary fibrosis in SSc. Soluble CXCL16 does not represent the total CXCL16 expression, because another significant portion is still membrane-bound[6]. Alveolar macrophages express CXCL16 protein, whereas CXCL16 levels in either bronchoalveolar lavage fluid or blood are not elevated in patients with sarcoidosis, asthma, or interstitial lung diseases compared with healthy controls[30]. Therefore, the relationship between membrane-bound CXCL16 expression in the lung and the severity of pulmonary fibrosis in patients with SSc should be examined. In addition, the reason that serum CXCL16 levels reflect only the extent of skin sclerosis in SSc is still unclear. It has been proposed that oxidative stress may play an important role in the development of SSc[31]. Ischemia and reperfusion injury following Raynaud's phenomenon can generate reactive oxygen species that may result in vascular endothelial damage[32,33]. Consistent with this, circulating CXCL16 levels correlate with the severity of coronary artery stenosis[34]. Therefore, it is likely that increased amounts of CXCL16 are released from infiltrating dermal mononuclear cells into circulation in response to oxidative stress-induced vascular damage. Further studies are needed to determine how CXCL16 is released into circulation and elicits inflammatory cell recruitment. Because no therapy has proven effective in suppressing or improving skin sclerosis in SSc, blockade of CXCL16-CXCR6 interaction might be a therapeutic target in patients with SSc who have severe skin sclerosis.

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