Elevated Circulating CD40L Concentrations in Patients with Systemic Sclerosis

KAZUHIRO KOMURA, SHINICHI SATO, MINORU HASEGAWA, MANABU FUJIMOTO, and KAZUHIKO TAKEHARA

ABSTRACT. ***Objective.*** B cell activation, fibrosis, and expression of adhesion molecules on endothelial cells are regulated by soluble CD40L (sCD40L)/CD40 interactions. Since these effects are characteristic in patients with systemic sclerosis (SSc), serum concentrations of sCD40L were determined in patients with SSc.

Methods. Fifty-two Japanese patients with SSc were examined. They were grouped into 24 patients with limited cutaneous SSc (lSSc) and 28 with diffuse cutaneous SSc (dSSc). Serum sCD40L levels were examined by ELISA. As a disease control, serum samples from 20 patients with systemic lupus erythematosus (SLE) were also examined. In addition, a retrospective longitudinal study was performed in 71 serum samples from 18 patients with SSc.

Results. Serum sCD40L levels were elevated in SSc patients compared with healthy controls ($p < 0.001$). Levels of sCD40L in patients with SSc were higher than in patients with SLE ($p < 0.001$) that had elevated sCD40L levels compared with healthy controls. Among SSc subsets, there were no differences in sCD40L levels between lSSc and dSSc. sCD40L levels correlated positively with C-reactive protein levels in SSc patients ($p < 0.0001$, $r = 0.449$). In a cross-sectional study and a longitudinal study, serum sCD40L levels in dSSc patients were persistently elevated, although those in lSSc patients were temporarily elevated at the early phase of the disease process.

Conclusion. Patients with SSc exhibited elevated sCD40L levels that may correlate with disease activity. These results suggest that CD40/CD40L interactions may be potential therapeutic targets in SSc. (J Rheumatol 2004;31:514–9)

Key Indexing Terms:
SOLUBLE CD40L SYSTEMIC SCLEROSIS LONGITUDINAL STUDY

Systemic sclerosis (SSc) is a connective tissue disorder characterized by fibrosis and vascular changes in the skin and internal visceral organs, with autoimmune background. Although the molecular basis for SSc is unknown, studies have attempted to elucidate the relationship between the features of fibrosis, vascular changes, and autoimmunity in SSc. It has been suggested that some cytokines or growth factors regulate the induction and development of fibrosis and vascular changes by stimulating the synthesis of extracellular matrix components[1,2]. These cytokines and growth factors are produced partly by leukocytes infiltrating the inflammatory sites[1-3]. The migration of leukocytes into inflammatory sites is fundamentally regulated by expression of a series of adhesion molecules on endothelium and surfaces of inflammatory cells[3]. In patients with SSc, abnormal expression of adhesion molecules and cytokines appears to be a hallmark: soluble forms of various adhesion molecules and cytokines are significantly elevated in sera from patients with SSc[3]. Thus, abnormal expression of adhesion molecules and cytokines could explain both fibrosis and vascular changes in SSc. It was also reported that a chronic activation of B lymphocytes is critical not only for induction of autoantibodies but also for the development of skin fibrosis in an animal model of SSc[4]. Nonetheless, the relationship between chronic B cell activation and abnormal expression of adhesion molecules or fibrosis in SSc remains unclear.

The interaction of CD40 ligand (CD40L), transiently expressed on activated CD4+ T lymphocytes, with CD40 on B cells contributes to the generation of humoral immune responses[5,6]. Further, soluble CD40L (sCD40L), released from activated CD4+ T cells, is biologically active by binding its receptor of CD40[7]. CD40 is also expressed on endothelial cells[8] and dermal fibroblasts[9]. CD40/CD40L interactions modulate upregulation of adhesion molecules on endothelial cells that express CD40[8,10]. In addition, CD40/CD40L interactions induce fibroblast proliferation[11,12]. Thus, CD40/CD40L interactions activate B cells, upregulate endothelial adhesion molecules, and induce fibrosis.

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These findings of immunoregulatory and fibrogenetic effects of CD40/CD40L interactions led us to investigate the roles of these molecules in the development of SSc. To determine whether serum concentrations of sCD40L reflect disease activity and clinical features in patients with SSc, we examined serum concentrations of sCD40L and related these results to clinical features. In addition, we performed a retrospective longitudinal study of sCD40L concentrations in some of these patients with SSc.

MATERIALS AND METHODS

Serum samples. Serum samples were obtained from 52 Japanese patients with SSc (47 female, 5 male). All patients fulfilled the criteria for SSc proposed by the American College of Rheumatology[13]. These patients were between 2 and 77 years old (mean age 50 yrs). Patients were grouped according to the classification system proposed by LeRoy, *et al*[14]: 24 patients (24 females) had limited cutaneous SSc (lSSc) and 28 patients (23 female, 5 male) had diffuse cutaneous SSc (dSSc). The disease duration of patients with lSSc and dSSc was 2.4 ± 1.4 and 3.2 ± 3.3 years, respectively. Five patients had been treated with low dose steroids (prednisolone, 5–20 mg/day) and 4 patients with low dose D-penicillamine (100–500 mg/day) at the first visit. No SSc patient had received immunosuppressive therapy or had a recent history of infection or other inflammatory disease. Since it was reported that plasma levels of sCD40L in patients with rheumatoid arthritis (RA) were elevated[15], SSc patients with RA were excluded. As a disease control, we also examined serum samples from 20 patients with systemic lupus erythematosus (SLE) that fulfilled the American College of Rheumatology criteria[16]. Twenty-eight healthy Japanese persons (25 female, 3 male) were used as normal controls; they were between 7 and 71 years old (mean age 47 yrs).

For a retrospective longitudinal study, patients whose serum samples were taken more than 3 times were analyzed. They included 71 serum samples from 18 SSc patients (17 female, 1 male) out of 52 SSc patients. These patients were classified into 9 patients (all female) with lSSc and 9 (8 female, 1 male) with dSSc. They were between 9 and 71 years old (mean age 54 yrs). Their disease duration at their first visit was 2.3 ± 3.2 years. These patients had been followed for 3.6 ± 1.7 years (0.6–5.8 yrs) at 3.9 ± 1.0 (3–6) different time points. At the first visit, no patient had been treated with steroids or D-penicillamine. All 9 dSSc patients received low dose steroids (prednisolone, 5–20 mg/day), and one dSSc patient received low dose D-penicillamine (100 mg/day) after the first visit. Treatment with steroids or D-penicillamine was not started in any patient with lSSc, and no patient with SSc received immunosuppressive therapy throughout the followup period. A peripheral venous blood sample was drawn into pyogen-free blood 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, with limited evaluations during followup visits. Organ system involvement was defined as described[17,18]: lung = 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 function testing, including vital capacity (VC) and diffusion capacity for carbon monoxide (DLCO), was also carried out. When DLCO and VC were < 75% and < 80%, respectively, of predicted normal values, they were considered abnormal. The protocol was approved by the Kanazawa University School of Medicine and Kanazawa University Hospital.

ELISA. Specific ELISA kits were used for measuring serum sCD40L levels (Bender Medsystems, Vienna, Austria), according to the manufacturer's protocol. Each sample was tested in duplicate. These kits detect trimers of sCD40L and the detection limit of the assay is 0.095 ng/ml.

Statistical analysis. Statistical analysis was performed using Mann-Whitney U test for comparison of sCD40L levels, Fisher's exact probability test for comparison of frequencies, and Bonferroni's test for multiple comparisons. Spearman's rank correlation coefficient was used to examine the relationship between 2 continuous variables. In the longitudinal study, K-mean clustering analysis was conducted to divide the sample according to the feature of the serial change of sCD40L levels. A p value less than 0.05 was considered statistically significant. All data are shown as means ± SD.

RESULTS

Serum sCD40L levels were elevated in SSc patients. Serum sCD40L levels were significantly higher in patients with SSc (5.3 ± 2.5 ng/ml) than normal controls (1.0 ± 1.4 ng/ml; $p < 0.001$) and patients with SLE (1.5 ± 1.2 ng/ml; $p < 0.001$; Figure 1). As reported[19,20], serum sCD40L levels in SLE patients were significantly higher than in normal controls ($p = 0.0001$; Figure 1). In the SSc subgroups, sCD40L levels in both lSSc (5.1 ± 2.4 ng/ml) and dSSc (5.4 ± 2.6 ng/ml) patients were significantly higher than in normal controls ($p < 0.0001$ and $p < 0.001$, respectively). However, serum sCD40L levels were similar for patients with lSSc and those with dSSc. We could not completely exclude secondary activation of platelets *ex vivo* since recent studies have revealed transient expression of CD40L on human activated platelets[21]. However, in this study the possibility was considered low because the sera were isolated immediately after phlebotomy and the sCD40L levels were normal in almost all healthy subjects.

The patient sample was divided into 2 groups with sCD40L levels > 5.0 ng/ml (the mean + 2 SD of the control serum samples) or ≤ 5.0 ng/ml; 50% (26/52) of SSc patients were thus classified as having elevated levels of sCD40L. The frequency of elevated C-reactive protein (CRP) in SSc patients with elevated sCD40L levels was higher than in those with normal sCD40L levels (27% vs 0%; $p < 0.001$; Table 1). Further, sCD40L levels correlated positively with CRP in patients with SSc ($p < 0.0001$, $r = 0.449$; data not shown). Since a single outlying point may significantly affect both correlation coefficient and significance of the result in correlation analyses, the single outlier points at 10 ng/ml sCD40L and > 3 mg/dl CRP were eliminated. However, sCD40L levels still correlated positively with CRP ($p < 0.03$, $r = 0.402$; Figure 2). Levels of sCD40L were elevated in patients with shorter disease duration ($p < 0.05$; Table 1). Moreover, dSSc patients with disease duration < 2 years and those with duration of 2–5 years had significantly elevated sCD40L levels compared to those with duration > 5 years ($p < 0.05$; Figure 3A), although sCD40L levels in patients with duration < 2 years were similar to those with duration of 2–5 years. By contrast, lSSc patients with duration < 2 years had significantly elevated levels of sCD40L compared to those with duration of 2–5 years ($p < 0.05$;

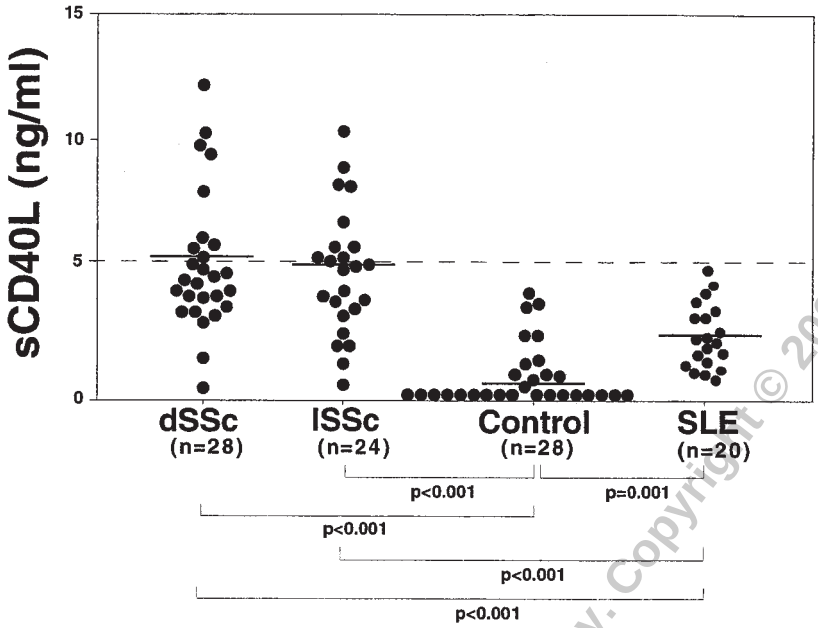

Figure 1. Serum concentrations of sCD40L in patients with lSSc, dSSc, and SLE, and normal controls. Serum sCD40L concentrations were determined by a specific ELISA. Short bars indicate the mean value in each group. Broken line indicates the cutoff value (mean + 2 SD of control samples).

Table 1. Clinical and laboratory data of patients with SSc showing elevated serum sCD40L concentrations.

	Elevated sCD40L*, n = 26	Normal sCD40L, n = 26
Age at onset, yrs, mean ± SD	53 ± 14	41 ± 19
Sex, M:F	3:23	2:24
Duration, yrs, mean ± SD	2.0 ± 1.9**	3.7 ± 3.0
Clinical features, %		
Pitting scars	38	46
Contracture of phalanges	46	62
Diffuse pigmentation	50	69
Organ involvement, %		
Lung	42	35
Decreased %VC	35	38
Decreased %DLCO	58	73
Esophagus	62	81
Heart	15	12
Kidney	0	4
Joint	15	27
Muscle	15	15
Laboratory findings, %		
Anti-topoisomerase I antibody	46	35
Anticentromere antibody	31	35
Anti-U1RNP antibody	4	4
Increased IgG	35	42
Elevated ESR	23	31
Elevated CRP	27**	0

* Values of clinical features, organ involvement, and laboratory findings are percentages. ** $p < 0.05$ vs SSc patients with normal sCD40L levels.

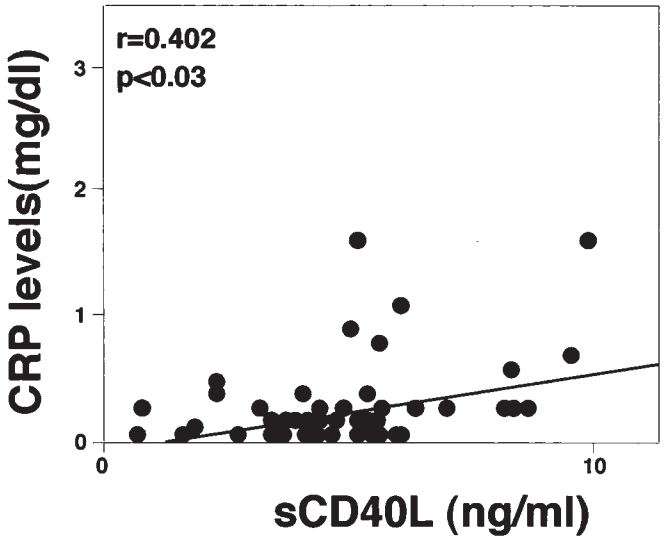

Figure 2. Correlation of CRP concentrations against serum levels of sCD40L in patients with SSc. Serum sCD40L levels were determined by a specific ELISA.

Figure 3B) and those with duration > 5 years ($p < 0.01$). Therefore, it appeared that serum levels of sCD40L were persistently elevated in dSSc patients, although they were temporarily elevated in lSSc patients.

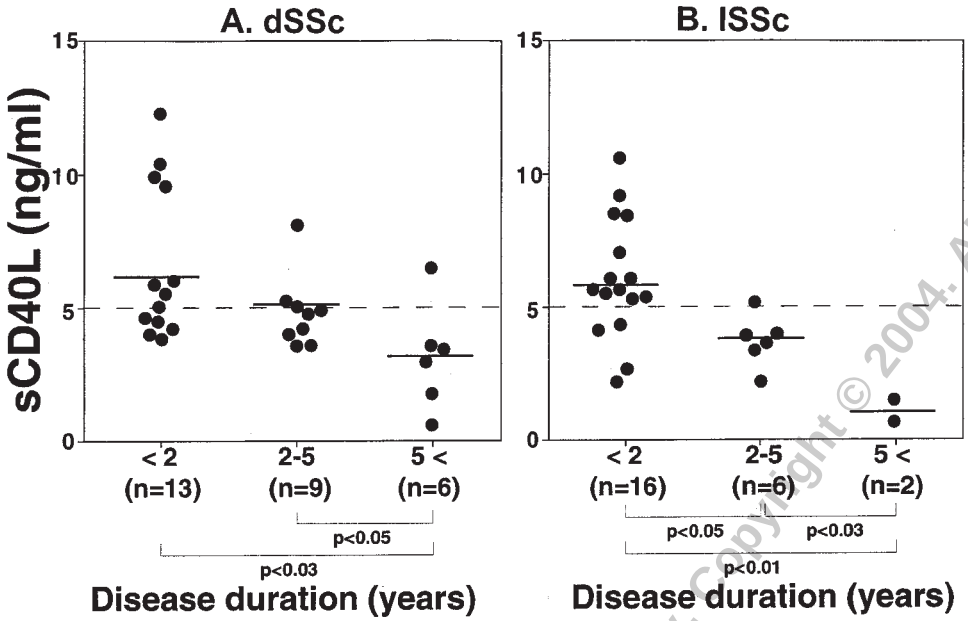

Figure 3. Correlation of disease duration with serum sCD40L concentrations in patients with dSSc (A) and lSSc (B). Serum sCD40L levels were determined by a specific ELISA. Broken lines indicate the cutoff value.

Longitudinal study of sCD40L levels. To assess changes in serum sCD40L levels over time, 71 serum samples from 18 patients with SSc were analyzed (Figure 4). At the first visit, no patient had been treated with steroids or D-penicillamine. We conducted K-mean clustering analysis on the data. According to K-mean clustering analysis, the dSSc patient sample was divided into 2 subgroups: dSSc patients with sCD40L levels > 5.0 ng/ml during the followup period (dSSc with elevated sCD40L, Figure 4A) or dSSc patients with normal sCD40L levels during the followup period (dSSc with normal sCD40L, Figure 4B). Levels of CD40L were elevated in dSSc patients with shorter duration ($p < 0.03$). No other clinical or laboratory difference between the subgroups was observed. In clinical profiles of 5 patients with elevated sCD40L, Patient 1, a relatively early SSc patient who died of subacute interstitial pneumonitis, showed gradual elevation of the sCD40L level. Patients 2, 3, 4, and 5, in the relatively early phase of SSc with progressive skin sclerosis, showed improvements of skin sclerosis and transition to the atrophic phase after normalization of serum sCD40L levels. It was noted that, except for one case (Patient 1), their elevated levels were normalized after more than 2 years. The remaining 4 patients with dSSc had normal serum sCD40L levels throughout the followup period. All 9 dSSc patients received low dose steroids, whereas one received low dose D-penicillamine, after their first visits. There were no differences in treatment between patients with increased sCD40L levels and those showing normal sCD40L levels during the followup time. By K-mean clustering analysis, the lSSc patient sample was divided into 2 subgroups, i.e., with sCD40L levels > 5.0 ng/ml during the followup period (lSSc with elevated sCD40L, Figure 4C) or normal sCD40L levels during the followup period (lSSc with normal sCD40L, Figure 4D). Levels of CD40L were elevated in lSSc patients with shorter duration ($p < 0.03$). No other clinical and laboratory difference was not observed between the subgroups. Five of 9 lSSc patients showed elevated serum sCD40L levels during the followup period, although the levels were normalized within one year. During the observation period, no lSSc patient received steroids or D-penicillamine, or had worsening skin sclerosis or developed new organ involvement.

DISCUSSION

We observed that serum sCD40L concentrations were elevated in patients with SSc (Figure 1). Because of its central role in contact-dependent signaling during the immune response, it has been believed that CD40L is involved in the development of autoimmunity[22]. Indeed, CD40/CD40L interaction has been shown to be required for the antimyelin basic protein response in experimental allergic encephalomyelitis[23], the initiation of insulitis and diabetes in non-obese diabetic mice[24], lupus-associated autoantibody production and nephritis[25], autoimmune thyroiditis[26], and collagen-induced arthritis[27]. In SSc, it has been known that the immune profile of patients is characterized by chronic T and B cell activation[28]. Increased concentrations of T cell-produced cytokines have been described in SSc patients, possibly contributing to antibody production and inflammation[1]. Moreover, augmented expression of CD40L in activated CD4+ T cells was reported[29]. Taken together, these results suggest that

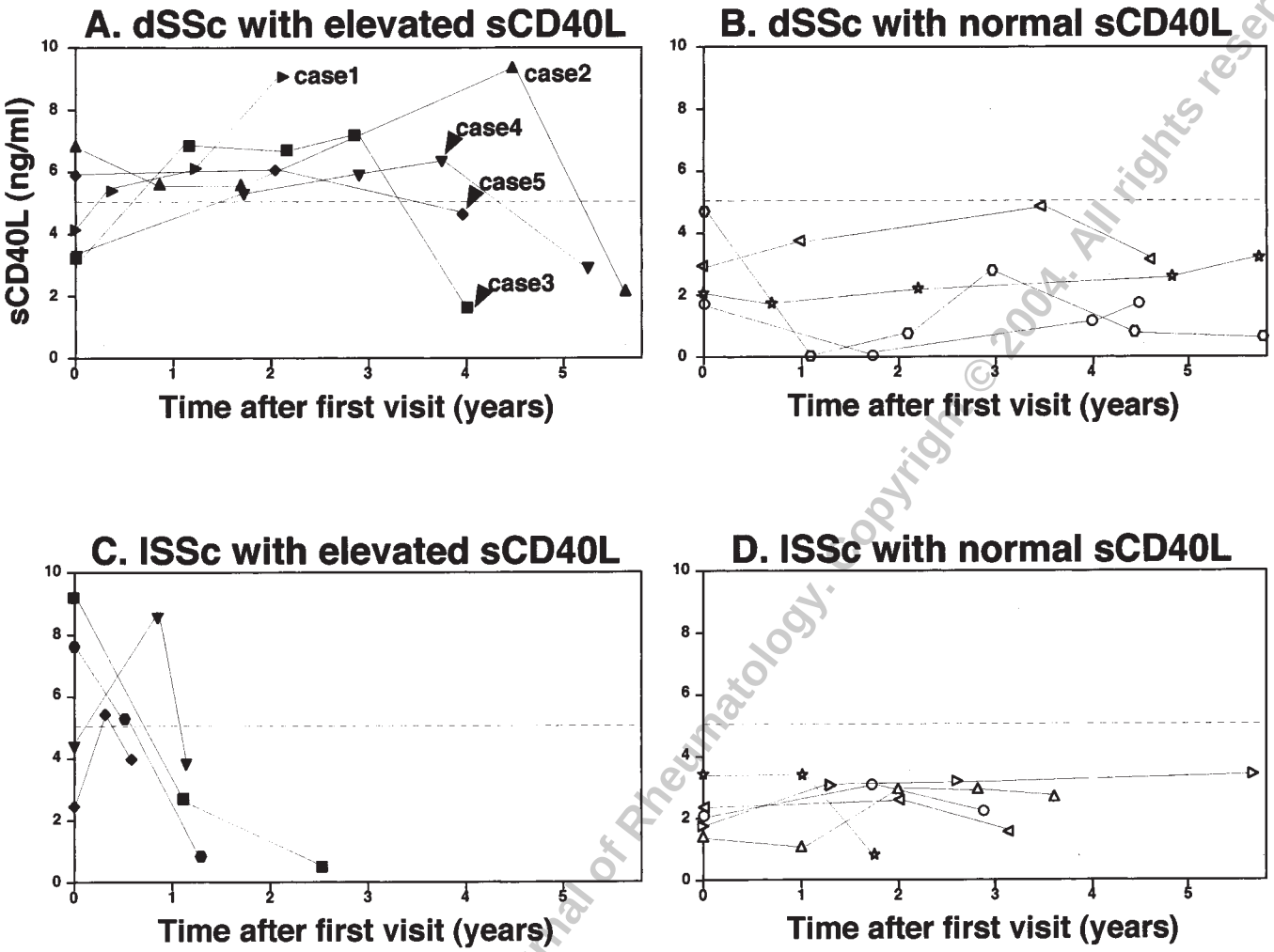

Figure 4. Serial changes of serum sCD40L concentrations during the followup period in SSc patients with dSSc (A, B) and lSSc (C, D). According to K-mean clustering analysis, the dSSc patient sample was divided into 2 subgroups: dSSc patients with sCD40L levels > 5.0 ng/ml during followup (dSSc with elevated sCD40L, A) or dSSc patients with normal sCD40L levels during followup (dSSc with normal sCD40L, B). Similarly, the lSSc patient sample was divided into 2 subgroups with sCD40L levels > 5.0 ng/ml during followup (lSSc with elevated sCD40L, C) or normal sCD40L levels during followup (lSSc with normal sCD40L, D). Open symbols: normal serum sCD40L levels during followup; closed symbols: elevated serum sCD40L levels. Serum sCD40L levels were determined by a specific ELISA. Broken lines indicate the cutoff value.

CD40/CD40L interactions play important roles in the development of SSc.

Our study showed that patients with dSSc exhibited relatively persistent elevations of sCD40L concentrations, whereas temporary elevations were observed in lSSc patients during followup (Figure 4). In addition to transient expression on activated CD4+ T cells, CD40L can be cleaved from the cell surface of activated T cells[7]. Released sCD40L has profibrotic effects[11,12], activates B cells with autoantibody production[7], and upregulates expression of vascular endothelial adhesion molecules[8,10,30]. Since all these functions are related to the characteristic features of SSc, it is suggested that sCD40L correlates with the induction of SSc, and enhances disease severity in patients with dSSc. However, further studies analyzing expression of CD40 on endothelium or fibroblasts in patients with SSc are needed.

Since there are few established basic therapies for skin sclerosis and lung fibrosis in SSc, new therapeutic agents have been researched. Recently, interactions of CD40/CD40L were shown to be a therapeutic target in SLE[31]. It has been reported that serum sCD40L concentrations are increased and reflect disease severity in SLE[19,20]. Moreover, sCD40L plays a predominant role in augmented proliferative response of normal B cells by SLE sera[20]. In addition, it was reported that anti-CD40L antibody was therapeutically useful to inhibit nephritis in a mouse model of SLE[32] and patients with SLE[31].

Our finding that serum sCD40L concentrations of SSc patients were elevated suggests that inhibition of CD40/CD40L interactions could be a therapeutic target in SSc as well as SLE. CD40L transgenic mice in which CD40L expression is targeted to the basal keratinocytes of

epidermis consistently develop SSc-like systemic autoimmunity. Specifically, these mice exhibit positive antinuclear antibody, skin fibrosis, and inflammation in skin and lung[33]. Moreover, blockade of CD40L by anti-CD40L antibody in cultured T and B cells from SSc patients inhibited anti-topoisomerase I antibody production[34]. In addition, blocking CD40/CD40L interactions with antibody prevented increased collagen deposition in the lungs during hapten-induced pulmonary interstitial fibrosis[11]. Therapy of anti-CD40L antibodies showed a low risk in one human study[31].

These findings together with our own suggest that CD40/CD40L interaction may be a potential target in the therapy of SSc.

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