# Increased Serum Soluble CD40 Levels in Patients with Systemic Sclerosis

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*ABSTRACT.* ***Objective.*** To determine serum levels of soluble CD40 (sCD40) and clinical association in patients with systemic sclerosis (SSc).

***Methods.*** Serum sCD40 levels were examined by ELISA in 49 patients with SSc, 15 patients with systemic lupus erythematosus, and 26 healthy individuals. sCD40 levels in plasma samples, which were obtained at the same time, were also determined. SSc patients were grouped into 22 patients with limited cutaneous SSc (lcSSc) and 27 patients with diffuse cutaneous SSc (dcSSc).

***Results.*** There was no significant difference between sCD40 levels of sera and those of plasma. Serum sCD40 levels were significantly elevated in patients with SSc compared to patients with systemic lupus erythematosus and controls ($p < 0.001$). Serum sCD40 levels were higher in patients with lcSSc than in those with dcSSc ($p < 0.001$). There was no correlation between sCD40 and sCD40 ligand levels in patients with SSc.

***Conclusion.*** Elevated serum sCD40 levels were associated with lcSSc. These results suggest that the blockade of CD40/CD40 ligand interaction could be a potential therapeutic strategy in SSc. (First Release Jan 15 2007; J Rheumatol 2007;34:353–8)

*Key Indexing Terms:*
SYSTEMIC SCLEROSIS CD40 SYSTEMIC LUPUS ERYTHEMATOSUS

Interaction between CD40 and CD40 ligand (CD40L, CD154) is essential for humoral and cellular immune response[1]. CD40 is a 50-kDa type I transmembrane protein that belongs to the tumor necrosis factor receptor superfamily. CD40 is expressed on the surface of immune cells including B cells, dendritic cells, and monocytes/macrophages[1], as well as other cell types, including endothelial cells[2] and fibroblasts[3]. CD40 signaling triggered by CD40L regulates various functions including B cell survival, immunoglobulin (Ig) class switching, and cytokine production[1]. CD40/CD40L interactions are likely to play a significant role in the development of autoimmune diseases[4]. Increased expression of CD40L has been detected on T and B cells from patients with active systemic lupus erythematosus (SLE)[5,6]. An increased serum level of soluble CD40L has been also reported in various collagen diseases such as SLE[7,8], systemic sclerosis (SSc)[9,10], and rheumatoid arthritis[11]. Further, autoantibody to CD40L has been demonstrated in SLE[12]. Therefore, CD40/CD40L interaction is an attractive therapeutic target in autoimmune diseases, and anti-CD40L monoclonal antibodies (Ab) have been used in clinical trials[13,14].

SSc is a connective tissue disease characterized by excessive extracellular matrix deposition in the skin and other visceral organs. The presence of autoantibodies is a central feature of SSc since > 90% of patients with SSc have autoantibodies reacting with intracellular components, such as DNA topoisomerase I (topo I), centromere, and RNA polymerases. Furthermore, patients with SSc exhibit hyper-γ-globulinemia, polyclonal B cell hyperactivity, skewed B cell phenotype, and altered B cell homeostasis characterized by expanded naive B cells and activated but diminished memory B cells[15]. Although the etiology of SSc remains unknown, these B cell abnormalities may be related to the disease development[16,17], in which CD40 may have an important role. For example, anti-topo I Ab production is dependent on CD40-CD40L interaction[18]. Increased expression of CD40L in activated CD4+ T cells[19] as well as increased circulating soluble CD40L concentrations have been detected in patients with SSc[9]. Roles of CD40 in SSc are intriguing since CD40 is expressed not only in B cells but also in fibroblasts and endothelial cells[2,3]. Indeed, CD40 expression is augmented on SSc fibroblasts compared to normal fibroblasts[20]. Collectively, CD40 possibly regulates various aspects of the pathogenesis in SSc.

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The soluble form of CD40 (sCD40), which can interfere with CD40-CD40L interaction, is produced by shedding from CD40-expressing cells[21]. While circulating sCD40 levels are reported to be elevated in patients with chronic renal failure, chronic liver diseases, and Alzheimer disease[22-25], the prevalence and clinical significance of sCD40 in autoimmune diseases have not been clarified. We measured the levels of sCD40 in sera from patients with SSc as well as patients with other autoimmune diseases.

## MATERIALS AND METHODS

*Serum samples*. Serum samples were obtained from 49 Japanese patients with SSc (44 female and 5 male). All patients fulfilled the criteria for SSc proposed by the American College of Rheumatology[26]. These patients were 2–76 years old (mean age 50). They were grouped according to the classification system proposed by LeRoy, *et al*[27]: 22 patients (all female) had limited cutaneous SSc (lcSSc) and 27 patients (22 female and 5 male) diffuse cutaneous SSc (dcSSc). The disease duration of patients with lcSSc and dcSSc was 9.9 ± 9.6 and 3.1 ± 3.4 years, respectively. Five patients had been treated with low-dose corticosteroids (prednisolone, 5–20 mg/day) and 4 patients with low dose D-penicillamine (100–500 mg/day) at the first visit. No patient with SSc had received immunosuppressive therapy, or had a recent history of infection or other inflammatory diseases. All patients with SSc except one were treated with antiplatelet agents, such as aspirin and/or prostaglandin $I_2$. As a disease control, we also examined serum samples from 15 patients with SLE that fulfilled the American College of Rheumatology criteria[28]. SLE patients with more than 10 SLE Disease Activity Index points were included in this study. Since patients with reduced renal function were reported to have elevated sCD40 levels, SLE patients with renal involvement were excluded in this study. No patient with SLE was treated with antiplatelet agents. Twenty-six healthy Japanese persons (23 female and 3 male) were used as controls. Controls were 7–70 years old (mean age 46).

For a retrospective longitudinal study, patients whose serum samples were taken more than 3 times were analyzed. They included 65 serum samples from 16 patients with SSc (all female) out of 49. These patients were classified into 8 patients with lcSSc and 8 with dcSSc. They were 9–71 years old (mean age 54). Their disease duration at their first visit was 2.2 ± 3.4 years. These patients had been followed up for 3.6 ± 1.5 years (1.3–5.8 yrs) with 4.1 ± 1.0 (3–6) different timepoints. At the first visit, none had been treated with corticosteroids or D-penicillamine. All 8 patients with dcSSc received low-dose corticosteroids (prednisolone, 5–20 mg/day), and one received low-dose D-penicillamine (100 mg/day) after the first visit. Treatment with corticosteroids or D-penicillamine was not started in any patients with lcSSc, and no patient with SSc received immunosuppressive therapy throughout the followup period. Peripheral venous blood sample was drawn into pyogen-free blood collection tubes without additives, immediately immersed in melting ice, and allowed to clot one hour before centrifugation (1,500 g at 4°C for 10 minutes). All samples were stored at –70°C prior to use.

*Plasma samples*. Serum and plasma samples were simultaneously obtained from 8 patients with SSc, 3 with SLE, and 5 controls. The EDTA-treated blood samples were immediately immersed in melting ice and centrifuged at low speed (300 g at 4°C for 5 minutes), and the plasma fraction was recovered. The plasma samples were then centrifuged again (1,000 g at 4°C for 30 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. Skin score was measured by the scoring technique of the modified Rodnan total skin thickness score (TSS)[29]. Organ system involvement was defined as described previously: 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 without any other explanation; and muscle = proximal muscle weakness and elevated serum creatine kinase. The protocol was approved by the Kanazawa University School of Medicine and Kanazawa University Hospital.

*Enzyme linked immunosorbent assay (ELISA)*. Specific ELISA kits were used for measuring serum sCD40 levels (Medsystems Diagnostics, Vienna, Austria), according to the manufacturer's protocol. Each sample was tested in duplicate. Levels of anticentromere or anti-topo I Abs were assessed using specific ELISA (Medical & Biological Laboratories, Nagoya, Japan).

*Statistical analysis*. Statistical analysis was performed using Mann-Whitney U-test for comparison of sCD40 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. A p value less than 0.05 was considered statistically significant. All data are shown as means ± standard deviation (SD). In the longitudinal study, it was difficult to separate into clusters of each disease subset and conduct cluster analysis.

## RESULTS

*Comparison of sCD40 levels between serum and plasma samples*. In several soluble factors, serum levels do not reflect their synthesis by peripheral tissues, and they are not representative of circulating levels at the time of sampling. Moreover, platelets constitutively express CD40. Therefore, first we determined sCD40 levels in serum samples and plasma samples, which were obtained simultaneously (Figure 1). In patients with SSc, sCD40 levels in plasma were slightly higher than those in sera, although there was no statistical difference (Figure 1). sCD40 levels in controls and those in SLE were similar between serum and plasma samples. The results suggest that elevated serum and plasma sCD40 levels in patients with SSc were not reflected by platelet releasing during sampling.

*Serum sCD40 levels were elevated in patients with SSc*. Then we assessed sCD40 levels using preserved serum samples from patients with SSc. Serum sCD40 levels were significantly higher in patients with SSc than in controls ($p < 0.001$) and patients with SLE ($p < 0.001$, Figure 2). Concerning subgroups of SSc, sCD40 levels were significantly elevated in patients with lcSSc compared with those with dcSSc ($p < 0.001$). By contrast, serum sCD40 levels were similar between patients with SLE and controls. While the expression of CD40 has been reported to increase with some cancers, including leukemias, non-Hodgkin's lymphoma, multiple myeloma, and lung cancer[30,31], no patient with SSc in this study had malignant diseases.

When values higher than the mean + 3 SD (34.1 ng/ml) of the control serum samples were considered to be elevated in this study, sCD40 levels were elevated in 80% (39/49) of patients with SSc. Elevated sCD40 levels were found in 95% (21/22) of patients with lcSSc, significantly more frequently than in patients with dcSSc (18/27, 66%; $p < 0.05$). Accordingly, sCD40 levels were elevated in patients with lower TSS ($p < 0.05$, Table 1). Further, sCD40 levels correlated negatively with TSS at the first evaluation ($r = –0.458$, $p <$

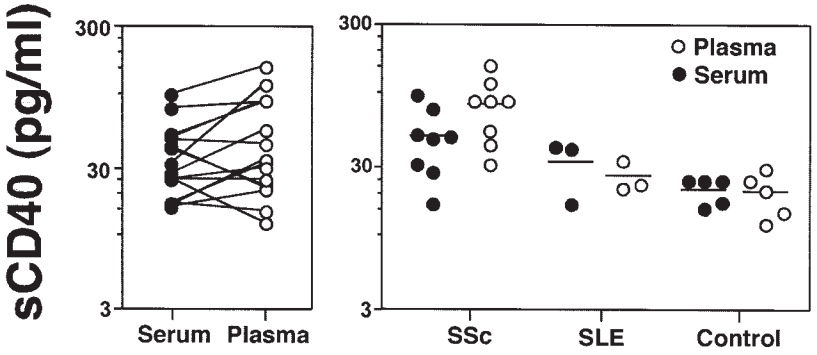


*Figure 1*. Serum and plasma levels of sCD40 in patients with systemic sclerosis (SSc) or systemic lupus erythematosus (SLE) and controls. Serum sCD40 levels were determined by a specific ELISA. Short bar indicates the mean value in each group.

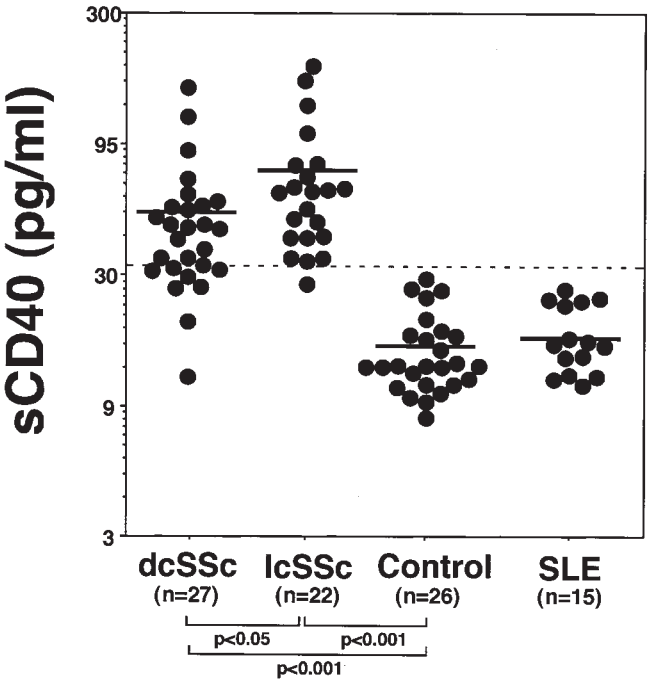


*Figure 2*. Serum levels of sCD40 in patients with diffuse cutaneous SSc (dcSSc), limited cutaneous SSc (lcSSc), or SLE and controls at the first evaluation. Serum sCD40 levels were determined by a specific ELISA. Short bar indicates the mean value in each group. Broken line indicates the cutoff value (mean + 3 SD of the control samples).

*Table 1*. Clinical and laboratory features of patients with SSc showing elevated serum sCD40 levels. Unless noted otherwise, values are percentages.

| | Elevated sCD40 (n = 39) | Normal sCD40 (n = 10) |
|---|---|---|
| Age at onset, yrs, mean ± SD | 50.6 ± 15.6 | 51.4 ± 16.0 |
| Sex, male:female | 3:36 | 0:10 |
| Duration, yrs, mean ± SD | 6.2 ± 7.4 | 4.1 ± 3.7 |
| Clinical features | | |
| dcSSc | 46* | 90 |
| lcSSc | 54* | 10 |
| TSS, points, mean ± SD | 11.6 ± 8.9* | 19.7 ± 4.2 |
| Pitting scars | 44 | 40 |
| Contracture of phalanges | 49 | 80 |
| Diffuse pigmentation | 54* | 100 |
| Organ involvement | | |
| Lung | 36 | 50 |
| Esophagus | 74 | 70 |
| Heart | 13 | 20 |
| Kidney | 3 | 0 |
| Joint | 21 | 30 |
| Muscle | 10 | 30 |
| Laboratory findings | | |
| Anti-topoisomerase I | 31 | 40 |
| Anticentromere | 38 | 10 |
| Increased IgG | 41 | 30 |
| Elevated ESR | 26 | 40 |
| Elevated CRP | 13 | 20 |
| Elevated sCD40L | 49 | 60 |

dcSSc: diffuse cutaneous systemic sclerosis; lcSSc: limited cutaneous SSc; TSS: modified Rodnan total skin thickness score; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein. * $p < 0.05$ vs. SSc patients with normal sCD40 levels.

0.03, Figure 3A). TSS in patients with normal sCD40 levels was significantly higher than those with elevated sCD40 levels ($p < 0.03$, Figure 3B). In addition, SSc patients with elevated sCD40 levels had less frequency of diffuse pigmentation than those with normal sCD40 levels ($p < 0.05$). One patient with renal failure showed elevated sCD40 level (Table 1). Nonetheless, there was no statistical difference between renal function and sCD40 levels, since almost all patients with SSc had normal renal function. By contrast, sCD40 levels did not significantly correlate with serum levels of anti-topo I Abs by ELISA, anticentromere antibodies by ELISA, IgG, IgA, IgM, CH50, C3, C4, C-reactive protein, or erythrocyte sedimentation rates (Table 1 and data not shown). Serum levels of sCD40 did not correlate with serum sCD40L levels, which were also elevated in patients with SSc (Table 1)[9]. Collectively, elevated sCD40 levels were associated with lcSSc.

*A longitudinal study of sCD40 levels*. To assess changes in

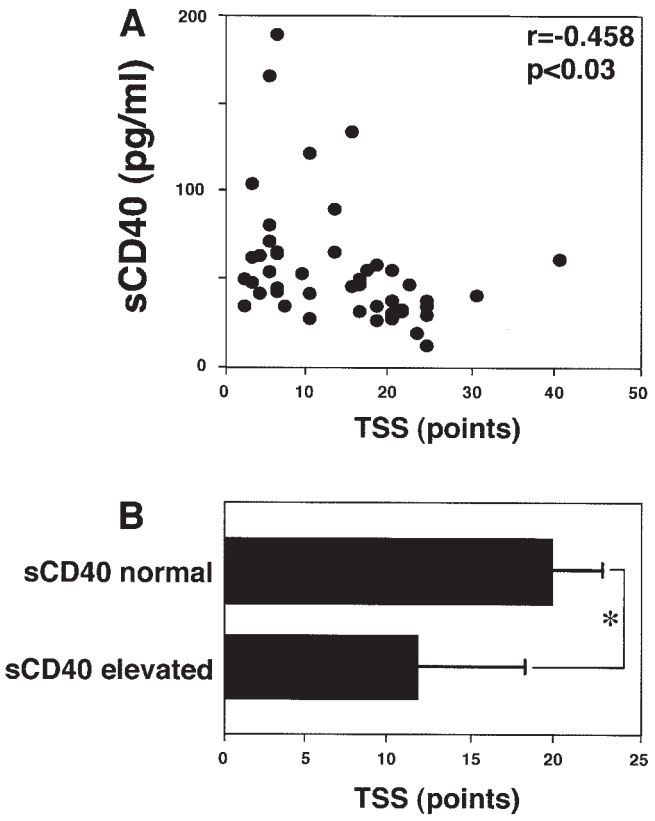


*Figure 3*. A. The correlation of total skin thickness score (TSS) against serum levels of sCD40 in patients with SSc at the first evaluation. Serum sCD40 levels were determined by a specific ELISA. TSS = modified Rodnan TSS. B. TSS in patients with normal sCD40 levels or elevated sCD40 levels. *p < 0.05.

serum sCD40 levels over time, 65 serum samples from 16 patients with SSc were analyzed (Figure 4). At the first visit, no patient had been treated with corticosteroids or D-penicillamine. Six of 8 dcSSc patients with elevated sCD40 levels at their first visit had stable or slightly decreased levels during the followup period. The remaining 2 patients with dcSSc had normal sCD40 levels at their first visit. In one of those patients sCD40 level was stable within normal range during the followup. The other showed temporal elevation, which returned to the normal range after 3 years' followup. Nonetheless, there was no clinical difference between patients showing stable or slightly increased sCD40 levels and a patient showing decreased sCD40 levels during the followup. All 7 patients with lcSSc showed elevated sCD40 levels at their first visit. Five of 7 patients with lcSSc showed gradually elevated sCD40 levels during followup, while 2 showed stable sCD40 levels. There was no clinical and therapeutic difference. No patient with lcSSc received steroids or D-penicillamine or had worsening skin sclerosis, or developed new organ involvement during the observation period.

## DISCUSSION

While circulating sCD40 can modulate CD40/CD40L interaction in immune response or may at least reflect abnormal CD40 regulation, sCD40 levels had not been assessed in collagen diseases. Our results show that serum sCD40 levels were specifically elevated in patients with SSc. In particular, sCD40 levels were significantly elevated in patients with lcSSc compared to those with dcSSc. In longitudinal studies, several patients with lcSSc showed gradually elevated sCD40 levels without worsening skin sclerosis or new organ involvement during followup (data not shown). Together with the fact that anti-CD40L monoclonal Ab appears effective in some autoimmune diseases, these results suggest that the persistent elevation of serum sCD40 levels plays a protective role from the progression of SSc.

In our study, serum levels of sCD40 did not correlate with serum sCD40L levels. Furthermore, longitudinal analysis of sCD40 in patients with SSc in our study contrasts with that of sCD40L levels we demonstrated previously[9]. As for sCD40L levels, patients with dcSSc exhibited persistent elevations, while those with lcSSc showed temporary elevations at the early timepoints. These results suggest that sCD40 and sCD40L levels are regulated independently. Since CD40L expression is augmented in activated T cells from patients with SSc[19], the interaction between CD40L on T cells and CD40 on fibroblasts/endothelial cells may contribute to

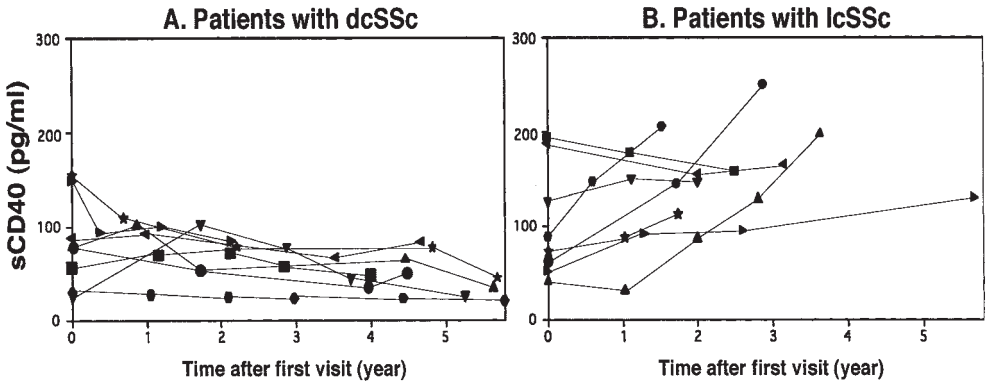


*Figure 4*. Serial changes of serum sCD40 levels during the followup period in patients with dcSSc (A) and those with lcSSc (B). Serum sCD40 levels were determined by a specific ELISA.

pathogenesis in SSc. In this process, sCD40L may trigger CD40 signaling pathway, while sCD40 may play an antagonistic role, which may be a similar function of anti-CD40L Ab.

sCD40 is produced through cleavage from CD40-expressing cells[21]. High concentration of sCD40 in patients with chronic renal failure who are on dialysis[22,23] suggests that circulating sCD40 is constitutively produced and that the level is tightly regulated. In addition, since CD40 engagement is shown to induce sCD40 release, CD40 activation may also contribute to the elevated sCD40 levels. Thus, increased sCD40 in SSc is likely to result from increased expression and/or activation of CD40 on B cells, fibroblasts, and endothelial cells. Increased CD40 expression in SSc fibroblasts has been demonstrated[20], although the results that patients with lcSSc had higher levels of sCD40 may lead to the speculation that the major source is not fibroblasts. The CD40 pathway, especially in B cells and/or endothelial cells, may be more strongly activated in lcSSc than in dcSSc. Alternatively, considering that CD40 engagement downregulates cell-surface CD40 expression at the same time[21], sCD40 release may be suppressed when CD40 pathway is activated too strongly. If this is the case, it may partly explain normal serum sCD40 levels in patients with active SLE as well as in those with dcSSc in our study.

CD40/CD40L interaction appears to play important roles in the pathogenesis of SSc. CD40L transgenic mice in which CD40L overexpression is seen on the basal keratinocytes of epidermis develop skin fibrosis and inflammation in skin and lung in addition to antinuclear Ab production[32]. Blockade of CD40L by anti-CD40L Ab prevented increased collagen deposition in the lungs during hapten-induced intestinal fibrosis[33] and *in vitro* anti-topo I Ab production in cultured T and B cells from patients with SSc[18]. Collectively, the CD40/CD40L-signal blockade may be a potential strategy for the therapy of SSc as well. Our results suggest that sCD40 levels were elevated in a milder subset of SSc in comparison to a severe subset. Therefore, plenty of sCD40, which can block CD40/CD40L interactions, may prevent the development of SSc, although further studies are needed. Since anti-CD40L monoclonal Ab therapy has caused several complications[34], administration of sCD40 may be a potential alternative strategy for treating autoimmune diseases in which CD40/CD40L interaction may play a role.

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