immune response from a Treg cell response toward a pathogenic Th17 cell response (16). Consistent with these findings, the results of the present study showed that treatment with topo I and CFA increased Th17 cell frequencies and decreased Treg cell frequencies in parallel with increased IL-6 and TGFβ1 overproduction (Figures 2 and 6). In contrast, treatment with topo I and IFA increased Treg cell frequencies and decreased Th17 cell frequencies. Furthermore, lack of IL-6 expression inhibited accumulation of Th17 cells and increased numbers of Treg cells in mice treated with topo I and CFA (Figure 6).

Recently, other studies suggested that IL-6 also promotes Th2 cell differentiation and simultaneously inhibits Th1 cell polarization (46,47). In the absence of any polarizing cytokine, IL-6 directs the differentiation of the CD4+ cells to a Th2 phenotype but not to a Th1 phenotype, since cells differentiated in the presence of IL-6 produce high amounts of IL-4 but not IFNγ (46,47). IL-4 promotes Th2 differentiation but inhibits Th1 differentiation, although IFNγ stimulates Th1 differentiation but suppresses Th2 differentiation (48–50). Indeed, in the present study, treatment with topo I and CFA increased Th2 cell frequencies and decreased Th1 cell frequencies in WT mice (Figure 6). Moreover, IL-6 deficiency inhibited augmentation of Th2 cell frequencies and increased Th1 cell frequencies. Collectively, treatment with topo I and CFA may lead to increased Th2 and Th17 cell frequencies in parallel with deteriorated skin and lung fibrosis, whereas topo I and IFA treatment leads to increased Th1 and Treg cell frequencies that are accompanied by inhibited fibrosis of skin and lung.

To date, few studies have addressed the role of induction of anti–topo I antibody production and its potential association with the pathogenesis of SSc. This is the first systematic study to reveal that treatment with topo I and CFA induces SSc-like dermal sclerosis, pulmonary fibrosis, and autoimmune abnormalities in mice. However, the exact role of anti–topo I antibody in fibrogenesis and overproduction of cytokines remains unclear. Future studies, in which anti–topo I antibodies are isolated from mice treated with topo I and adjuvant and transferred into recipient mice, will be needed to clarify the exact role of anti–topo I antibody in this pathogenesis. We also suggest that IL-6 plays important roles in the development of fibrosis and autoimmune abnormalities in this novel model of SSc induced by treatment with topo I and CFA. These results provide additional clues to understanding the complexity of the pathogenesis of SSc.

ACKNOWLEDGMENTS

We thank Ms M. Yozaki, Ms A. Usui, and Ms K. Shimoda for technical assistance.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Sato had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Yoshizaki, Sato.

Acquisition of data. Yoshizaki, Yanaba, Ogawa.

Analysis and interpretation of data. Yoshizaki, Ogawa, Asano, Kadono, Sato.

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Altered dynamics of transforming growth factor β (TGF-β) receptors in scleroderma fibroblasts

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Accepted 16 August 2010
Published Online First 15 November 2010

ABSTRACT

Objectives To investigate the difference in the dynamics of transforming growth factor β (TGF-β) receptors between normal and scleroderma fibroblasts.

Methods The cell surface expression levels of TGF-β receptors were determined by biotinylation and immunoprecipitation assay. The dynamics of TGF-β receptors on the cell surface was determined by the reversible biotinylation assay. The subcellular localisation of TGF-β receptors was determined by immunoprecipitation using antibodies against clathrin and caveolin.

Results Although the total expression levels of TGF-β receptors were elevated in scleroderma fibroblasts compared with normal fibroblasts, there was no significant difference in the cell surface expression levels of TGF-β receptors between these two groups. However, the internalisation rate of TGF-β receptors was higher in scleroderma fibroblasts compared with normal fibroblasts. Furthermore, caveolin constitutively made a complex with TGF-β receptors, while the interaction of clathrin with TGF-β receptors was marginal in scleroderma fibroblasts.

Conclusions The dynamics of TGF-β receptors on the cell surface is accelerated in scleroderma fibroblasts. Considering that the activation state of TGF-β signalling is regulated by a balance between the clathrin-dependent internalisation and the lipid raft-caveolar internalisation, the accumulation of TGF-β receptors in caveolin-positive vesicles may result in the deceleration of caveolin-dependent internalisation and subsequently lead to the relative acceleration of clathrin-dependent internalisation.

INTRODUCTION

Systemic sclerosis or scleroderma (SSc) is an acquired disorder typically resulting in fibrosis of skin and internal organs due to inflammation, autoimmune reaction and vascular damage.[1] However, cultured SSc fibroblasts free from such stimulation keep producing excessive amounts of extracellular matrix proteins,[2] suggesting that once activated, these cells establish a constitutive self-activation system. One of major cytokines involved in this process is transforming growth factor β (TGF-β)1.[3–6]

TGF-β1 initiates signalling through the ligand-dependent activation of TGF-β receptors. Upon binding TGF-β, the receptors rotate within the complex, resulting in the phosphorylation and activation of type I receptor (TβRI) by the constitutively active and autophosphorylated type II receptor (TβRII). The activated TβRI directly phosphorylates Smad 2/3, which subsequently associates with Smad4 and translocate to the nucleus, where the complex regulates transcriptional responses of target genes together with additional DNA-binding cofactors.[7] The inhibitory Smad7 associates with ligand-activated TβRI and interferes with the phosphorylation of Smad2/3.[8] In addition, Smad7 recruits ubiquitin ligases, such as Smad ubiquitination regulatory factor (Smurf)1/2, to the TGF-β receptor complex and promotes its degradation through proteasomal and lysosomal pathways.[9 10] Since the expression of Smad7 is induced by TGF-β1, Smad7 inhibits TGF-β signalling by a negative feedback system.[8]

Di Guglielmo *et al*[11] previously proposed a novel model of TGF-β signalling. The clathrin-dependent internalisation of TGF-β receptors into the early endosome antigen-1-positive endosome, where the anchor protein for Smad2 is abundant, promotes TGF-β signalling, while the lipid raft-caveolar internalisation pathway contains the Smad7–Smurfs-bound receptor and is required for rapid receptor turnover. These two pathways are mutually exclusive and the activation status of TGF-β signalling is regulated by the balance between them. We previously demonstrated that Smad7–Smurf2-mediated negative regulation of TGF-β signalling was impaired in SSc fibroblasts.[3] We also verified that Smad7 was upregulated and constitutively made complex with TGF-β receptors in cytoplasm and the degradation rate of TβRI was decreased in SSc fibroblasts. Collectively, we speculated that the increased stability of the Smad7–TβRI complex resulted in an accumulation of this complex in caveolin-positive vesicles and subsequently disturbed the efficient lipid raft-caveolar internalisation, and such an abnormality led to the relative acceleration of the clathrin-dependent internalisation. The purpose of this study is to assess this hypothesis.

METHODS

Cell cultures

Ten strains of SSc fibroblasts and closely matched control fibroblasts were prepared.[3 4]

Immunoblotting

Proteins levels were determined by immunoblotting as described previously.[3 4]

Biotinylation and immunoprecipitation

Cell surface expression levels of TGF-β receptors were determined by immunoprecipitation following biotinylation with membrane-impermeant NHS-LC-biotin as described previously.[12]

Reversible biotinylation assay

The internalisation rate of TGF-β receptors was determined using sulfo-NHS-SS-biotin and an impermeant reducing agent as described previously.[12]

Statistical analysis

Statistical analysis was carried out with the Mann–Whitney U test for comparison of means. p Values less than 0.05 were considered significant.

RESULTS

Cell surface expression levels of TGF-β receptors in normal and SSc fibroblasts

Previous reports demonstrated the increased expression of TGF-β receptors in SSc fibroblasts.[3 4] Therefore, we initially confirmed this notion by immunoblotting (top and middle panels in figure 1A). Consistently, TβRI was 2.96 times elevated and TβRII was 1.73 times elevated in SSc fibroblasts compared with normal fibroblasts. Since we performed immunoblotting using whole cell lysates, the levels of TGF-β receptors detected in these experiments means the total amount of TGF-β receptors (cell surface plus cytoplasm). Thus, we next investigated the cell surface expression levels of TGF-β receptors. To this end, cells were incubated in cold media for 30 min to inhibit completely the internalisation of TGF-β receptors, and cell surface proteins were subsequently labelled with biotins. Then, whole cell lysates were subjected to immunoprecipitation with antibodies against TGF-β receptors. As shown in bottom panels in figure 1A, cell surface expression levels of TGF-β receptors were comparable between normal and SSc fibroblasts. Collectively, these results indicate that TGF-β receptors located in intracellular regions, but not those on the cell surface, are elevated in SSc fibroblasts compared with normal fibroblasts.

The internalisation rate of TGF-β receptors in normal and SSc fibroblasts

To investigate the dynamics of TGF-β receptors on the cell surface, we performed immunoprecipitation using cell lysates prepared from cells labelled with biotin for 3 h at 37°C, where TGF-β receptors on the cell surface were continuously labelled. As shown in figure 2A, the level of TGF-β receptors labelled with biotin was elevated in SSc fibroblasts compared with normal fibroblasts. Accordingly, we speculated that the internalisation rate of TGF-β receptors on the cell surface was accelerated in SSc fibroblasts. To clarify this point, we performed the reversible biotinylation assay. As shown in figure 2B,C, the internalisation rate of TGF-β receptors was significantly elevated in SSc fibroblasts compared with normal fibroblasts. These results support the hypothesis described above.

The subcellular localisation of TGF-β receptors in SSc fibroblasts

We next investigated the subcellular localisation of TGF-β receptors in normal and SSc fibroblasts. To this end, cell surface proteins were labelled with biotins at 4°C and whole cell lysates were prepared. Cell surface proteins were then removed by immunoprecipitation with streptavidin-coupled beads. The comparable cell surface expression levels of TGF-β receptors between normal and SSc fibroblasts were confirmed by immunoblotting using these precipitants (figure 3A). Supernatant fractions were subjected to immunoprecipitation using anti-clathrin antibody or anti-caveolin antibody. Precipitated proteins were subjected to immunoblotting using antibodies for TGF-β receptors. As shown in lanes 3 and 4 in figure 3B, the constitutive complex formation of caveolin with TGF-β receptors was observed in SSc fibroblasts, while the complex of clathrin with TGF-β receptors was not detected. In contrast, we could not detect TGF-β receptors in precipitated proteins by anti-clathrin antibody or anti-caveolin antibody in normal fibroblasts untreated with TGF-β1 (lane 1 and 2 in figure 3) and those treated with TGF-β1 (data not shown). These results were consistent with

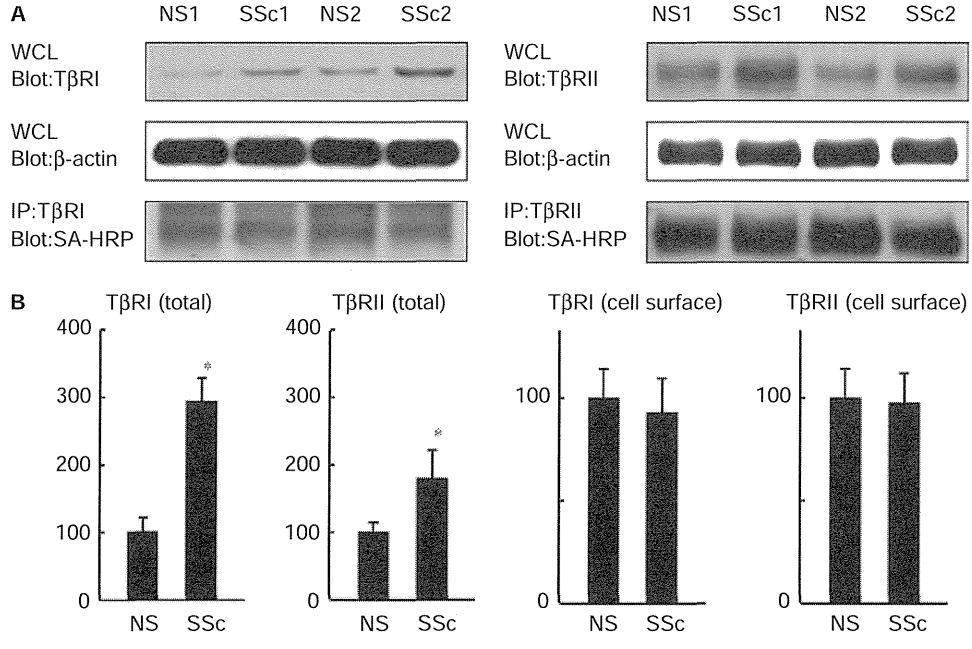

Figure 1 Expression levels of transforming growth factor β (TGF-β) receptors (TβR) in normal and scleroderma fibroblasts. (A) Whole cell lysates were electrophoresed through a 10% polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with the indicated antibodies (top and middle panels). To investigate the cell surface expression levels of TGF-β receptors, cell surface proteins were labelled with biotin at 4°C for 30 min and immunoprecipitation was performed with the indicated antibodies using the same amount of protein extracts. Precipitated proteins were subjected to immunoblotting using streptavidin coupled to horseradish peroxidase (SA-HRP) (bottom panels). One representative of five independent experiments is shown. (B) The protein levels quantified by scanning densitometry are shown relative to those in normal fibroblasts (100). Data are expressed as the mean ± SD of five independent experiments. NS, normal skin fibroblasts; SSc, systemic sclerosis or scleroderma.

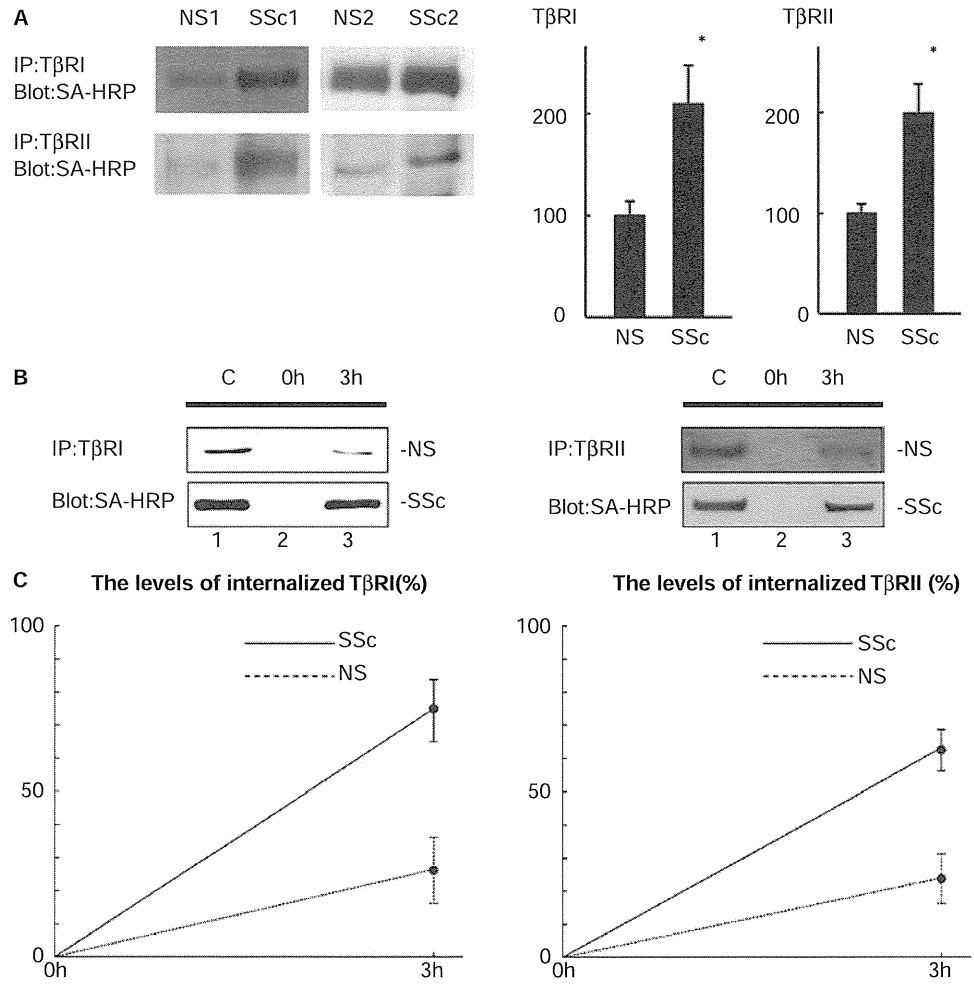

Figure 2 Comparison of the internalisation rate of transforming growth factor β (TGF-β) receptors (TβR) between normal and systemic sclerosis or scleroderma (SSc) fibroblasts. (A) Cell surface proteins were labelled with biotin at 37°C for 3 h. Immunoprecipitation was performed with the indicated antibodies using the same amount of protein extracts. Precipitated proteins were subjected to immunoblotting using streptavidin coupled to horseradish peroxidase (SA-HRP). One representative of five independent experiments is shown. The protein levels quantified by scanning densitometry are shown relative to those in normal fibroblasts (100). Data are expressed as the mean ± SD of five independent experiments. (B) Confluent quiescent fibroblasts were biotinylated at 4°C with sulfo-NHS-SS-biotin (0.5 mg/ml) and rewarmed. At the time points indicated, cells were treated with an impermeant reducing agent to remove the remaining biotin. Whole cell lysate (1 mg) was subjected to immunoprecipitation using anti-TβRII antibody and the Western blots were probed with SA-HRP. Lanes 1, cells untreated with reducing agent at time 0; lanes 2, cells treated with reducing agent at time 0; lanes 3, cells treated with reducing agent at 3 h. (C) Quantitative analysis of the rate of endocytosis of TGF-β receptors. Data are expressed as percent cell surface TβRI (left panel) or TβRII (right panel) at time 0. The mean ± SD of five experiments is shown. NS, normal skin fibroblasts.

previous reports that demonstrated that the complex of TGF-β receptors with clathrin or caveolin could not be detected in normal fibroblasts because the interaction of these molecules is transient.[11] These results suggest that the turnover of the complex of caveolin with TGF-β receptors is decelerated in SSc fibroblasts, which is consistent with our previous report.[3]

DISCUSSION

The biological effect of cytokines is mainly determined by the occurrence of cytokine–receptor interaction, which is modulated by the concentration and the activity of cytokines and/or their receptors. Therefore, the evidence that there is no significant difference in the levels of total (active plus latent) and active TGF-β protein between normal and SSc fibroblast cultures[4 13] suggests that the upregulated expression of TGF-β receptors contributes to the establishment of autocrine TGF-β signalling in SSc fibroblasts. In fact, the total level of TGF-β receptors (cell surface plus intracellular) is elevated in SSc fibroblasts[3–5] and the transient overexpression of TβRI or TβRII increases type I collagen production in normal fibroblasts.[5 14–16] In this study, however, we showed that the cell surface expression level of TGF-β receptors was comparable between normal and SSc fibroblasts. Furthermore, the internalisation of TGF-β receptors was accelerated in SSc fibroblasts. These results indicate that the turnover rate of TGF-β receptors on the cell surface is increased in SSc fibroblasts and that such an abnormality may be involved in the establishment of autocrine TGF-β signalling in those cells.

In SSc fibroblasts, Smad7 constitutively makes a complex with TGF-β receptors and the degradation of TGF-β receptors is decelerated.[3] The present observation that caveolin, but not clathrin, constitutively makes a complex with TGF-β receptors suggests that the increased stability of the Smad7–TβRI complex induces an accumulation of this complex in caveolin-positive vesicles. Since clathrin and caveolin pathways are mutually exclusive and the activation state of TGF-β signalling is regulated by a balance between them, the accumulation of TGF-β receptors in caveolin

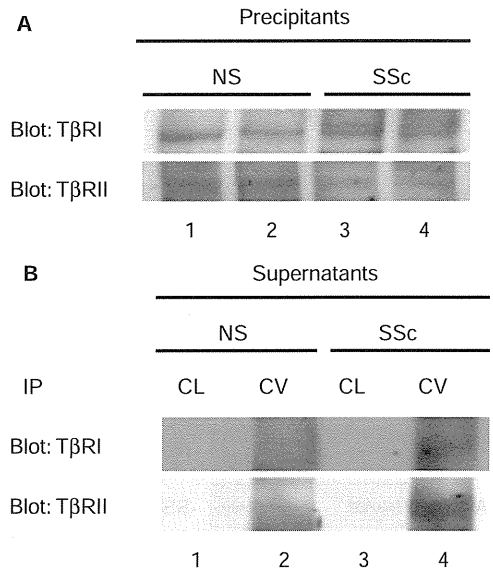

Figure 3 The subcellular localisation of transforming growth factor β (TGF-β) receptors (TβR) in systemic sclerosis or scleroderma fibroblasts. Cell surface proteins were labelled with biotin at 4°C for 30 min. Whole cell lysates (1 mg) were subjected to immunoprecipitation with streptavidin-coupled beads. (A) Precipitated proteins were subjected to immunoblotting using anti-TβRI antibody or anti-TβRII antibody. (B) Supernatant fractions were subjected to immunoprecipitation using anti-clathrin antibody or anti-caveolin antibody. Precipitated proteins were subjected to immunoblotting using anti-TβRI antibody or anti-TβRII antibody. One representative of five independent experiments is shown. NS, normal skin fibroblasts; SSc, systemic sclerosis..

pathway may disturb the efficient lipid raft-caveolar internalisation and such an abnormality may lead to the relative acceleration of the clathrin-dependent internalisation in SSc fibroblasts. This preliminary hypothesis is supported by the following present and previous findings: (1) The internalisation rate of TGF-β receptors is elevated in SSc fibroblasts; and (2) TGF-β signalling is constitutively activated in SSc fibroblasts.

The rapid turnover of cell surface TGF-β receptors requires an increase in the occurrence of interaction between TGF-β and its receptors. Such interaction is modulated by the concentration and the activity of TGF-β and/or its receptors. However, the cell surface expression level of TGF-β receptors was not elevated in SSc fibroblasts. Furthermore, there was almost equal amount of active and total TGF-β protein in normal and SSc fibroblast culture. Collectively, these findings suggest that cell surface molecules recruiting endogenous TGF-β to its receptors may be a promising candidate involved in the establishment of autocrine TGF-β signalling in SSc fibroblasts. Our previous reports indicated that latent TGF-β receptors, such as integrin αvβ5[17] and thrombospondin-1[18] were upregulated, and such abnormalities contribute to the establishment of autocrine TGF-β signalling in SSc fibroblasts.[12 13 18] These cell surface molecules and impaired Smad7–Smurfs-mediated negative regulation of TGF-β signalling may co-ordinately contribute to the establishment of autocrine TGF-β signalling in SSc fibroblasts.

Acknowledgements This study was supported in part by a grant for scientific research from the Japanese Ministry of Education (10770391) and by project research for systemic sclerosis from the Japanese Ministry of Health and Welfare.

Ethics approval This study was conducted with the approval of the University of Tokyo.

Competing interests None.

Provenance and peer review Not commissioned; externally peer reviewed.

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Clin Rheumatol (2011) 30:231–237
DOI 10.1007/s10067-010-1610-4

ORIGINAL ARTICLE

Serum chemokine and cytokine levels as indicators of disease activity in patients with systemic sclerosis

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Received: 2 April 2010 / Revised: 20 September 2010 / Accepted: 19 October 2010 / Published online: 4 November 2010

Abstract To determine the clinical utility of serum levels of chemokines and cytokines for the evaluation of disease activity in patients with systemic sclerosis (SSc), concentrations of four chemokines (interferon γ-inducible protein-10 [IP-10, CXCL10], monokine induced by interferon γ [MIG/CXCL9], monocyte chemoattractant protein-1 [MCP-1/CCL2], interleukin 8 [IL-8/CXCL8]) and six cytokines (IL-2, IL-4, IL-6, IL-10, tumor necrosis factor [TNF]-α, interferon [IFN]- γ) were measured using cytometric beads array kits in serum samples from 31 Japanese patients with SSc and 20 normal controls. Clinical and laboratory data and serum chemokine and cytokine levels were assessed for each patient at their first visit and each subsequent year for 3 years. Among these chemokines and cytokines, serum levels of IP-10, MIG and MCP-1 were significantly elevated in SSc patients compared with normal controls at their first visit. Serum MCP-1 levels declined year and year, along with improvement for skin sclerosis. The variations of MCP-1, but not IP-10 and MIG, were significantly associated with the variations of skin thickness score and vital capacity during 3 years. These results suggest that MCP-1 is a serological indicator of the activity of skin and lung involvement in patients with SSc. However, a longer-term prospective study in a larger population will be needed to confirm its clinical utility as predictors of outcomes.

Keywords Chemokines · Cytokines · Marker · MCP-1 · Scleroderma

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Introduction

Systemic sclerosis (SSc) is a multi-system disorder with an autoimmune background of connective tissues that is characterized by excessive fibrosis and vascular changes in the skin and various internal organs [1–3]. Monocytes/macrophages and T cells show increased numbers or activation in the circulation or tissues of patients with SSc [4, 5]. Infiltration of these cells in SSc may promote endothelial damage and fibrosis, probably through the production of soluble mediators, including cytokines. Recent investigations have identified many potential molecules, including chemokines, that regulate the migration and recruitment of specific leukocytes to regions of inflammation. A large literature on SSc has reported chemokine abnormalities that might explain the altered accumulation of effector leukocyte subsets in affected tissues [6, 7]. Among the various chemokines, monocyte chemoattractant protein-1 (MCP-1/CCL2) most likely plays a critical role in tissue fibrosis in SSc [6, 8, 9].

Microarray analysis has revealed that genes are not differentially expressed in SSc fibroblasts and normal fibroblasts [10], suggesting exogenous fibroblast activation by immunocompetent cells. Macrophages can produce various cytokines, including interleukin (IL)-1, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, IL-6, transforming growth factor (TGF)-β and platelet derived growth factor (PDGF), that can regulate inflammation and tissue

fibrosis [11]. T cells also appear to play a role in the disease process through activation of macrophages and the direct release of inflammatory and pro-fibrogenic cytokines. Thus, the secreted cytokines from infiltrating leukocytes appear to be involved in the development of SSc tissue fibrosis via stimulating collagen synthesis by fibroblasts.

There are no definitive serum markers to estimate the disease activity in the skin or the internal organs in SSc. In most patients, severe organ involvement occurs within the first 3 years of disease onset and skin sclerosis seldom progresses after 5 or 6 years [12]. Therefore, clarifying the disease activity is particularly important for SSc patients with a short disease duration [13]. In the present study, we sought to determine if serum chemokines and cytokines were associated with disease activity in SSc patients with a short disease duration.

Materials and methods

Patients

Thirty-one Japanese patients with SSc (21 females, ten males; age[mean ± SD] = 48 ± 16 years) who had visited Kanazawa University Hospital between January 1, 2002 and January 1, 2005 were included in this study. All patients fulfilled the criteria proposed by the American College of Rheumatology [14]. Patients were grouped according to the degree of skin involvement, based on the classification system proposed by LeRoy et al. et al [15] (diffuse cutaneous SSc (dcSSc) and limited cutaneous SSc (lcSSc)). To evaluate the clinical utility of cytokines and chemokines as biomarkers of disease activity, early SSc patients (disease duration; <3 years) who have diffuse cutaneous SSc (dcSSc) or interstitial pneumonia were included in this study (18 patients were dcSSc with interstitial pneumonia, three patients were dcSSc without interstitial pneumonia, and ten patients were lcSSc with interstitial pneumonia). The disease duration (mean±SD) of patients was 21±15 months. With respect to anti-nuclear antibodies (Ab), 23 patients were positive for anti-topoisomerase I Ab, two patients were positive for anti-centromere Ab, and four patients were positive for anti-RNA polymerase I/III Ab. Twenty healthy subjects (14 females, six males; meanage = 46 ± 15 years) were also evaluated. The protocols were approved by the Kanazawa University Graduate School of Medical Science, and informed consent was obtained from all patients.

Clinical assessments

All patients had a physical examination and laboratory tests at their first visit and each subsequent year for 3 years. A modified Rodnan total skin thickness score (MRSS) was used as a semi-quantitative measure of skin sclerosis [16]. Organ system involvement was defined as described previously [17, 18]: lung = bibasilar fibrosis on chest radiography and high resolution computed tomography; isolated pulmonary hypertension = clinical evidence of pulmonary hypertension and increased mean pulmonary arterial pressure (>35 mmHg) documented by echocardiography, in the absence of severe pulmonary interstitial fibrosis; esophagus = hypomotility shown by barium radiography; joint = inflammatory polyarthralgias or arthritis; heart = pericarditis, congestive heart failure, or arrhythmias requiring treatment; kidney = renal crisis defined as malignant hypertension and rapidly progressive renal failure without any other explanation; and muscle = proximal muscle weakness and elevated serum creatine kinase. Pulmonary function tests included vital capacity (VC) and diffusion capacity for carbon monoxide (DLco). When the DLco and VC were <75% and <80%, respectively, of the predicted normal values, they were considered to be abnormal. Routine laboratory markers including C-reactive protein and erythrocyte sedimentation rate were also assessed.

Serum cytokine and chemokine assays

Fresh venous blood samples were taken at the patients' first visit (baseline) and each subsequent year for 3 years. Samples were centrifuged shortly after clot formation. All serum samples were stored at −70°C prior to assays. Serum levels of chemokines, including interferon γ-inducible protein-10 (IP-10, CXCL10), monokine induced by interferon γ (MIG/CXCL9), MCP-1 and IL-8/CXCL8, and cytokines (IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ) were measured by cytometric beads array kit (BD PharMingen, San Diego, CA) using a FACScan flow cytometer (BD PharMingen).

To determine whether the longitudinal changes of serum cytokines and chemokine levels correlated with the change of clinical features, we analyzed the variation of each chemokine or cytokine levels and the variation of clinical data including MRSS and %VC during 3 years. The variation was defined as '(the value at the third year/the value at the baseline) × 100 (%)'.

Statistical analysis

JMP® Statistically Discovery Software (SAS institute, Cary, NC) was used for analysis. Because the distributions of biomarker concentrations were not normally distributed, Wilcoxon rank sum tests were used for comparisons of biomarker concentrations. Spearman's rank correlation coefficient was used to examine the relationship between two continuous variables. A p value<0.05 was considered statistically significant.

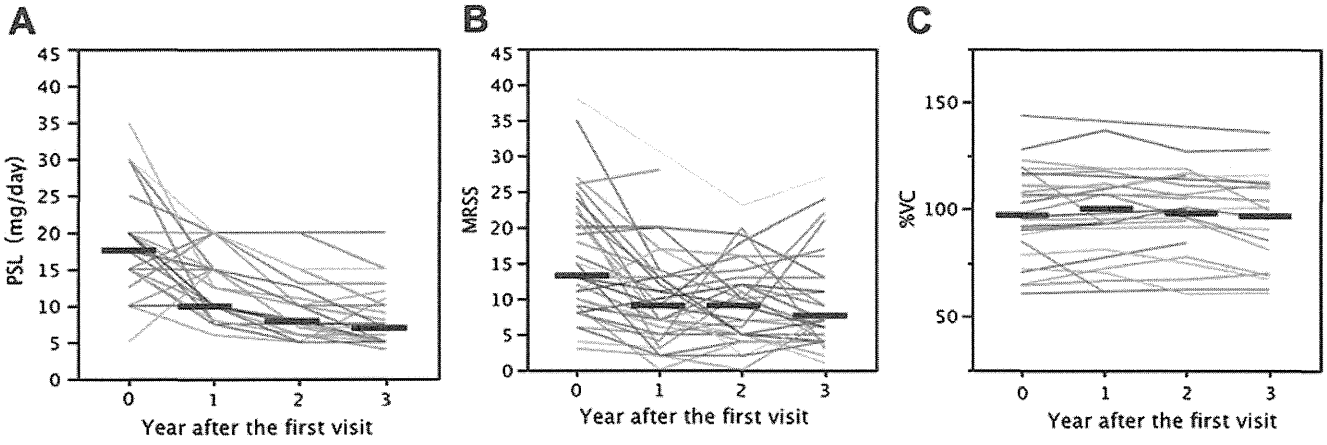

Fig. 1 Longitudinal data for each case during the 3 years. **a** The dose of oral prednisolone (PSL, mg/day), **b** MRSS, **c** %VC. *Short bars* indicate median values at each year

Results

At the time of first visit, 15 patients (48%) had been receiving immunosuppressive treatment. Ten patients had been taking oral prednisolone for treating skin sclerosis. In addition to oral prednisolone therapy, four patients had a history of cyclophosphamide pulse therapy and one patient had been taking oral cyclosporine A for the treatment of active interstitial pneumonia. After their first visits, 15 patients began to take oral prednisolone for treating

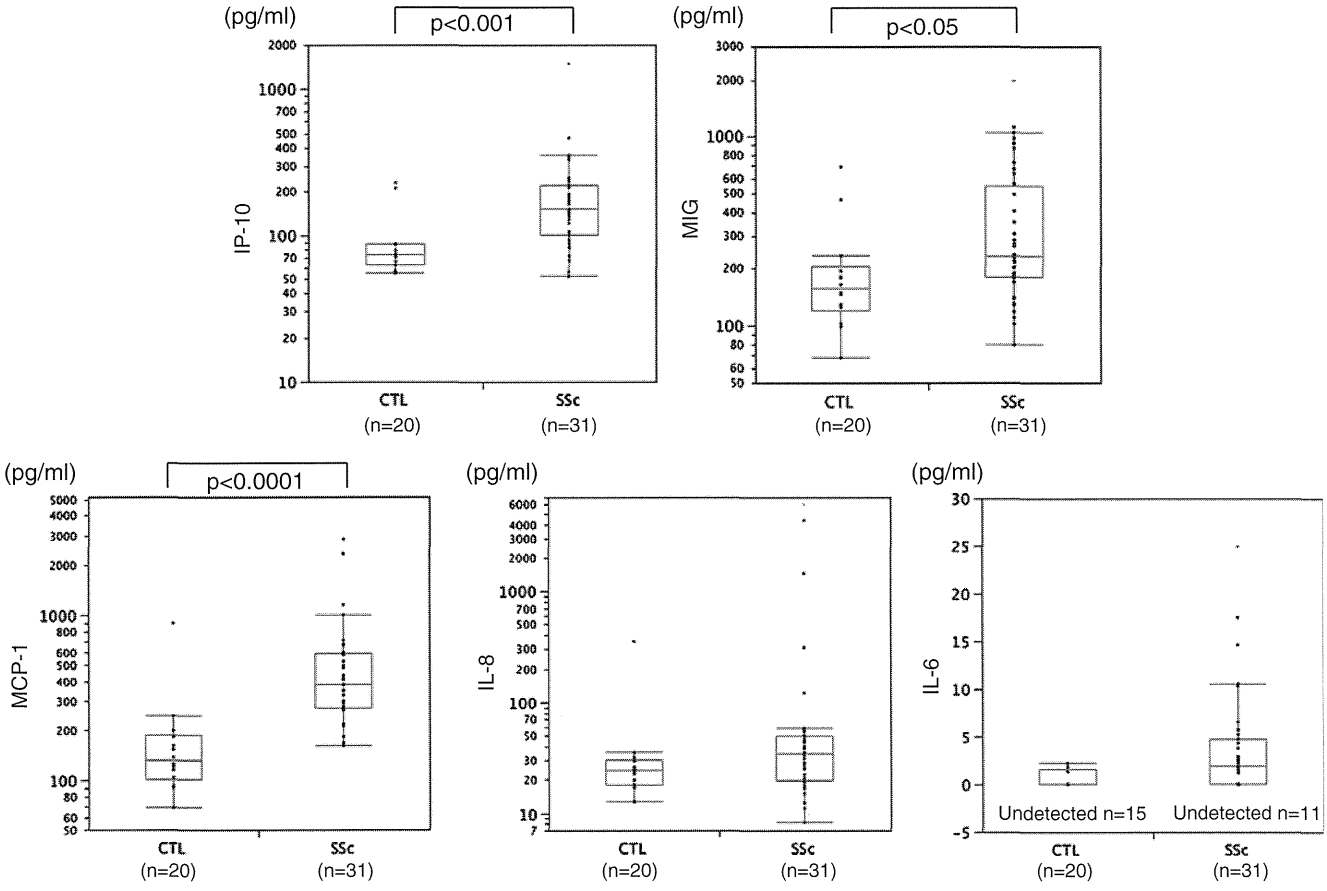

Fig. 2 Serum levels of chemokines and cytokines in SSc patients at their first visit and in normal controls (*CTL*). *Lines inside the boxes* indicate median values; *outer borders of the boxes* indicate the 25th and 75th percentiles; *bars extending from the boxes* indicate the 10th and 90th percentiles. *p* values are from Wilcoxon rank sum tests

progressing skin sclerosis. Thus, all the patients, except for one, were treated with oral prednisolone during the follow-up period.

The median values for prednisolone were 17.5 mg/day at first visit, 10 mg at 1 year, 8 mg at 2 years and 7 mg at 3 years after their first visit (Fig. 1a). Three patients with active interstitial pneumonia started cyclophosphamide pulse therapy after their first visit. Two patients began cyclophosphamide pulse therapy due to aggravation of interstitial pneumonia during the 3 years. One patient died of interstitital pneumonia during the follow-up period. No patients developed renal crisis during the study period.

During the 3 years, the median MRSS was 13 at patients' first visit, nine at the end of both years 1 and 2, and 7 at 3 years after their first visit (Fig. 1b). The median value of % VC did not change during the 3 years: 99.5% at first visit, 100.8% at the end of the first year, 99.8% at the end of the second year and 99.6% at the end of the third year (Fig. 1c).

Serum IP-10 levels were significantly increased in the SSc patients compared with normal controls ($p<0.001$, Fig. 2). Serum MIG levels were also significantly elevated in the SSc patients compared with normal controls ($p<0.05$). Serum MCP-1 levels were markedly increased in the SSc patients compared with normal controls ($p<0.0001$). Although the SSc patients showed slightly increased IL-8 levels compared with controls, the difference was not significant. Serum IL-6 levels were detected in 20/31 patients (65%) and 5/20 controls (25%). The median values were not significantly different between the patients and controls. IL-2, IL-4, IL-10, TNF-α and IFN-γ were not detected in most of the patients and normal controls (data not shown). Serum levels of these chemokines and cytokines were similar between patients who had been taking prednisolone and/or other immunosuppressive drugs and patients who had not been taking them (data not shown). There were no significant differences in the levels of chemokines and cytokines between dcSSc and lcSSc patients (data not shown). Otherwise, no significant associations were found in the patients between any of these chemokine or cytokine levels and any clinical or laboratory findings.

The yearly changes of serum chemokine and cytokine levels for each case are shown in Fig. 3. The median value of IP-10 declined during the first year and then gradually increased to a level similar to that at their first visit. The median value of MIG declined during the first year and remained at the same level thereafter. Serum MCP-1 declined, especially during the first year, and then declined gradually thereafter. IL-8 declined slightly during the first

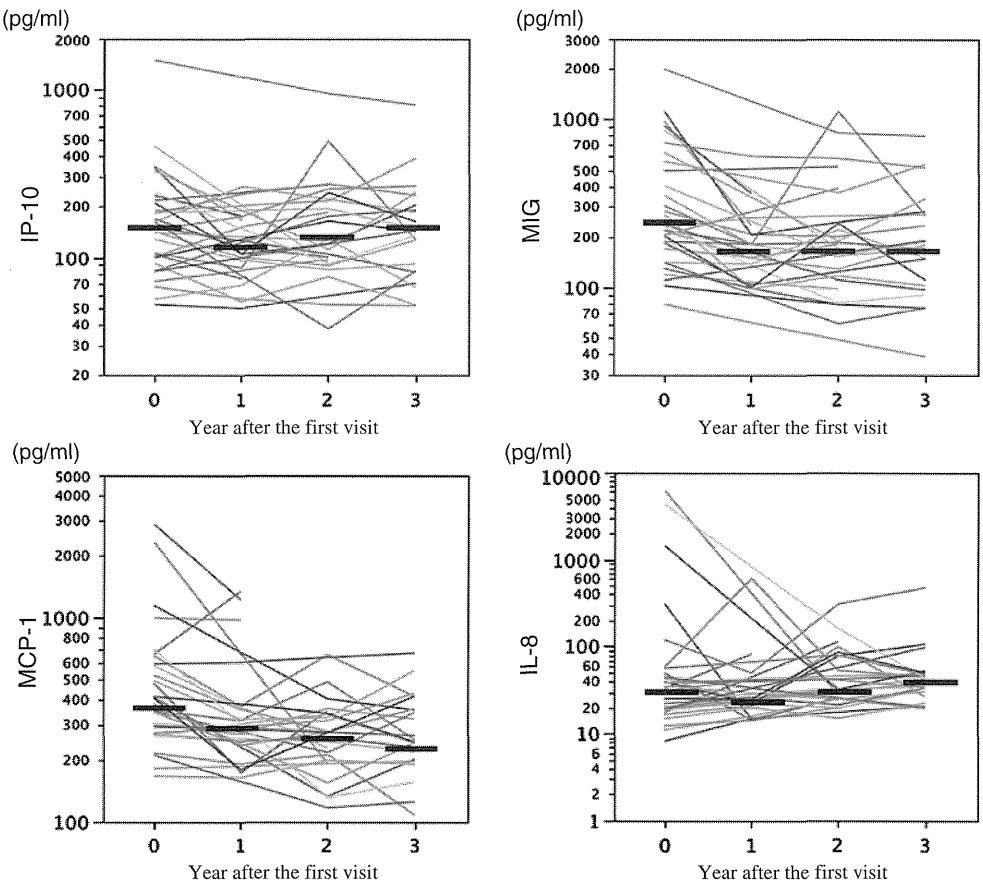

Fig. 3 Variations in serum chemokine levels for each case during the 3 years. *Short bars* indicate the median values at each year

year and then gradually increased to above the level of their first visit. IL-6 was not detected in most patients after the first year (data not shown).

Next, we assessed associations between the variation of each chemokine and cytokine level and the progression of skin sclerosis and internal organ involvement. The progression or improvement of skin sclerosis was estimated from changes of MRSS during the 3 years. Among assessed chemokines and cytokines, only the variation of MCP-1 was significantly associated with changes of skin scores during the 3 years (Fig. 4a, $r=0.42$, $p<0.05$). Interstitial pneumonia activity was evaluated by increases and decreases of %VC during the 3 years. Among examined chemokines and cytokines, only the variation of MCP-1 was significantly inversely associated with variation of %VC during the 3 years (Fig. 4b, $r=-0.44$, $p<0.05$). Furthermore, the variation of MCP-1 significantly correlated with the change of MRSS/%VC (Fig. 4c, $r=0.54$, $p<0.05$). Otherwise, the variation of chemokines and cytokines including IP-10 and MIG were not significantly associated with the change of clinical data including MRSS and %VC (Fig. 4; data not shown).

Discussion

In the present study, we assessed several chemokines and cytokines for possible associations with the activity of skin sclerosis or internal organ involvement during 3 years. At their first visit, serum levels of IP-10, MIG, and MCP-1 were significantly elevated in SSc patients compared with controls (Fig. 2). However, there were some overlaps in the ranges of chemokine levels between SSc patients and controls. In addition, the patients' treatment histories might have affected the results at baseline, as 48% of the patients had been taking immunosuppressive therapy. Although serum IL-6 and IL-8 levels were also higher or more

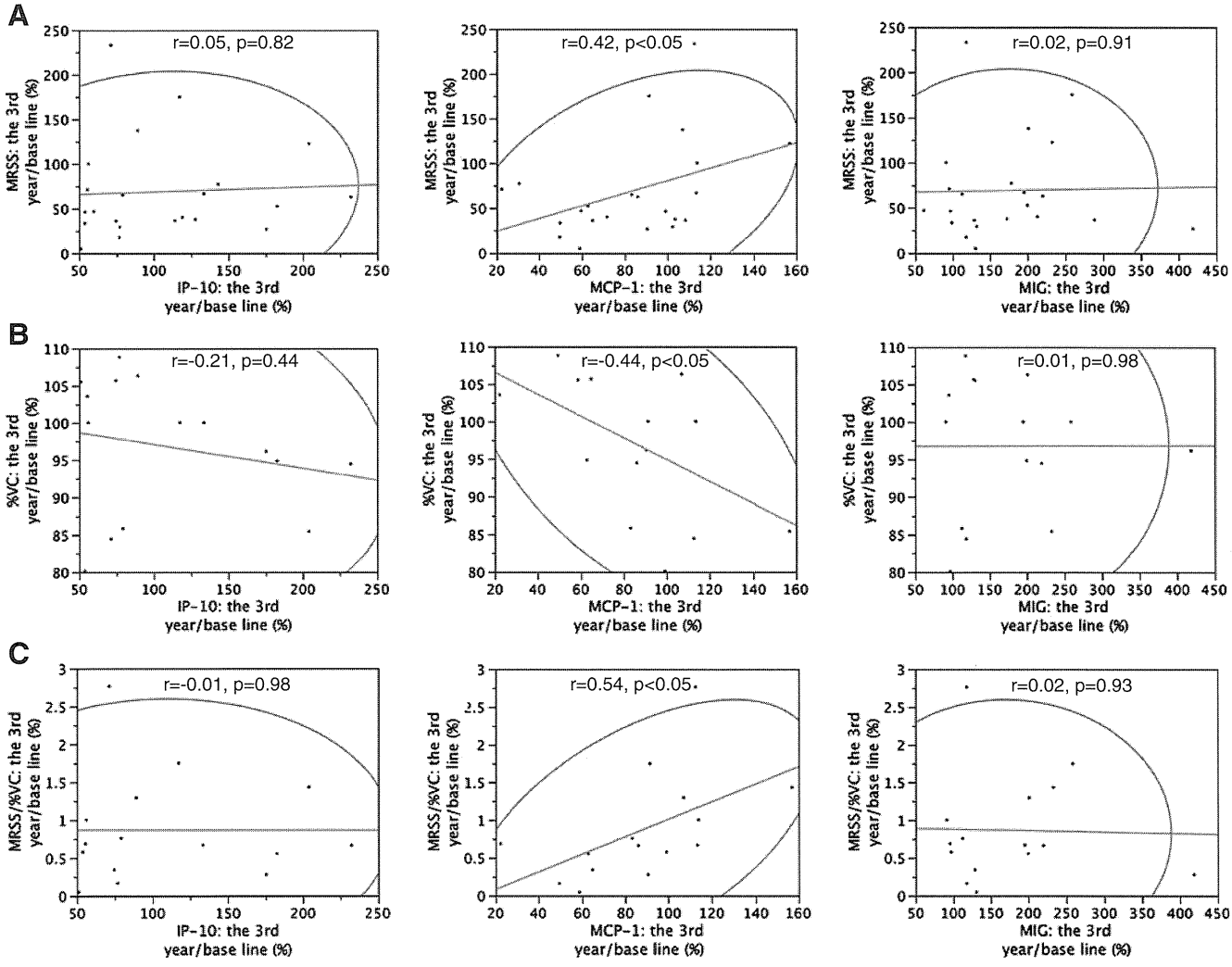

Fig. 4 Relationships between the variations of serum chemokine levels (levels at the third year/levels at baseline) × 100 (%) and the variations of **a** MRSS, **b** %VC, or **c** MRSS/%VC (numerical value at the third year/numerical value at baseline) × 100 (%) during the 3 years. *Straight lines* are regression lines; *ellipses* surround 95% confidence intervals

frequently detected in SSc patients than in healthy controls, those differences were not significant (Fig. 2). Therefore, the clinical utility of biomarkers at baseline may be questionable. Instead, serial measurements or longitudinal study of serum chemokines and cytokines are more likely to be useful for the evaluation of disease activity in individual SSc patients.

Our findings indicate that the serum MCP-1 concentration is a candidate for monitoring the activity and therapeutic effect of skin sclerosis or interstitial pneumonia. MCP-1 is well-known as one of the most pathogenic chemokines during the development of inflammation and fibrosis in SSc [6, 8, 9]. Serum MCP-1 levels declined year and year, along with improvements for skin sclerosis (Figs. 1 and 3). The variation of MCP-1 during 3 years was significantly associated with the change of MRSS during the 3 years (Fig. 4a). That is, patients with consistently increasing MCP-1 tended to show intractable skin sclerosis. When interstitial pneumonia progression was evaluated by variations of %VC, the variations of %VC during the 3 years were inversely associated with change of MCP-1 (Fig. 4b). Furthermore, the variation of serum MCP-1 levels was significantly associated with the variation of MRSS/%VC (Fig. 4c). MCP-1 is produced by macrophages, fibroblasts, endothelial cells and other cells. In addition to its chemoattractant activities for monocytes and T cells, MCP-1 induces Th2 cell polarization [19] and stimulates collagen production by fibroblasts via specific receptors and endogenous upregulation of TGF-β expression [20]. We have previously shown that serum MCP-1 levels are elevated and its expression in skin and lung is augmented in SSc patients [21]. It has been reported that cultured SSc dermal fibroblasts display augmented expressions of MCP-1 mRNA and protein [22, 23]. Furthermore, stimulation with PDGF significantly enhanced MCP-1 expression in dermal fibroblasts from SSc patients [22, 24].

On the other hand, serum levels of IP-10 and MIG are not likely as useful as MCP-1 for evaluating disease activity in SSc patients. The findings of elevated serum IP-10 levels (Fig. 1) are consistent with the results of previous reports [25, 26]. In one of those studies, high values of IP-10 were associated with a more severe clinical phenotype, such as lung and kidney involvement [25]. In this study, serum MIG levels were significantly elevated in SSc patients than in controls (Fig. 1). In contrast to this, the serum MIG level was not significantly elevated in the SSc patients compared to normal controls in a previous study [26]. A possible explanation for this discrepancy may be the lower sensitivity of the method used in the previous study, as most of the serum samples assayed were under the detection limits for patients and controls. It is also possible that the differences in the results may be due to the inclusion of early SSc patients with diffuse skin sclerosis or interstitial pneumonia only in this study. In either case, the every year reductions of these chemokines along with improvement for skin sclerosis were not as clear as that of MCP-1 (Figs. 1 and 3). Additionally, the relationship between the variation of IP-10 or MIG and the change of clinical parameters was not apparent in this study (Fig. 4). Furthermore, the roles of IP-10 and MIG in SSc remain unclear, although these Th1 chemoattractants are secreted by various cells, including monocytes and other cells, in response to IFN-α [27, 28].

In this study, most patients showed moderate MRSS despite the presence of anti-topoisomerase I Ab or anti-RNA polymerase I/III Ab at their first visit (Fig. 1). In addition, these MRSS declined further during the 3 years. A possible explanation for the good outcomes in our study may be due to racial differences, as Japanese patients usually show milder skin sclerosis compared with patient reports from Europe and the United States. In addition, prednisolone therapy might have attenuated the skin sclerosis in our patients. Corticosteroids are usually avoided, particularly in early dcSSc patients, due to fears of inducing scleroderma renal crisis [29]. However, renal crisis is rare in Japanese SSc patients [30]. Therefore, we frequently use them to suppress the progression of skin sclerosis. Additional studies will be needed to clarify the efficacy and safety of corticosteroid treatment in Japanese patients.

Several limitations exist in our study. The cytometric beads array kit was beneficial, as several different kinds of chemokines and cytokines could be measured using a small amount of serum. However, serum levels of IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ were undetectable in some or most patients and controls by this method. More sensitive methods should be used to evaluate these cytokines. Furthermore, the number of cases, especially severe cases, was small and the follow-up period was not very long. In addition, the heterogeneity of the treatments might have biased the analyses. A longer-term prospective study using multivariate analysis for a larger, more homogeneous population will be needed to determine which chemokines and cytokines might predict outcomes for early SSc patients.

Acknowledgments This work was supported by funds for research on intractable diseases from the Ministry of Health, Labour and Welfare of Japan. We thank Tomoko Hayashi, Masako Matsubara and Yuko Yamada for their technical assistance.

Disclosures None.

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Use of Serum Clara Cell 16-kDa (CC16) Levels as a Potential Indicator of Active Pulmonary Fibrosis in Systemic Sclerosis

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ABSTRACT. ***Objective.*** To clarify the clinical significance of concentrations of serum Clara cell 16-kDa protein (CC16; previously denoted CC10) in the diagnosis and monitoring of pulmonary fibrosis (PF) in patients with systemic sclerosis (SSc); and to compare CC16 levels with levels of the current most reliable serum markers for PF, such as Krebs von den Lungen-6 (KL-6) antigen and surfactant protein-D (SP-D).

Methods. Serum levels of CC16, KL-6, and SP-D were determined by ELISA in 92 patients with SSc, 20 patients with systemic lupus erythematosus (SLE), and 20 healthy controls. In a retrospective longitudinal study, correlation of serum CC16 levels with the activity of PF was assessed in 16 SSc patients with PF.

Results. Although CC16 levels were higher in patients with SSc than in SLE patients or healthy controls, the difference was not significant. Increased serum CC16 levels were associated with involvement of PF, especially active PF, as well as KL-6 and SP-D. Receiver operating characteristic curve analysis revealed that the utility of CC16 is slightly inferior to KL-6, but was comparable with that of SP-D for detecting PF in patients with SSc. In the longitudinal study, serum levels of CC16, KL-6, and SP-D were significantly decreased in the inactive disease phase compared to the active disease phase.

Conclusion. CC16 levels can be used as a potential serum biomarker for PF in addition to KL-6 and SP-D in patients with SSc. (First Release Jan 15 2011; J Rheumatol 2011;38:877–84; doi:10.3899/jrheum.100591)

Key Indexing Terms:
SERUM CLARA CELL 16-kDa (CC16) PULMONARY FIBROSIS
SYSTEMIC SCLEROSIS BIOMARKER

Systemic sclerosis (SSc) is a connective tissue disease characterized by tissue fibrosis in the skin and internal organs. Pulmonary fibrosis (PF) develops in more than half of patients with SSc and is one of the major SSc-related causes of death[1,2]. To assess fibrotic activity, high-resolution computed tomography and pulmonary function tests are performed. However, lung-specific serological markers may provide an easier and less invasive approach for closely monitoring the activity of PF in patients with SSc.

Krebs von den Lungen-6 (KL-6) and surfactant protein-D (SP-D) are currently the most reliable serum markers for PF. KL-6 antigen is expressed mainly by alveolar type II pneumocytes and respiratory bronchiolar epithelial cells including Clara cells[3], whereas SP-D is produced and secreted by alveolar type II pneumocytes and Clara cells[4]. Recent studies revealed that levels of KL-6 and SP-D are elevated in serum from patients with PF, including SSc-related PF[3,5,6]. These studies suggested that serum levels of KL-6 and SP-D are serologic markers of the severity and activity of PF in SSc[7,8,9]. However, some SSc patients with active PF showed discrepancies in the serum levels of these markers. Serum KL-6 levels can be increased in patients with adenocarcinoma of the lung, breast, or pancreas[10]. Elevated serum levels of SP-D can be observed in patients with bacterial pneumonia and pulmonary tuberculosis[11,12,13]. Further, KL-6 or SP-D does not necessarily reflect the activity of PF in some patients. Therefore, an

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Supported by funds for research on intractable diseases from the Ministry of Health, Labor and Welfare of Japan.

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Accepted for publication December 1, 2010.

additional serum marker may be helpful for reliable monitoring of PF.

Clara cell 16-kDa protein (CC16) is a 15.8-kDa homodimeric protein secreted throughout the tracheobronchial tree, especially in the terminal bronchioles where Clara cells are localized. This protein was previously referred to as the 10-kDa CC10 because of an underestimation of its molecular weight[4]. Although the exact function of CC16 remains to be clarified, studies suggest that CC16 plays a protective role in the respiratory tract during oxidative stress and inflammatory responses[14]. CC16 has been shown to modulate the production and activity of various mediators of the inflammatory response, including phospholipase A_2, interferon-γ, and tumor necrosis factor-α *in vitro*[15]. In addition, CC16 has been considered a peripheral biomarker for assessing the integrity of the lung epithelium. Increased CC16 concentrations have been found in serum from patients with lung injury such as sarcoidosis and idiopathic PF[4,16]. In contrast, serum CC16 levels are decreased in patients with asthma[17].

These findings suggest that CC16 is a candidate serum biomarker for PF in SSc. To test this hypothesis, we evaluated serum levels of CC16 and examined the correlation with clinical features in patients with SSc.

MATERIALS AND METHODS

Patients. Serum samples were obtained from 92 Japanese patients with SSc (77 women, 15 men). All patients fulfilled the criteria proposed by the American College of Rheumatology (ACR; formerly, the American Rheumatism Association)[18]. Patients were grouped according to the degree of skin involvement, based on the classification system proposed by LeRoy, *et al*[19]. Thirty-five patients (31 women, 4 men) had limited cutaneous SSc (lcSSc) and 57 patients (49 women, 8 men) had diffuse cutaneous SSc (dcSSc). The average age of the SSc patients was 52.3 ± 13.5 years (mean ± SD). Disease duration was 5.5 ± 6.6 years in patients with lcSSc and 3.3 ± 7.3 years in patients with dcSSc. Fifty-six patients (61% of total SSc patients, 8 lcSSc and 48 dcSSc) had PF. None of the SSc patients received any treatment, including corticosteroids, D-penicillamine, or other immunosuppressive therapy, at their first visit.

Antinuclear antibodies were determined by indirect immunofluorescence using HEp-2 cells as a substrate. Autoantibody specificities were further assessed by ELISA and immunoprecipitation. Anticentromere antibodies were present in 29 patients, antitopoisomerase I antibodies were present in 39 patients, anti-U1 RNP antibodies were present in 2 patients, anti-U3 RNP antibodies were present in 2 patients, anti-RNA polymerase I and III antibodies were present in 6 patients, anti-Th/To antibodies were present in 2 patients, and antinuclear antibodies of unknown specificity were present in 6 patients. Six patients tested negative for autoantibodies. Twenty patients with systemic lupus erythematosus (SLE; 17 women, 3 men; mean age 51.7 ± 15.9 yrs) who fulfilled the ACR criteria[20] and did not have PF were also evaluated as disease controls. No patients with SLE were treated with corticosteroid or immunosuppressive agents at this timepoint. In addition, 20 healthy age- and sex-matched Japanese volunteers (17 women, 3 men; mean age 54.2 ± 16.1 yrs) served as controls. All SSc and SLE patients and healthy controls involved in this study were nonsmokers and had no other respiratory diseases, including asthma and sarcoidosis, or impaired renal function.

To determine whether the change in serum pneumoprotein levels correlated with the activity of PF, we analyzed serum samples obtained at the time of active and inactive phase of PF in 16 SSc patients (11 women, 5 men). Five patients had lcSSc and 11 had dcSSc. The age of these patients was 53.7 ± 11.6 years and the disease duration was 3.1 ± 1.7 years. Nine patients with antitopoisomerase I antibodies and no patients with anticentromere antibody were included. These patients initially exhibited active PF. Twelve patients received oral corticosteroid therapy (prednisolone ~20 mg/day) and intravenous cyclophosphamide pulse therapy (500~1000 mg, once per month × 6) for treatment of active PF. The other 4 patients were treated with oral corticosteroid therapy (prednisolone ~20 mg/day). Activity of PF was stabilized by the treatment during the followup period in all patients (3.2 ± 2.0 yrs).

Samples of venous blood were drawn and allowed to clot, and centrifuged shortly after clot formation. Sera were removed, and all samples were stored at –70°C prior to use.

Clinical assessments. Complete medical histories, physical examinations, and laboratory tests were conducted on all patients. The degree of skin involvement was determined according to the modified Rodnan skin thickness score, as described[21]. Organ system involvement was defined as described[22] with some modifications: pulmonary fibrosis = bibasilar interstitial fibrosis on high-resolution computed tomogram (HRCT); pulmonary hypertension = clinical evidence of pulmonary hypertension and increased mean pulmonary arterial pressure (> 40 mm Hg) documented by echocardiography, in the absence of severe pulmonary interstitial fibrosis; 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; joint = inflammatory polyarthralgias or arthritis; and muscle = proximal muscle weakness and elevated serum creatine kinase. Pulmonary function, including vital capacity (VC) and diffusing capacity for carbon monoxide (DLCO), was also tested. Erythrocyte sedimentation rates (ESR) and C-reactive protein (CRP) were considered elevated when each value was higher than 20 mm/h and 0.5 mg/dl, respectively.

PF activity was initially determined by HRCT of the chest and pulmonary function testing. Specifically, PF was considered to be active when the following 2 criteria were met: (1) a ground-glass appearance or reticular pattern on HRCT of the chest[23]; and (2) > 10% change in VC or > 15% change in DLCO within 1 year[24]. PF activity was monitored by serial HRCT scans of the chest and by pulmonary function testing, as described[25,26,27].

The study protocol was approved by the Kanazawa University Graduate School of Medical Science. Informed consent was obtained from all study participants.

Measurement of serum CC16 concentrations. Serum levels of CC16 were measured in duplicate with a specific competitive ELISA kit (APC Biomaterials, Rockville, MD, USA), according to the manufacturer's protocol. Briefly, horseradish peroxidase (HRP) conjugated to recombinant human CC16 is captured by the anti-CC16 antibody coating the wells, generating a signal (A_{450}) proportional to the amount of CC16-HRP conjugate bound. The CC16-HRP conjugate is premixed with the sample to be assayed. The assay thus measures a decrease in signal as CC16 in the sample competes with the CC16-HRP conjugate for binding sites. The detection limit of this assay is 10 ng/ml.

Measurement of serum KL-6 and SP-D levels. Serum levels of KL-6 and SP-D were measured with specific ELISA kits (Eitest KL-6, Eisai, Tokyo, Japan; SP-D kit, Yamasa, Chiba, Japan), according to the manufacturers' protocols. Briefly, 96-well plates were coated with monoclonal antibodies to KL-6 or SP-D and diluted serum samples were added to duplicate wells. After washing, bound antibodies were detected with peroxidase-conjugated monoclonal antibodies against KL-6 or SP-D. The detection limits of KL-6 and SP-D are 50 U/ml and 1.56 ng/ml, respectively.

Statistical analysis. Statistical analyses were performed using the Mann-Whitney U test and Wilcoxon's signed-rank test for comparison of sample means, 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 contin-

uous variables. The concentrations of CC16, KL-6, and SP-D were analyzed using receiver-operating characteristic (ROC) curves in order to find cutoff values for optimal discriminative accuracy. Statistical analyses were performed using JMP® 7.01 software (SAS Institute, Cary, NC, USA). P values less than 0.05 were considered statistically significant. All values are reported as the mean ± SD.

RESULTS

Serum concentrations of CC16 in SSc patients at initial presentation. Although SSc patients had higher serum levels of CC16 at the initial presentation (mean 71.7 ± 118.6 ng/ml) compared with the levels of healthy controls (27.7 ± 17.1 ng/ml) and SLE controls (30.6 ± 19.0 ng/ml), the difference was not significant (Figure 1A).

Serum CC16 levels were significantly elevated in SSc patients with PF (n = 56) compared with SSc patients without PF (n = 36, 90.8 ± 110.7 vs 42.1 ± 80.7 ng/ml; $p < 0.01$; Figure 2A). Although several cases of SSc without PF showed increased CC16 levels, these patients did not show any characteristic difference. Serum levels of KL-6 were significantly elevated in SSc patients with PF compared with SSc patients without PF (711.0 ± 535.1 vs 275.2 ± 330.4 U/ml; $p < 0.001$; Figure 2A). Serum levels of SP-D were also significantly elevated in SSc patients with PF compared with SSc patients without PF (146.2 ± 94.6 vs 88.9 ± 75.0 ng/ml; $p < 0.01$; Figure 2A).

Further, SSc patients with active PF (n = 18) showed significantly elevated serum CC16 levels compared to patients with inactive PF (n = 38, 168.8 ± 161.5 vs 53.8 ± 95.0 ng/ml; $p < 0.01$; Figure 3A). SSc patients with active PF also showed significantly elevated serum levels of KL-6 and SP-D compared to patients with inactive PF (1225.1 ± 630.7 vs 467.5 ± 231.2 ng/ml; $p < 0.001$; and 207.7 ± 122.6 vs 117.1 ± 60.8 U/ml; $p < 0.01$, respectively; Figure 3A).

Serum CC16 levels were significantly associated with SP-D ($r = 0.40$, $p < 0.0001$), but not with KL-6 ($r = 0.22$, $p = 0.10$, data not shown).

Thus, serum levels of CC16 as well as KL-6 and SP-D were elevated in SSc patients with PF, especially active PF.

ROC curve analysis. To evaluate the value of CC16 for diagnosis of PF in patients with SSc, ROC curve analysis was performed and the result was compared with that of KL-6 and SP-D. For this analysis, serum CC16 levels in SSc patients with PF were compared to serum CC16 levels observed in SSc patients without PF. KL-6 had excellent diagnostic capacity as demonstrated by an area under the curve (AUC) of 0.89 (Figure 2B). KL-6 level of 302 U/ml or higher was diagnostic of PF with a sensitivity of 85.5% and specificity of 85.3%. AUC of SP-D was 0.72 and SP-D level of 91.0 ng/ml or higher was diagnostic of PF with a sensitivity of 71.4% and specificity of 77.2%. AUC of CC16 was 0.76 and the value was inferior to that of KL-6 but was comparable with that of SP-D (Figure 2B). CC16 level of 46.0 ng/ml or higher was diagnostic of PF with a sensitivity of 51.8% and specificity of 88.8% (Figure 2B).

Similar analysis was also assessed for diagnosis of active PF in SSc patients with PF. For this analysis, serum pneumoprotein levels in SSc patients with active PF were used and compared to levels observed in SSc patients with inactive PF. The AUC of KL-6 (0.94) was higher compared with that of CC16 (0.82) and SP-D (0.75, Figure 3B). Cutoff levels set as the closest point to 100% sensitivity and 100% specificity were 46.0 ng/ml for CC16 (sensitivity 94.4%, specificity 68.4%), 729.0 U/ml for KL-6 (sensitivity 88.9%, specificity 89.2%), and 147.0 ng/ml for SP-D (sensitivity 72.5%, specificity 82.9%).

Although KL-6 seems to be the best marker of PF, this is still not perfect. While 2 patients without PF showed markedly elevated KL-6 (Figure 2A), these patients were

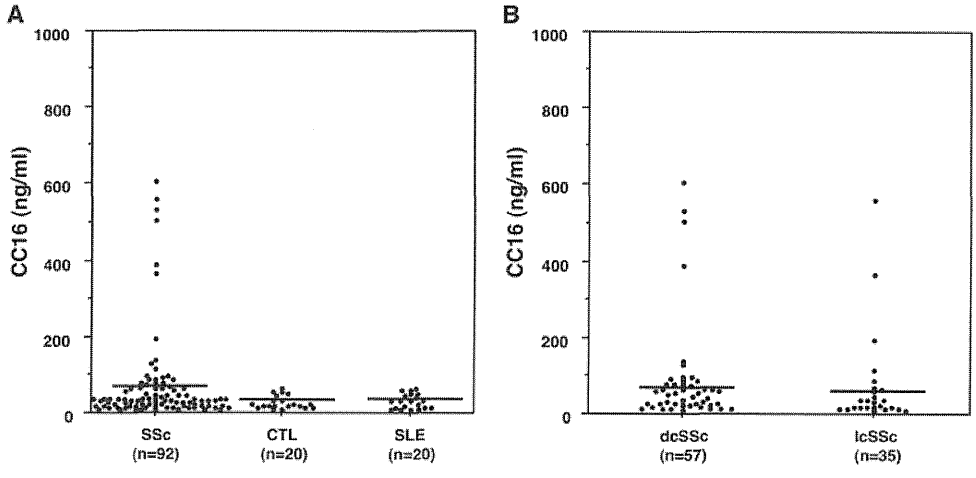

Figure 1. A. Levels of CC16 in serum samples from patients with systemic sclerosis (SSc), and systemic lupus erythematosus (SLE) as well as healthy controls (CTL). B. Serum CC16 levels in patients with diffuse cutaneous SSc (dcSSc) and limited cutaneous SSc (lcSSc). Serum CC16 levels were determined by competitive ELISA. Bars show group means.

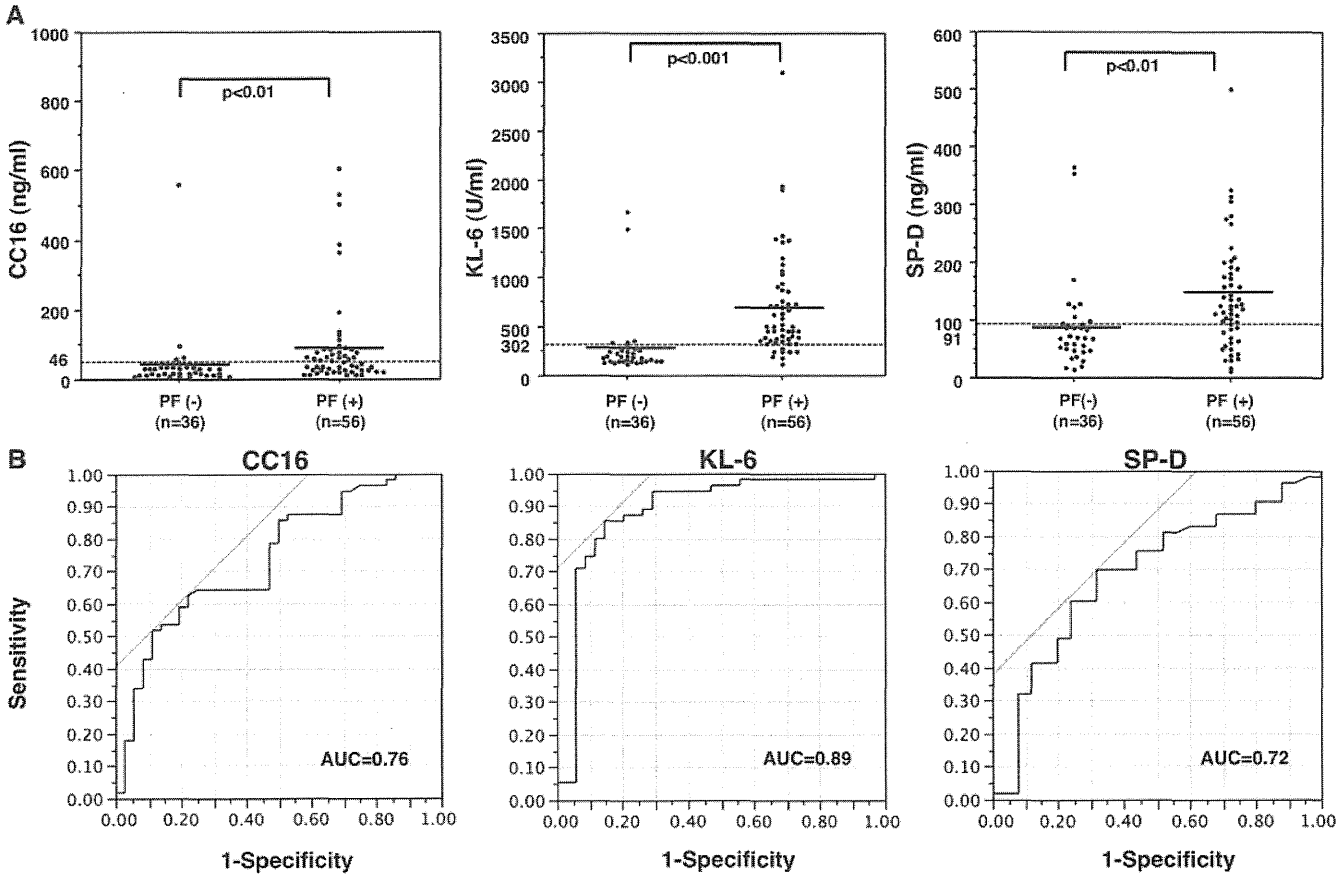

Figure 2. A. Serum levels of CC16, KL-6, and SP-D from SSc patients with pulmonary fibrosis (PF) and without PF. Bars show group means. B. ROC curves show the sensitivity and 1 – specificity of CC16, KL-6, and SP-D for detection of PF in patients with SSc. AUC: area under the ROC curve.

negative for CC16 and positive for SP-D (data not shown). Among 8 patients with PF that showed negative KL-6 (Figure 2A), 2 cases were positive for both CC16 and SP-D, and 3 cases were positive only for CC16, and 3 cases were positive only for SP-D (data not shown). These findings suggest that use of CC16 and SP-D in combination with KL-6 is valuable as an indicator of PF. Thus, ROC curve analysis suggests that CC16 is as useful as SP-D for diagnosis of PF or active PF, although it may not be as diagnostic as KL-6.

Serum CC16 levels and correlation with clinical features in SSc. Serum levels of CC16 were comparable in dcSSc and lcSSc patients (78.3 ± 124.3 vs 60.8 ± 109.5 ng/ml, Figure 1B). When the cutoff value was determined as 46 ng/ml as described above, elevated serum CC16 levels were observed in 35.9% of the SSc patients (33 of 92; Table 1). SSc patients with elevated CC16 levels had PF more frequently than patients with normal CC16 levels (88% vs 46%, respectively; $p < 0.001$; Table 1). Patients with elevated serum CC16 levels showed significantly decreased VC and DLCO values (predicted percentages) relative to those in SSc patients with normal CC16 levels ($p < 0.01$ for both comparisons). Antitopoisomerase I antibodies were present more frequently in SSc patients with elevated levels of CC16 than in those with normal levels, whereas anticentromere antibodies were found less frequently ($p < 0.05$, both comparisons). It is well known that patients with antitopoisomerase I antibodies frequently develop severe PF, whereas patients with anticentromere antibodies usually do not develop PF. Serum CC16 levels and the type of autoantibody present do not likely have a direct association, since serum CC16 levels were also found to be closely correlated with PF among patients with antitopoisomerase I antibodies or patients without anticentromere antibodies (data not shown).

Serum levels of CC16 were significantly inversely correlated with percentage VC ($r = –0.28$, $p < 0.05$) or percentage DLCO ($r = –0.20$, $p < 0.05$) values in patients with SSc (Table 2). The direct correlation of CC16 with percentage VC and percentage DLCO was modest compared with that of KL-6 ($r = –0.33$, $p < 0.01$, and $r = –0.46$, $p < 0.0001$, respectively), but was comparable with that of SP-D ($r = –0.21$, $p < 0.05$, and $r = –0.36$, $p < 0.01$, respectively). No other significant correlations were detected between serum CC16 levels and clinical or laboratory findings, as shown in Table 1. Thus, serum CC16 levels were specifically associated with the involvement of PF.

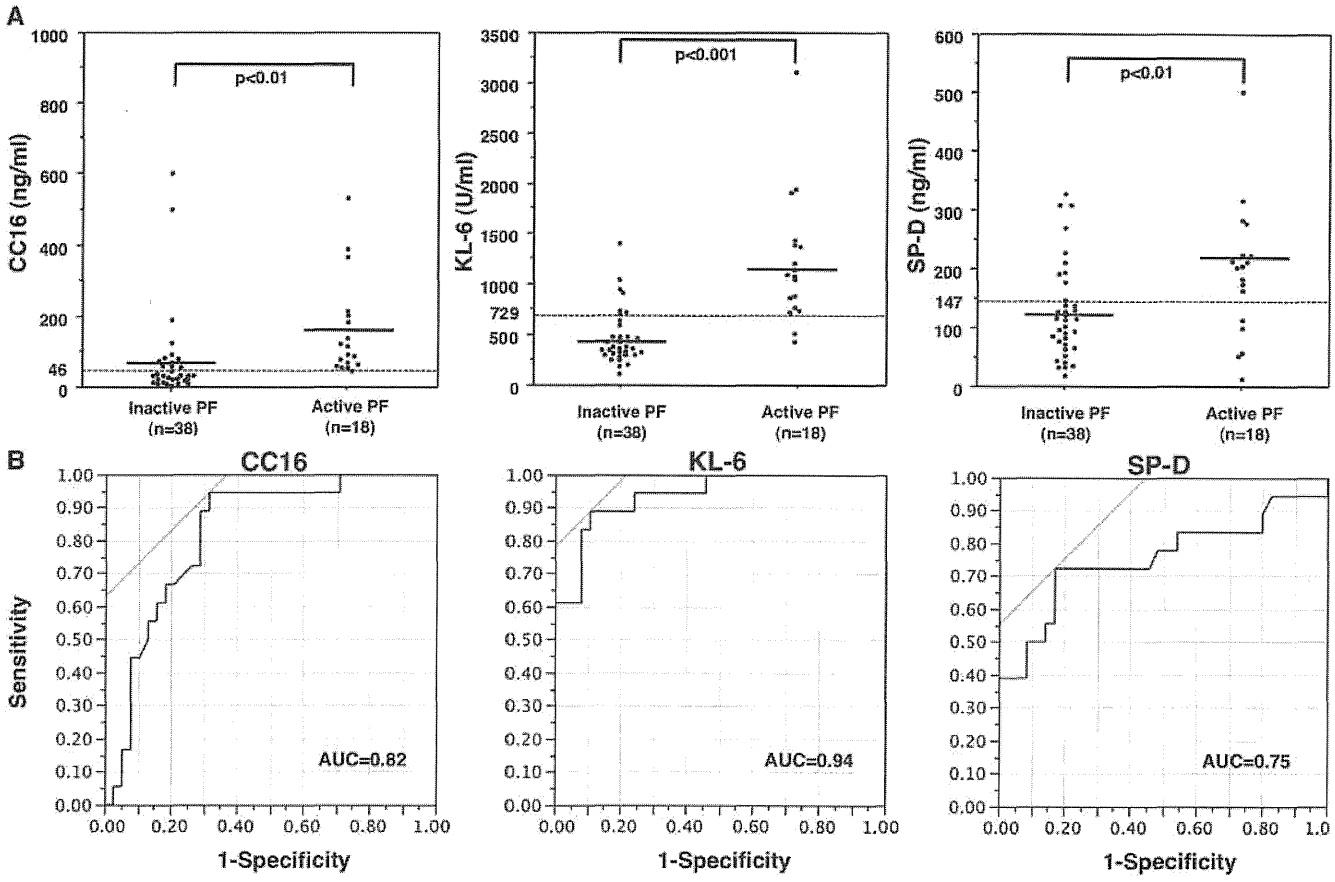

Figure 3. A. Serum levels of CC16, KL-6, and SP-D from SSc patients with active pulmonary fibrosis (PF) and inactive PF. Bars show group means. B. ROC curves show the sensitivity and 1 – specificity of CC16, KL-6, and SP-D for the detection of active PF in SSc patients with PF. AUC: area under the ROC curve.

Correlation between serum CC16 levels and activity of PF. Changes in serum CC16 levels in 16 SSc patients with active PF were also compared with changes in serum KL-6 and SP-D levels (Figure 4). Serum levels of CC16 were decreased significantly in parallel with the suppression of PF activity ($p < 0.05$). Serum levels of KL-6 were also significantly reduced in the inactive phase ($p < 0.05$). Further, serum SP-D levels were significantly decreased in association with the stabilization of PF activity. There were a few cases that showed increased levels of CC16, KL-6, or SP-D after stabilization of PF. In these patients, either 1 or 2 pneumoprotein levels were reduced in association with the disease activity (data not shown). These findings suggest that the utility of each marker for monitoring disease activity can be different in each patient. Thus, serum CC16 levels may indicate the PF activity as well as KL-6 or SP-D.

DISCUSSION

To our knowledge, this study is the first to evaluate serum levels of CC16 in patients with SSc. We found not only that serum CC16 levels were elevated in SSc patients with PF, but also that serum CC16 levels were remarkably higher in patients with active PF compared to patients with inactive PF. Further, CC16 levels were significantly reduced after the stabilization of PF in SSc patients. ROC curve analysis demonstrated that CC16 is as useful as SP-D for the diagnosis of PF or active PF in patients with SSc. Although serum CC16 may be slightly inferior as a marker for diagnosing PF or evaluating the severity of PF compared to KL-6, our data show it can be useful as an additional marker of PF activity in patients with SSc.

Ohnishi, *et al*[28] demonstrated a clear superiority of KL-6 to SP-D, SP-A, and monocyte chemoattractant protein-1 as a diagnostic marker of PF in terms of accuracy, sensitivity, specificity, and likelihood ratio in patients with idiopathic PF and collagen vascular disease-related interstitial pneumonia[28]. In SSc patients, the utility of KL-6 and SP-D has been reported for the evaluation of PF[7,8,9]. In a previous comparative study, we concluded that combined use of these 2 markers would be more effective for diagnosis and monitoring of PF activity in SSc than single use of each marker[9]. Another group's study supports these findings[8]. Our current results suggest that CC16 is another candidate protein for evaluating PF in SSc patients. Although the sensitivity and

Table 1. Clinical and laboratory features of patients with systemic sclerosis according to serum CC16 levels. Except where indicated otherwise, values are percentages.

Characteristic	Elevated CC16, n = 33	Normal CC16, n = 59
Sex, male:female	6:27	9:50
Age, mean ± SD yrs	55.2 ± 10.9	50.3 ± 14.4
Disease duration, mean ± SD yrs	5.4 ± 12.0	3.3 ± 4.0
Clinical features		
MRSS, mean ± SD	12.6 ± 9.7	12.3 ± 10.5
Digital pitting scars/ulcers	37	42
Contracture of phalanges	45	41
Diffuse pigmentation	48	42
Organ involvement		
Pulmonary fibrosis	88**	46
%VC, mean ± SD	93.8 ± 19.3**	105.8 ± 17.5
%DLCO, mean ± SD	57.1 ± 16.9**	70.0 ± 14.5
Pulmonary hypertension	21	10
Esophagus	63	73
Heart	3	2
Kidney	0	0
Joint	19	20
Muscle	7	22
Laboratory findings		
Antitopoisomerase I antibody	61*	32
Anticentromere antibody	3*	32
Elevated ESR	67	39
Elevated CRP	19	12

* $p < 0.05$, ** $p < 0.01$ vs normal serum CC16 levels. MRSS: modified Rodnan skin thickness score; VC: vital capacity; DLCO: diffusing capacity for carbon monoxide; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein.

Table 2. Correlations between serum pneumoprotein levels and values of respiratory function tests in patients with systemic sclerosis.

	VC, %	DLCO, %
CC16	r = –0.28, p < 0.05	r = –0.20, p < 0.05
KL-6	r = –0.33, p < 0.01	r = –0.46, p < 0.0001
SP-D	r = –0.21, p < 0.05	r = –0.36, p < 0.01

specificity were limited compared to KL-6, they were comparable with that of SP-D. Similarly, the sensitivity and specificity for active PF were slightly inferior to KL-6 and were at least comparable to that of SP-D. Further, our retrospective longitudinal study suggested that CC16 is as effective as KL-6 and SP-D to evaluate the activity of PF.

In addition to other important functions, the lung epithelium produces complex secretions, including mucus, surfactant proteins, and several proteins important for host defense[29]. Although CC16 is secreted by Clara cells into bronchoalveolar lavage (BAL) fluid, a significant correlation between CC16 levels in BAL fluid and serum has been reported[30]. Therefore, serum CC16 could potentially provide a less invasive method for assessing airway damage[31]. The exact physiological function of CC16 in the lung is not known, but it is believed to play a role in reducing inflammation in the airways[32] and protecting the respiratory tract from oxidative stress[15,31,32]. Serum CC16 has been shown to be elevated in several conditions related to impairment of the air-blood barrier, including idiopathic PF[16,29]. In our study, the CC16 serum levels as well as SP-D levels were found to be significantly associated with measurements of lung function such as percentage VC, but the correlation factors of CC16 and SP-D were slightly lower than that of KL-6. Serum CC16 levels were significantly associated with SP-D levels but not with KL-6 levels. The molecular weights of CC16 and SP-D (15.8 kDa and 43 kDa, respectively) are lower than that of KL-6 (> 200 kDa). Therefore, CC16 and SP-D may more easily leak into the circulation compared with KL-6 despite considerable lung-blood barrier destruction and subsequent fibrosis[3,15,33]. CC16 and SP-D are secretory proteins, whereas KL-6 is basically a structural component of cell membrane. Additionally, some proteinase to cleave its extracellular domain would be needed for KL-6 to leak into peripheral blood. Further, high molecular weight KL-6 may require more destruction of the lung-blood barrier or regeneration of lymph vessels compared with the other 2 pneumoproteins to leak into the circulation. These differences may explain why KL-6 is a more specific marker for detecting PF and evaluating the severity of PF compared with CC16 and SP-D in patients with SSc. On the other hand, these features may suggest that CC16 and SP-D are more sensitive to monitor the activity of interstitial pneumonia compared with KL-6. Although our study could not clarify this, previous studies demonstrated that SP-D is a more sensitive marker of alveolitis than KL-6 in patients with SSc[8,9].

There are some limitations to this study. This was not a prospective study and the study groups were not large, especially in the longitudinal study. Further, CC16 is not necessarily a perfectly specific marker for PF as much as KL-6 and SP-D. Reductions in levels of serum CC16 of approximately 30% have been found in smokers[17], and circulating CC16 levels were increased in patients with sarcoidosis and decreased in patients with asthma as compared with healthy controls[17]. Since CC16 is eliminated by glomerular filtration, the concentration of CC16 in serum can be used as a biomarker of lung epithelial injury only when the renal function is normal or moderately decreased[14]. Therefore, smokers and people with asthma, sarcoidosis, or impaired renal function were excluded from this study. The effects of these conditions on CC16 levels need to be considered when evaluating the effectiveness of using CC16 for monitoring PF. In addition, serum CC16 levels were elevated in several SSc patients without PF. Although we could not find any characteristic features in these patients, the kinds of factors other than PF that affect serum CC16 levels should be clarified in future studies.

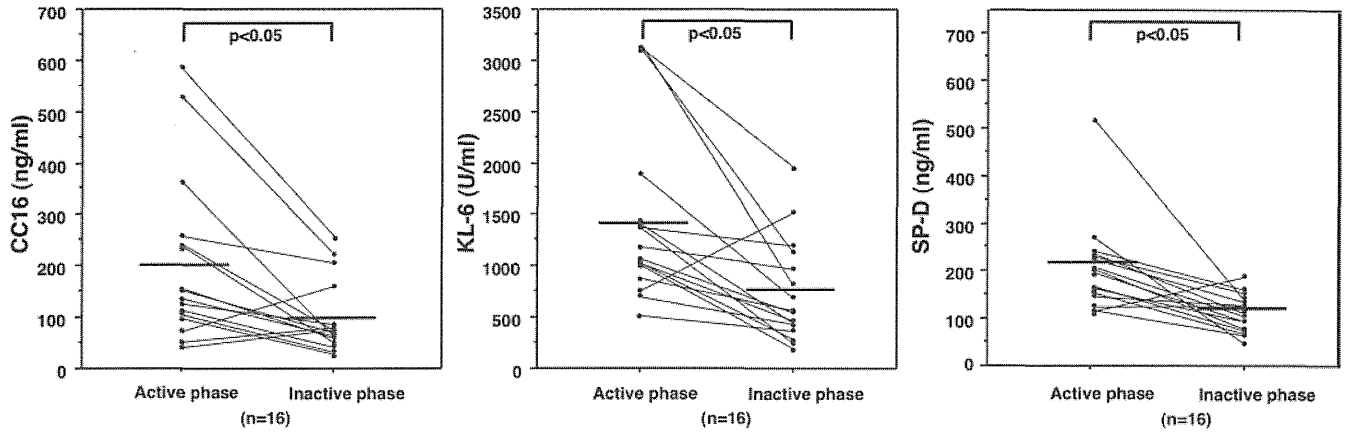

Figure 4. Change in serum levels of CC16, KL-6, and SP-D during active and inactive PF in patients with SSc. Serum samples were obtained during an active phase and an inactive phase of PF in each patient. Bars show group means.

Our data indicate that higher serum CC16 levels reflect increased activity of PF in patients with SSc. KL-6 and SP-D levels are currently the best serum markers of PF, but are still not perfect. Therefore, use of CC16 combined with KL-6 and SP-D may be a more valuable approach to monitor PF in SSc patients. Further prospective and comparative studies in larger populations will be needed to confirm these studies.

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