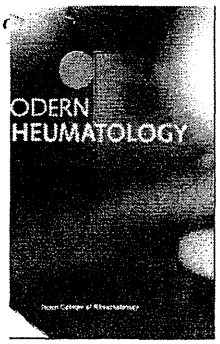

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ORIGINAL ARTICLE

Immune complexome analysis of antigens in circulating immune complexes isolated from patients with IgG4-related dacryoadenitis and/or sialadenitis

Kaname Ohyama[1,2], Miyako Baba[1], Mami Tamai[3], Motohisa Yamamoto[4], Kunihiro Ichinose[3], Naoya Kishikawa[1], Hiroki Takahashi[4], Atsushi Kawakami[3], and Naotaka Kuroda[1]

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Abstract

Objectives. IgG4-related disease (IgG4-RD) is characterized by various serological abnormalities. Some patients with IgG4-RD present with hypergammaglobulinemia, hypocomplementemia, and autoantibodies recognizing rheumatoid factors and nuclear factors. However, whether IgG4-RD is an autoimmune disease remains unclear.

Methods. Here, we used immune complexome analysis to comprehensively identify constituent antigens of circulating immune complexes isolated from 10 patients with IgG4-related dacryoadenitis and/or sialadenitis (IgG4-RDS) which is one condition associated with IgG4-RD.

Results. We detected each of 125 distinct antigens in independent samples from two or more patients with IgG4-RDS. Of them, 17 antigens were found to be specific to patients with IgG4-RDS by comparing 125 antigens with all the antigens found in other connective tissue diseases (antineutrophil cytoplasmic antibody-associated vasculitis, Takayasu's arteritis, mixed connective tissue disease, dermatomyositis, Sjögren's syndrome, systemic scleroderma, and systemic lupus erythematosus) or healthy donors. The number of disease-specific antigens associated with IgG4-RDS was comparable to those autoimmune diseases.

Conclusions. Studies of the 17 IgG4-RDS-specific antigens might lead to a better understanding of the pathogenesis of IgG4-RD, but further study of the prevalence of these CIC-associated antigens that involves a selective and sensitive assay will be needed to determine their potential as diagnostic or pathogenic biomarkers.

Abbreviations

ANCA antineutrophil cytoplasmic antibody-associated vasculitis, *CICs* circulating immune complexes, *DM* dermatomyositis, *ICs* immune complexes, *IgG4-RD* IgG4-related disease, *IgG4-RDS* IgG4-related dacryoadenitis and/or sialadenitis, *MCTD* mixed connective tissue disease, *nano-LC–MS/MS* nano-liquid chromatography-tandem mass spectrometry, *RA* rheumatoid arthritis, *SLE* neuropsychiatric systemic lupus erythematosus, *SS* Sjögren's syndrome, *SSc* systemic scleroderma, *TA* Takayasu's arteritis

Keywords

Autoimmune disease, IgG4-related disease, Immune complex, Immune complexome analysis, Tandem mass spectrometry

History

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Introduction

IgG4-related disease (IgG4-RD) is a novel clinical disease entity characterized by elevated IgG4 concentration in serum and prominent infiltration of plasmacytes expressing IgG4 into the lacrimal and salivary glands. IgG4-related dacryoadenitis and/or sialadenitis (IgG4-RDS, or previously called Mikulicz's disease) represents a unique condition involving persistent enlargement of the lacrimal and salivary glands that is characterized by good responsiveness to glucocorticoids [1,2], and is one condition associated with IgG4-RD. Previously, IgG4-RDS was considered a condition associated with primary Sjögren's syndrome (SS), but the aforementioned features are not evident with the connective tissue diseases, including SS, systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA) [3]. Elevated serum IgG4 can be used to differentiate IgG4-RDS from SS [3].

The pathogenesis of IgG4-RD remains unknown and whether IgG4-RD is an autoimmune disease is also unknown. The presence of disease-specific autoantigens can be a determining factor in categorizing a disease as an autoimmune disease. Immune complexes (ICs) are products of reactions that involve non-covalent interactions between antibody molecules and foreign antigens or autoantigens. Importantly, the identification of IC-associated antigens would have very different clinical significance than identification of free antigens because ICs are the direct and real-time

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products of an immune response. We developed a proteomic strategy, called immune complexome analysis, in which ICs are separated from patient samples (serum or some other sample type) and then subjected to direct tryptic digestion and nano-liquid chromatography-tandem mass spectrometry (nano-LC–MS/MS) to comprehensively identify and profile the constituent antigens in ICs [4]; previously and in the current study, we used this method to identify circulating IC (CIC)-associated antigens in serum samples. Of the CIC-associated antigens identified in serum from patients with established or early RA by this method, CIC-associated thrombospondin-1 was highly specific and sensitive for RA [4,5]. Subsequently, we used immune complexome analysis to study seven distinct connective tissue diseases: antineutrophil cytoplasmic antibody-associated vasculitis (ANCA), Takayasu's arteritis (TA), mixed connective tissue disease (MCTD), dermatomyositis (DM), SS, systemic scleroderma (SSc), and SLE; we identified and cataloged 468 distinct CIC-associated antigens in that analysis [6].

Here, we applied immune complexome analysis to the study of patients with IgG4-RDS to determine what kinds of antigens are incorporated into CICs and whether there are one or more disease-specific CIC-associated antigens in serum from patients with IgG4-RDS. We also sought to compare the CIC-associated antigens in IgG4-RDS serum with the CIC-associated antigens identified in our aforementioned study of connective tissue diseases and healthy donors [6]. We used either of two types of magnetic beads, beads coated with Protein G or beads coated with Protein A, to collect CICs from serum samples because Protein G and Protein A differ in their affinities for immunoglobulin isotypes. Consequently, we were able to profile a wide range of CIC-associated antigens.

Methods

Serum samples were collected from the patients with IgG4-RDS ($n = 10$; 47–80 years; 4 female) at Sapporo Medical University School of Medicine who fulfill the comprehensive diagnostic criteria for IgG4-RD [7]. Whole blood was collected into tubes containing coagulation accelerator. After removing the clot by centrifugation at 1300 *g* for 10 min at 4°C, the resulting supernatant (serum) was stored at − 80°C until analysis. Each serum sample was subdivided and subjected to two independent analyses. All the experiments were performed in accordance with the Helsinki Declaration with approval from the institutional ethics committees of the Graduate School of Biomedical Sciences at Nagasaki University and Sapporo Medical University School of Medicine. Each patient provided written informed consent for their participation in this study. Immune complexome analyses of serum samples were performed as described previously [4–6]. The specific antigens were defined as antigens which were found in IgG4-RD patients but not in other connective tissue disease (ANCA, TA, MCTD, DM, SS, SSc, and SLE) or healthy donors.

Results and discussion

Although IgG4-RD can be diagnosed, it is still unclear whether IgG4-RD should be categorized as an autoimmune disease entity. The presence of disease-specific antigens may be indicative of an autoimmune disease. Yamamoto et al. used surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) to analyze the CIC-associated antigens isolated from patients with IgG4-RD or SS and from healthy donors [8]. However, TOF-MS can determine molecular weights of proteins of interest, but TOF-MS cannot provide the names of those proteins.

In this study, 125 CIC-associated antigens were isolated from patients with IgG4-RDS, via immune complexome analysis; 43 and 82 of those were recovered with Protein G or Protein A beads, respectively. Each of the 125 CIC-associated antigens was recovered from both subsamples from two or more patients with IgG4-RDS. Of these 125 CIC-associated antigens identified, 59 and 62

Table 1. Summary of disease-specific antigens in CICs isolated from patients with IgG4-RDS.

Accession	Description	IgG4-RD ($n = 10$) Frequency	Positive patient No.	Protein G Or Protein A	Involved organs
IPI00031605.1	AHNAK protein	3	1, 5, 7	G	Lachrymal submandibular thyroidal renal pelvis
IPI00894488.1	23-kDa protein	3	3, 6, 10	G	Lachrymal submandibular renal pelvis liver lung
IPI00217512.4	Probable G-protein-coupled receptor 115	3	4, 5, 7	A	Lachrymal submandibular vertebral body thyroidal renal pelvis
IPI00396341.1	Isoform 1 of Uncharacterized protein C2orf67	2	4, 7	G	Lachrymal submandibular vertebral body renal pelvis
IPI00445543.1	Conserved hypothetical protein	2	1, 7	G	Lachrymal submandibular renal pelvis
IPI00642533.1	Isoform 4 of ATPase WRNIP1	2	1, 10	G	Lachrymal submandibular lung
IPI00644892.4	Isoform 2 of Rootletin	2	3, 6	G	Lachrymal submandibular renal pelvis liver
IPI00845359.1	Isoform 2 of Coiled-coil domain-containing protein 73	2	6, 8	G	Lachrymal submandibular lung
IPI00555812.5	Vitamin D-binding protein isoform 1 precursor	2	1, 10	G	Lachrymal submandibular lung
IPI00017696.1	Complement C1s subcomponent	2	2, 8	G	Lachrymal submandibular lung
IPI00017431.1	PRED15 protein (Fragment)	2	3, 4	A	Lachrymal submandibular renal pelvis liver vertebral body
IPI00169307.6	rho GTPase-activating protein 21	2	3, 4	A	Lachrymal submandibular renal pelvis liver vertebral body
IPI00718811.1	cDNA FLJ43766 fis, clone TESTI2049246	2	7, 8	A	Lachrymal submandibular renal pelvis lung
IPI00513768.2	ATPase family AAA domain-containing protein 3C	2	2, 6	A	Lachrymal submandibular
IPI00884904.1	Isoform 3 of Protein AHNAK2	2	6, 7	A	Lachrymal submandibular renal pelvis
IPI00902596.1	cDNA FLJ40505 fis, clone TESTI2045562, highly similar to HEAT SHOCK-RELATED 70-kDa PROTEIN 2	2	3, 7	A	Lachrymal submandibular renal pelvis liver
IPI01010122.1	43-kDa protein	2	5, 10	A	Lachrymal submandibular thyroidal lung

were identical to CIC-associated antigens recovered from samples taken from patients with SS or SLE, respectively, and 49 of the 125 antigens were found in IgG4-RDS, SS, and SLE serum samples. Protein A has moderate affinity for IgA, IgD, IgE, and IgM, but Protein G does not have any affinity of IgA, IgD, IgE, and IgM. Approximately 2-fold more IgG4-RDS-associated antigens were detected with Protein A beads (82 antigens) than with Protein G beads (43 antigens). This finding indicated that there were more CICs associated with IgA, IgD, IgE, or IgM than those associated with IgG. This phenomenon was also seen with CIC-associated antigens isolated from ANCA, TA, MCTD, or DM samples; in contrast, most CIC-associated antigens isolated from SS, SSc, or SLE serum were recovered specifically with Protein G beads [6]. Therefore, IgG4-RDS may not be closely related to SS. Notably, 17 of the 125 CIC-associated antigens were specific to IgG4-RDS serum and not present in samples from any other patient group—ANCA, TA, MCTD, DM, SS, SSc, SLE, or healthy donors (Table 1). Based on these observations, IgG4-RDS can be categorized as an autoimmune disease.

Using SELDI-TOF-MS, Yamamoto *et al.* detected a 13.1-kDa protein in each sample taken from any patient with IgG4-RD, but not in any samples taken from patients with SS or from healthy subjects [8]. Notably, this 13.1-kDa protein was not among the 125 antigens identified in the present study. This may be because the 13.1-kDa autoantigen is a peptide fragment of a protein and this fragmentation may induce autoimmune reactivity; if this is the case, our LC–MS/MS method would have identified the autoantigen in the parental form of an intact protein, while SELDI-TOF-MS identifies the antigen in its antigenic even though both methods detect the same peptide.

Each of the 17 IgG4-RDS-specific antigens was detected in no more than 3 of the 10 patients. The sensitivity of each of the 17 disease-specific antigens was $\leq 30\%$. For example, probable G-protein-coupled receptor 15 was found in only three of the 10 patients with IgG4-RDS ($3/10 = 30\%$); in contrast, thrombospondin-1 was present in 81% of samples taken from patients with established RA and 55% of the samples taken from patients with early RA [5,6]. Future studies that involve a larger number of patients with IgG4-RDS are warranted to determine the clinical benefit of using any of these 17 disease-specific antigens as novel biomarkers. Also, the sensitivities could be improved by developing an enzyme immune assay (e.g., ELISA) method specifically detecting a certain IC [9]. Ultimately, these disease-specific antigens might become promising diagnostic and pathogenic biomarkers of IgG4-RD.

Conclusions

To our knowledge, this study is the first to comprehensively identify the antigens incorporated into CICs in patients with IgG4-RDS. We recovered 125 CIC-associated antigens, and of them, 17 antigens were found to be specific to patients with IgG4-RDS by comparing 125 antigens with all the antigens found in other connective tissue diseases (ANCA, TA, MCTD, DM, SS, SSc, and SLE). Studies of these 17 IgG4-RDS-specific antigens might lead to a better understanding of the pathogenesis of IgG4-RD, but further study of the prevalence of these CIC-associated antigens that involves a selective and sensitive enzyme immunoassay will be needed to determine their potential as diagnostic or pathogenic biomarkers.

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Conflict of interest

Research Funding: K. Ohyama, Eisai Co., Ltd.

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Decreased levels of regulatory B cells in patients with systemic sclerosis: association with autoantibody production and disease activity

Takashi Matsushita[1], Yasuhito Hamaguchi[1], Minoru Hasegawa[2], Kazuhiko Takehara[1] and Manabu Fujimoto[3]

Abstract

Objective. B cell abnormalities characterized by autoantibody production and polyclonal B cell activation play an important role in the pathogenesis of SSc. IL-10 producing regulatory B (Breg) cells also play an important role in the negative immune response. We identified a human Breg cell subset that was predominantly found within the $CD24^{hi}CD27^{+}$ B cell subpopulation. However, the role of Breg cells in SSc remains unknown. The aim of this study was to investigate the clinical association of Breg cells in SSc patients.

Methods. Blood IL-10 producing Breg cell levels were determined by FACS in 35 SSc patients and 30 healthy subjects. In a follow-up study, we analysed six individual dcSSc patients before and after treatment.

Results. The frequency of blood Breg cells was significantly lower in SSc patients than in healthy controls ($P < 0.0001$). Similarly, the frequency of $CD24^{hi}CD27^{+}$ B cells was significantly lower in SSc patients than in healthy controls ($P < 0.0001$). SSc patients with decreased Breg cell levels often had interstitial lung disease ($P < 0.05$). Furthermore, Breg cell levels correlated negatively with the titre of anti-topo I antibody (Ab) and anticentromere Ab in SSc patients. For a follow-up study, Breg cell levels in dcSSc patients after treatment were found to be significantly increased compared with those before treatment ($P < 0.05$), accompanied by decreased disease activity. Thus, Breg cell levels were inversely correlated with disease activity of SSc.

Conclusion. These results suggest that decreased Breg cell levels may contribute to the development of SSc.

Key words: systemic sclerosis, regulatory B cell, IL-10.

Rheumatology key messages

- Regulatory B cells are decreased in patients with SSc.
- Decreased levels of regulatory B cells may accelerate autoantibody production in patients with SSc.
- Regulatory B cell levels are inversely correlated with disease activity of SSc.

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Introduction

SSc is a connective tissue disorder characterized by fibrosis in the skin and various internal organs, with an autoimmune background. The two subsets were termed dcSSc and lcSSc [1]. Autoantibodies are positive in over 90% of patients, and these autoantibodies (Abs) react to various intracellular components. The autoantibodies associated with SSc include anti-DNA topo I and

anticentromere Abs. It has been reported that SSc patients have distinct abnormalities of blood B lymphocyte compartments characterized by expanded naive B cells and activated memory B cells [2]. Furthermore, B cell depletion therapy suppresses the development of skin fibrosis in the tight-skin mouse [3, 4]. In addition, several recent studies have revealed a possible beneficial effect of antihuman CD20 Ab (rituximab) therapy for SSc patients [5].

Regulatory B (Breg) cells that produce IL-10 are now recognized as an important component of the immune system. Remarkably, Breg cells are potent negative regulators of inflammation and autoimmunity in mouse models of disease *in vivo*. Our recent study revealed that Breg cells play an inhibitory role in murine sclerodermatous chronic graft-*vs*-host disease (Scl-cGVHD), a mouse model of scleroderma [6]. Breg cells in humans normally represent <1% of peripheral blood B cells [7]. The phenotype of human Breg cells is reported to be $CD24^{hi}CD27^{+}$ B cell subset [7] or $CD19^{+}CD24^{hi}CD38^{hi}$ B cells [8]. It has been reported that pan-B cell depletion, including regulatory B cell depletion, worsens autoimmunity in humans [9]. Pan-B cell depletion is not always a beneficial effect in autoimmunity. Therefore, it is necessary to investigate Breg cells in SSc.

Patients and methods

Patients

Serum samples were obtained from 35 Japanese SSc patients (29 females and 6 males). All patients fulfilled the criteria for SSc proposed by the ACR [10]. Their median (range) age was 57 (18–71) years and the median (range) disease duration was 2.0 (0.1–24) years. These patients were grouped according to the classification system proposed by LeRoy *et al.* [1]: 16 patients had dcSSc and 19 patients had lcSSc. Anti-topo I Abs were positive for 14 patients; anticentromere Abs for 11; and anti-RNA polymerases I/III Abs for 3. The disease duration was calculated from the time of the first clinical event (other than Raynaud's phenomenon) that was a clear manifestation of SSc. Five patients were treated with a low-dose prednisolone (1–10 mg/day). Eight patients were treated with an intermediate-dose prednisolone (11–20 mg/day). In addition to an intermediate-dose of steroid treatment, two patients were treated with ciclosporin (2–3 mg/kg/day) and two patients were treated with i.v. CYC pulse (500 mg/m^2). In a follow-up study, we analysed six individual patients with dcSSc before and after immunosuppressant agents. At the first blood sampling, one patient was treated with 12 mg/day of prednisolone, while the remaining five SSc patients did not receive any immunosuppressant agents. Afterwards, four patients were treated with 20–40 mg/day of prednisolone. Furthermore, two patients were treated with six cycles of i.v. CYC pulse (500 mg/m^2) in addition to 20 mg/day of prednisolone. The second blood samples were taken after treatment. As disease control in this study, we also examined serum samples from 10 patients with DM that fulfilled the ACR criteria [11] and the Bohan and Peter criteria [12]. Thirty age- and sex-matched healthy Japanese individuals were used as healthy controls. The study was approved by the ethics committee of the Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, and informed consent was obtained from all patients.

Clinical assessment

Complete medical histories, physical examinations and laboratory tests were conducted for all patients at the time of blood sampling, with limited evaluations during follow-up visits. Skin score was measured by the scoring technique of the modified Rodnan total skin thickness score [modified Rodnan total skin score (TSS)]. Organ system involvement was defined as described previously [13].

Antibodies and flow cytometric analysis

Anti-human mAbs included: CD19, CD24, CD27 from BD Biosciences (San Diego, CA, USA) and anti-IL-10 mAb from BioLegend (San Diego, CA, USA). Blood samples (50 μl) were stained at 4 °C using mAbs for 20 min. Cells were analysed on a BD FACSCant II flow cytometer (BD Biosciences).

Analysis of IL-10 production

Heparinized blood samples were collected. Peripheral blood mononuclear cells were resuspended (2×10^6 cells/ml) in medium and stimulated with CpG (ODN 2006, 10 μg/ml; Invivogen), CD40L (1 μg/ml; R&D Systems, Minneapolis, MN, USA), PMA (50 ng/ml; Sigma), ionomycin (1 μg/ml; Sigma), and Brefeldin A (1X solution/ml; BioLegend). Stained cells were fixed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences) and stained with anti-IL-10 mAb.

Statistical analysis

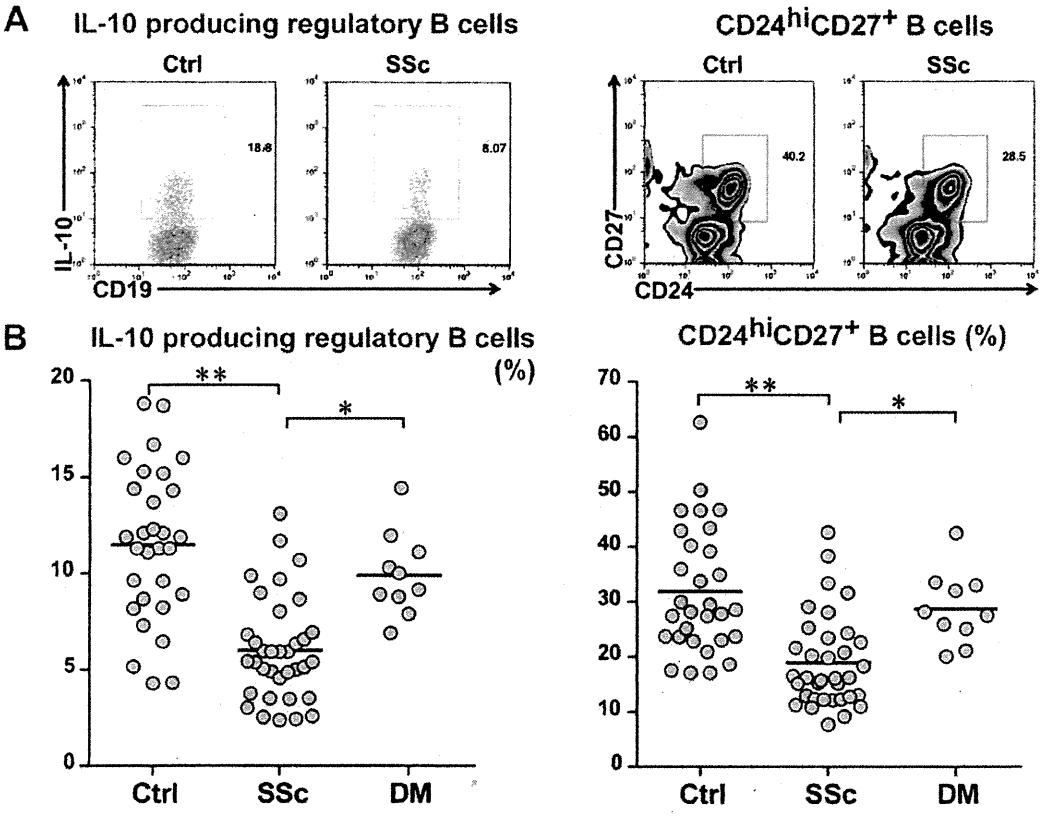
Statistical analyses were performed using Student's *t*-test for comparison of sample means between two groups or one-way analysis of variance with the Bonferroni *post hoc* test for comparisons of more than two groups. The Pearson product–moment correlation coefficient was used to examine the relationship between two continuous variables. The Wilcoxon matched paired rank test was used for matched paired samples. *P*-values <0.05 were considered to be statistically significant. The data were shown as the median (range) unless otherwise indicated.

Results

Breg cell levels in SSc

The frequency of IL-10 producing Breg cells was significantly lower in SSc patients [5.43 (2.38–13.10)%] than in healthy controls [11.60 (4.29–18.80)%, $P < 0.0001$; Fig. 1]. The absolute number of Breg cells was also significantly lower in SSc patients [1.68 (0.08–15.30) $\times 10^6$/l] than in healthy controls [4.31 (1.03–24.52) $\times 10^6$/l, $P < 0.0001$]. By contrast, the frequency of Breg cells in DM patients [9.57 (6.90–14.50)%] was comparable with that in healthy

Fig. 1 IL-10 producing regulatory B cell frequencies in SSc patients



(**A**, left panel) Representative B cell cytoplasmic IL-10 expression by control (Ctrl) individuals and SSc patients after *in vitro* 48 h CD40L+ CpG stimulation, with PMA, ionomycin, Brefeldin A added during the final 5 h of culture. (**A**, right panel) Representative $CD24^{hi}CD27^{+}$ B cells in Ctrl individuals and SSc patients. (**B**) IL-10 producing regulatory B cell and $CD24^{hi}CD27^{+}$ B cell frequencies of Ctrl individuals, SSc and DM patients. Horizontal bars indicate group means. Blood IL-10 producing regulatory B cell and $CD24^{hi}CD27^{+}$ B cell frequencies were determined by FACS. $^{*}P < 0.01$, $^{**}P < 0.0001$.

controls (Fig. 1B). Similarly, the frequency of $CD24^{hi}CD27^{+}$ B cells was significantly lower in SSc patients [16.2 (7.6–42.6)%] than in healthy controls [28.3 (17.1–62.7), $P < 0.0001$]. The absolute number of $CD24^{hi}CD27^{+}$ B cells was also significantly lower in SSc patients [3.43 (0.22–65.98) $\times 10^6$/l] than in healthy controls [12.99 (3.94–64.25) $\times 10^6$/l, $P < 0.01$].

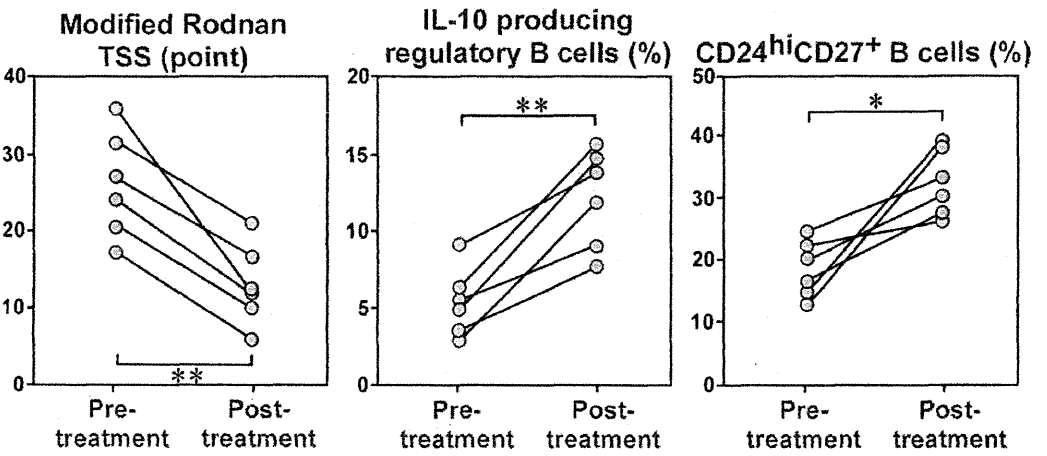
Clinical correlation of Breg cell levels in SSc

Breg cell levels were decreased in 26% (9/35) of SSc patients, in 26% (5/19) of dcSSc patients and in 25% (4/12) of lcSSc patients. There was no significant difference in disease duration between patients with decreased Breg cell levels and those with normal levels (supplementary Table S1, available at *Rheumatology* Online). In addition, there was no significant difference in modified Rodnan TSS and clinical features. SSc patients with decreased Breg cell levels more frequently had interstitial lung disease ($P < 0.05$). In contrast, there was no significant difference in the prevalence of oesophagus, heart, kidney, joint or muscle involvement. The frequency of elevated CRP levels was significantly increased in SSc patients with decreased Breg cell levels compared with normal levels ($P < 0.05$). Furthermore, Breg cell levels correlated negatively with CRP in SSc patients ($P < 0.05$, $r = -0.343$; supplementary Fig. S1, available at *Rheumatology* Online), although Breg cell levels did not correlate with CRP in healthy controls or other diseases accompanied by elevated CRP levels, such as cellulitis and erysipelas (data not shown). Collectively, Breg cell levels may not correlate with disease severity.

Clinical correlation of Breg cell levels in SSc

Autoimmunity plays an important role in the pathogenesis of scleroderma. It was reported that the titre of a scleroderma-specific autoantibody, such as anti-topo I Ab, reflects the severity of skin sclerosis in SSc patients [14]. Decreased levels of Breg cells might accelerate the autoimmunity of scleroderma. As a result, Breg cell levels were found to correlate negatively with the titre of anti-topo I

Fig. 2 Serial change of IL-10 producing regulatory B cell



The modified Rodnan total skin score, IL-10 producing regulatory B cell and $CD24^{hi}CD27^{+}$ B cell levels were analysed in six individual patients with dcSSc before and after immunosuppressive treatment. $^{*}P < 0.05$, $^{**}P < 0.01$.

and anticentromere Abs in SSc patients ($P < 0.05$, $r = -0.533$; $P < 0.05$, $r = -0.684$, respectively, supplementary Fig. S1 available at *Rheumatology* Online). Thus, decreased Breg cell levels were generally associated with the autoimmunity of SSc, rather than the severity of SSc.

Breg cells as a marker of disease activity

To determine whether Breg cell levels are inversely correlated with the disease activity of SSc, we analysed six individual patients with dcSSc before and after treatment. Disease activity was defined as modified Rodnan TSS. After treatment, disease activity in six patients with dcSSc had declined, since the modified Rodnan TSS in dsSSc patients was significantly decreased after treatment ($P < 0.01$, Fig. 2). Breg and $CD24^{hi}CD27^{+}$ B cell levels in dcSSc patients after treatment were found to be significantly increased compared with the levels before treatment ($P < 0.01$ and $P < 0.05$, respectively, Fig. 2), accompanied with decreased disease activity. To confirm whether immunosuppressant agents increase Breg cell levels, we compared Breg cell levels between the patients treated with immunosuppressants and those not treated with immunosuppressants. The Breg cell levels in SSc patients treated with immunosuppressants tended to be increased compared with those in SSc patients without treatment, while there was no significant difference (supplementary Fig. S2, available at *Rheumatology* Online). Taken together, Breg cell levels inversely correlated with the disease activity of SSc.

Discussion

This is the first report to reveal decreased Breg cell levels in SSc. Decreased Breg cell levels were associated with the prevalence of interstitial lung disease. In addition, Breg cell levels correlated negatively with the titre of anti-topo I and anticentromere Ab in SSc patients. Although Breg cell levels did not correlate with the severity of skin sclerosis, Breg cell levels were inversely correlated with the disease activity of SSc. Therefore, decreased Breg cell levels were generally associated with pathogenesis of SSc.

It has been suggested that Th1 cytokines, such as IFN-γ and IL-2, generally decrease extracellular matrix deposition, whereas Th2 cytokines, such as IL-4, IL-5, IL-6 and IL-13, increase it [15]. To date, B cells have primarily been studied in the roles of effector B cells, which positively regulate the immune response via Ab production and IL-6 secretion. However, Breg cells have been identified, and it was consequently revealed that B cells negatively control the immune response via IL-10 secretion [16]. Thus, B cells have two conflicting subsets. We elucidated the role of these two conflicting subsets in a mouse model of experimental autoimmune encephalomyelitis (EAE) [17]. EAE was exacerbated when B cells were depleted in the early phase, whereas it improved when B cells were depleted in the late phase. These results indicate that in the early phase, Breg cells play a major role, and the lack of Breg cells exacerbates EAE. On the other hand, in the late phase, effector B cells are dominant and the lack of effector B cells improves EAE. These results reveal that both effector and Breg cells are involved in autoimmune disease and that the weights of their roles differ according to the disease stage. These results also indicate that Breg cells are involved in the onset of autoimmune disease because they are important in the early phase. In the current study, Breg cell levels were lower in SSc patients than in healthy individuals; however, we found no correlation with severity. On the other hand, a decreased level of Breg cells correlated with a high autoantibody titre. Furthermore, Breg cell levels inversely correlated with disease activity of SSc. We recently reported that Breg cells are important in the suppression of Scl-cGVHD, a mouse model of SSc [6]. The lack of Breg cells induced severe

Scl-cGVHD, while replacement of Breg cells in the early phase restored the exacerbated Scl-cGVHD. However, replacement of Breg cells in the late phase had no effect on Scl-cGVHD. This suggests that Breg cells may play an important role in the onset of Scl-cGVHD. Thus, Breg cells are important for the pathogenesis of scleroderma.

Two large randomized controlled trials of Rituximab, which depletes human pan-B cells, were conducted with SLE patients with the expectation that it would be effective; however, it failed to achieve the primary endpoints [18, 19]. This ineffectiveness may have been due to the existence of Breg cells. If Breg cells are dominant in a patient with SLE, B cell depletion will worsen the condition of the patient with SLE. Thus, the effect of pan-B cell depletion depends on the balance of Breg cells *vs* effector B cells in the patient. A clinical trial is currently examining B cell depletion in SSc, so it will be necessary to consider the existence of Breg cells in the future. Selective B cell depletion, which retains Breg cells, is the ideal treatment for patients with autoimmune disease.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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Clinical and Immunologic Predictors of Scleroderma Renal Crisis in Japanese Systemic Sclerosis Patients With Anti–RNA Polymerase III Autoantibodies

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Objective. **To identify predictive factors for scleroderma renal crisis (SRC) in patients with anti–RNA polymerase III (anti–RNAP III) antibodies.**

Methods. **A total of 583 adult Japanese patients with systemic sclerosis (SSc) were screened for anti–RNAP III using a commercially available enzyme-linked immunosorbent assay (ELISA) kit. RNAP subsets were further identified by immunoprecipitation (IP) assays. The association of clinical and immunologic factors with SRC was examined by logistic analyses.**

Results. **In this cohort, 37 patients (6%) were positive for anti–RNAP III, as determined by anti–RNAP III–specific ELISA. Further IP assays revealed that 19 patients were positive for anti–RNAP I/III, 17 for anti–RNAP I/II/III, and 1 for anti-RNAP III. SRC occurred in a total of 17 (2.9%) of 583 patients, with a significantly higher frequency in anti–RNAP III–positive SSc patients (9 of 37 [24%]) than those without anti–RNAP III (8 of 546 [1%]) (odds ratio [OR] 21.6 [95% confidence interval (95% CI) 7.8–60.3], $P < 0.00001$). Our multivariate analyses using the Cox proportional hazards regression model revealed that anti–RNAP I/II/III positivity (OR 11.0 [95% CI 1.6–222.8], $P = 0.0118$) and an ELISA index for anti–RNAP III of ≥157 (OR 2.4 × 10^9 [95% CI 2.1–uncalculated], $P = 0.0093$) were independent factors associated with the development of SRC.**

Conclusion. **Our findings indicate that anti–RNAP III is associated with SRC, as reported previously. In addition, the presence of anti–RNAP II in combination with anti–RNAP I/III (anti–RNAP I/II/III) and a higher ELISA index for anti–RNAP III may be associated with the development of SRC in SSc patients with anti–RNAP III.**

Systemic sclerosis (SSc) is a multisystem connective tissue disorder characterized by excessive fibrosis of skin and internal organs and microvascular damage. While the etiology of SSc remains unclear, autoimmunity is considered to be involved in the pathophysiology. Serum antinuclear antibodies (ANAs) are detected in >90% of patients with SSc and are a hallmark of the disease (1). Many ANAs are specific for SSc, including antibodies against topoisomerase I (topo I), centromeres, RNA polymerase III (RNAP III), Th/To, and U3 RNP. Autoantibodies are present at diagnosis of the disease. These antibodies do not coexist in most patients, and patients' autoantibody profiles usually do not change during the disease course (2). Importantly, ANAs are closely associated with distinct clinical subsets, and thus detection of SSc-related antibodies is useful not only in the diagnosis of the disease, but also for predicting organ involvement and prognosis.

SSc is a heterogeneous disease with variable clinical presentations. Scleroderma renal crisis (SRC) is a major complication in patients with SSc (3). SRC occurs in 5% of patients with SSc, particularly in the first years of disease evolution, and typically in the diffuse

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form (4). It is characterized by accelerated hypertension and acute progressive renal impairment. Although the prognosis of SRC has been dramatically improved by the use of angiotensin-converting enzyme (ACE) inhibitors, long-term survival after SRC remains poor (5,6). Furthermore, SRC is one of the most rapidly progressive conditions among SSc complications. Therefore, identification of high-risk groups and early detection of SRC are critical for improving outcomes. While several indicators for SRC have been reported, anti–RNAP III antibodies are present in one-third of the patients who develop SRC. Nonetheless, the indicators for SRC within the anti–RNAP III–positive group have not been identified. Thus, in this study we examined factors that were associated with SRC in anti–RNAP III–positive SSc patients.

PATIENTS AND METHODS

Patients. We analyzed 583 consecutive Japanese patients with SSc (490 women and 93 men) who were evaluated between 1995 and 2012 at Kanazawa University Hospital or Social Insurance Chukyo Hospital. All patients met one of the following criteria (7): the American College of Rheumatology preliminary criteria for the classification of SSc (8); the presence of at least 3 of the 5 features of CREST syndrome (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasias); or the presence of definite Raynaud's phenomenon, abnormal nailfold capillaries, and scleroderma-specific autoantibodies (9). SSc patients were classified as having limited cutaneous SSc (lcSSc) or diffuse cutaneous SSc (dcSSc) according to the criteria proposed by LeRoy et al (10). All serum samples were obtained prior to the onset of SRC. Fresh venous blood samples were centrifuged shortly after clot formation. Samples were stored at −70°C until used. Written informed consent was obtained from all subjects, and the protocol was approved by Kanazawa University Hospital.

Clinical assessments. The modified Rodnan skin thickness score (MRSS) was used to semiquantitatively assess the degree of skin sclerosis (11), and the maximum score during the disease course was recorded. The skin thickness progression rate was defined as the MRSS on the first visit divided by the duration, in years, from when the patient reported or a physician judged or recorded the onset of skin thickening, in MRSS units per year (12). Organ system involvement was defined principally as previously described (13–17), and definitions are listed in Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38994/abstract.

Enzyme-linked immunosorbent assays (ELISAs). Samples were screened for anti–RNAP III by ELISA kits according to the recommendations of the manufacturer (MBL) (18,19).

Immunoprecipitation (IP) assays. IP assays were performed using extracts of the leukemia cell line K562, as previously described (20). Briefly, 10 μl of patient sera was mixed with protein A–Sepharose CL-4B (Pharmacia Biotech) in IP buffer (10 m*M* Tris HCl, pH 8.0, 500 m*M* NaCl, and 0.1% Nonidet P40) and incubated for 2 hours at 4°C, and then washed 5 times with IP buffer. For protein analysis, the antibody-coated Sepharose beads were incubated with ^{35}S-methionine–labeled K562 cell extracts at 4°C for 2 hours. After 9 washes, the immunoprecipitated materials were subjected to 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Radiolabeled polypeptide components were analyzed by autoradiography.

Statistical analysis. Comparisons between anti–RNAP III–positive SSc patients with SRC and those without SRC were made using a chi-square test with Yates' correction or Fisher's exact test, when appropriate. Continuous variables were divided into 2 groups based on a cutoff value that was determined by the receiver operating characteristic curve in a univariate analysis. For instance, the cutoff value for the anti–RNAP III ELISA index was 157, which yielded a maximum value of "sensitivity − (1 − specificity)" (see Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38994/abstract). Variables selected by the univariate analyses were further subjected to multivariate analyses using a Cox proportional hazards regression model to determine independent factors associated with SRC development. We used the Kaplan-Meier method to estimate SRC development between the 2 patient groups. The results are presented as the odds ratio (OR) and 95% confidence interval (95% CI). Statistical analyses were performed using JMP statistical software version 10.

RESULTS

Assessment of anti–RNAP III in SSc patients. Of the 583 SSc patients included in this study, 60% had lcSSc and 40% had dcSSc (352 and 231 patients, respectively). In this cohort, 37 patients (6%) (13 men and 24 women) were positive for anti–RNAP III, as determined by an anti–RNAP III–specific ELISA. Six of these patients had lcSSc, and 31 had dcSSc. Their mean ± SD maximum MRSS was 17.9 ± 10.8.

We examined the immunoprecipitation pattern of RNAP in the 37 anti–RNAP III–positive patients identified by ELISA. Sera from the 37 anti–RNAP III–positive patients were divided into 3 groups based on the immunoprecipitation of RNAP. Seventeen patients had both anti–RNAP I/III and anti–RNAP II (anti–RNAP I/II/III), 19 had anti–RNAP I/III, and 1 had anti–RNAP III alone. All 17 serum samples that were positive for anti–RNA I/III and II recognized both the phosphorylated and the nonphosphorylated forms of RNAP II (Figure 1). All 37 anti–RNAP III–positive patients were then classified into 2 groups based on the presence or absence of anti–RNAP II. Therefore, the one patient with anti-RNAP III alone was included in the anti–RNAP I/III patient group.

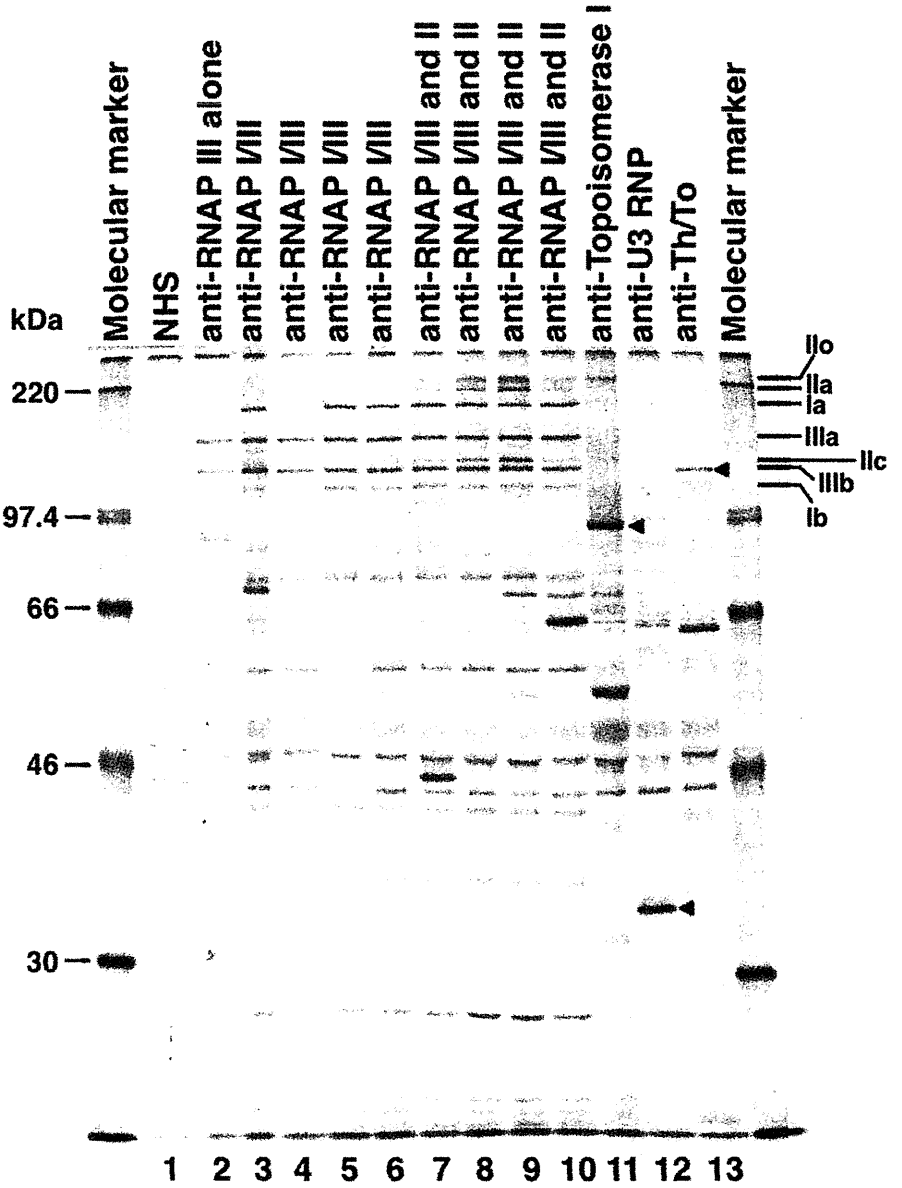

Figure 1. Immunoprecipitation assay of autoantibodies related to systemic sclerosis (SSc). Immunoprecipitation of ^{35}S-methionine–labeled K562 cell extracts was performed on sera from patients with SSc (lanes 2–13) and normal human serum (NHS; lane 1), separated by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and analyzed by autoradiography. RNA polymerase III (RNAP III) alone (lane 2), RNAP I/III (lanes 3–6), and RNAP I/III and II (lanes 7–10) proteins are shown by the indicated lines. Arrowheads indicate topoisomerase I (100 kd; lane 11), U3 RNP (34 kd; lane 12), and 7-2 RNA (120 kd; lane 13). All 17 serum samples that were positive for anti–RNA I/III and II recognized both the phosphorylated (IIo) and the nonphosphorylated (IIa) forms of RNAP II.

SRC frequency in Japanese SSc patients. SRC occurred in 17 (2.9%) of the 583 SSc patients (4 men and 13 women). All 17 patients had dcSSc. Consistent with the findings of previous studies (5), SRC occurred at a significantly higher frequency in anti–RNAP III–positive SSc patients (9 of 37 [24%]) than in those without anti–RNAP III (8 of 546 [1%]) (OR 21.6 [95% CI 7.8–60.3], $P < 0.00001$). Thus, we confirmed that anti–

Table 1. Clinical outcomes in the 37 anti–RNAP III–positive patients with SSc with or without SRC*

	Patients with SRC (n = 9)	Patients without SRC (n = 28)
Age at onset, years	54 ± 16	55 ± 13
Duration, years	1.8 ± 1.1	2.0 ± 1.8
Followup period, years	4.4 ± 2.8	7.8 ± 6.2
SSc subtype, no. with dcSSc/no. with lcSSc	9/0	22/6
MRSS	21 ± 11	17 ± 11
Skin thickness progression rate, MRSS units/year	19 ± 17	16 ± 18
Maximum oral steroid, mg/day	26 ± 15	19 ± 11
ELISA index for anti–RNAP III	250.4 ± 71.2	141.6 ± 84.2†

* Except where indicated otherwise, values are the mean ± SD. Anti–RNAP III = anti–RNA polymerase III; SSc = systemic sclerosis; dcSSc = diffuse cutaneous SSc; lcSSc = limited cutaneous SSc; MRSS = modified Rodnan skin thickness score; ELISA = enzyme-linked immunosorbent assay.
† $P = 0.0013$ versus patients with scleroderma renal crisis (SRC).

RNAP III positivity is characteristic of Japanese SSc patients with SRC. All 9 anti–RNAP–positive and 8 anti–RNAP–negative (7 with anti–topo I and 1 with anti–U3 RNP) patients with SRC had the diffuse form and typical SRC. No anti–RNAP III–positive patients with SRC died of SRC, but 2 of the 8 anti–RNAP III–negative patients with SRC died of SRC despite administration of ACE inhibitors. Of 9 anti–RNAP III–positive patients with SRC, 8 (89%) received corticosteroids, and the corticosteroid dosage was >15 mg/day in 7 (88%) of those 8 patients. Those 8 patients received the maximum dose of corticosteroids in the period preceding SRC. Of 28 anti–RNAP III–positive patients without SRC, 23 patients (82%) received corticosteroids, and 20 (87%) of 23 patients received corticosteroids >15 mg/day.

Clinical characteristics of SSc patients with anti–RNAP III antibodies. We compared 9 patients with anti–RNAP III who developed SRC and 28 patients with anti–RNAP III who did not. As shown in Table 1, there was no significant difference in age at onset, disease duration, or followup period between the 2 groups. Although the differences were not significant, the percentage of patients with dcSSc was higher in the group with SRC than in the group without SRC (100% versus 79%; $P = 0.3025$), and the MRSS was higher in those with SRC than in those without SRC (mean ± SD 21 ± 11 versus 17 ± 11, $P = 0.2778$). Skin thickness progression rate and maximum oral steroid dosage were not statistically significantly different between the 2 groups. However, the ELISA indexes for anti–RNAP III were significantly higher in patients with SRC compared to those without SRC (mean ± SD 250.4 ± 71.2 versus 141.6 ± 84.2; $P = 0.0013$). Additionally, we examined the association of an ELISA index for anti–RNAP III with the maximum skin score. ELISA indexes for anti–RNAP III were positively associated with the maximum skin score, although the correlation was mild ($P = 0.0413$, $R^2 = 0.11365$) (see Supplementary Figure 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38994/abstract).

Predictive factors for SRC and clinical course in anti–RNAP III–positive SSc patients. We examined the baseline clinical and immunologic factors associated with the development of SRC as indicated by logistic analysis. The steroid dosage used for analysis was the maximum dosage used during the patient's entire clinical course. We found that the factors associated with the development of SRC in the univariate analysis were anti–RNAP I/II/III positivity (OR 16.9 [95% CI 1.8–156.3], $P = 0.0055$) and an ELISA index for anti–RNAP III of ≥157 (OR 33.5 [95% CI 4.2–268.2], $P < 0.0001$) (Table 2).

For our multivariate analysis, in addition to anti–RNAP I/II/III positivity and an ELISA index for anti–RNAP III of >157, we added the factors of MRSS and oral steroid dosage, since previous studies indicated that these 2 factors were associated with the development of

Table 2. Univariate logistic regression analysis to predict SRC development*

Variable baseline factor	OR of SRC development (95% CI)
Age at onset >59 years	0.923 (0.204–4.179)
Male	0.900 (0.184–4.400)
MRSS >12	6.000 (0.659–54.667)
Skin thickness progression rate >7.3	3.033 (0.533–17.250)
Digital pitting scars	3.000 (0.525–17.159)
Digital ulcers	7.714 (0.608–97.846)
Interstitial lung disease	1.500 (0.310–7.247)
Gastrointestinal tract involvement	1.400 (0.238–8.240)
Heart involvement	3.000 (0.525–17.159)
Joint involvement	0.375 (0.040–3.551)
Skeletal muscle involvement	7.714 (0.608–97.846)
Malignancy	2.300 (0.424–12.465)
Oral steroids	1.739 (0.176–17.222)
Maximum oral steroid dosage >30 mg/day	2.639 (0.568–12.254)
Anti–RNAP I/II/III positivity	16.889 (1.825–156.282)†
ELISA index for anti–RNAP III ≥157	33.476 (4.178–268.216)‡

* SRC = scleroderma renal crisis; OR = odds ratio; 95% CI = 95% confidence interval; MRSS = modified Rodnan skin thickness score; anti–RNAP I/II/III = anti–RNA polymerase I/II/III; ELISA = enzyme-linked immunosorbent assay.
† $P = 0.0055$.
‡ $P < 0.0001$.

Table 3. Selected variables from the Cox proportional hazards regression model to predict SRC development*

Variable baseline factors	OR of SRC development (95% CI)
MRSS >12	4.743 (0.601–96.331)
Maximum oral steroid dosage >30 mg/day	1.790 (0.386–9.698)
Anti-RNAP I/II/III positivity	10.967 (1.617–222.846)†
ELISA index for anti-RNAP III ≥157	2.367×10^9 (2.122–uncalculated)‡

* SRC = scleroderma renal crisis; OR = odds ratio; 95% CI = 95% confidence interval; MRSS = modified Rodnan skin thickness score; anti-RNAP I/II/III = anti-RNA polymerase I/II/III; ELISA = enzyme-linked immunosorbent assay.
† $P = 0.0118$.
‡ $P = 0.0093$.

SRC (4,6). Our multivariate analyses using a Cox proportional hazards regression model revealed that anti-RNAP I/II/III positivity (OR 11.0 [95% CI 1.6–222.8], $P = 0.0118$) and an ELISA index for anti-RNAP III of ≥157 (OR 2.4×10^9 [95% CI 2.1–uncalculated], $P = 0.0093$) were the independent factors associated with the development of SRC (Table 3).

Based on our multivariate analysis, the development of SRC was further evaluated by Kaplan-Meier analyses of anti-RNAP I/II/III and anti-RNAP I/III (Figure 2A) and of an ELISA index of ≥157 and of <157 (Figure 2B). Eight (47%) of 17 patients with anti-RNAP I/II/III developed SRC, whereas only 1 patient had SRC in the anti-RNAP I/III–positive group ($P = 0.0023$ by log rank test). While 9 (47%) of 19 patients whose ELISA indexes for anti-RNAP III were ≥157 had SRC, none of the patients with an ELISA index of <157 developed SRC ($P = 0.0005$ by log rank test). Figure 3 summarizes the association between the development of SRC, anti-RNAP subtype, and ELISA index for anti-RNAP III. The difference in ELISA indexes for anti-RNAP III between patients with anti-RNAP I/II/III and those with anti-RNAP I/III was not significant ($P = 0.231$).

DISCUSSION

In this study, we demonstrated that coexistence of anti-RNAP II with anti-RNAP I/III (anti-RNAP I/II/III) and a higher ELISA index for anti-RNAP III were immunologic predictors of developing SRC in anti-RNAP III–positive patients with SSc. Moreover, we did not detect any contribution of skin thickness or oral prednisolone dosage to the risk of developing SRC.

RNAPs I, II, and III are major targets of autoantibody responses in SSc patients. Most SSc sera that react with RNAPs recognize more than one class of RNAP, with a pattern of RNAP I/III or RNAP I/II/III. Anti-RNAP II alone is detected at a low frequency in sera from SSc patients, often in a combination with anti-topo I, and also in sera from some patients with systemic lupus erythematosus or overlap syndrome (21,22). Thus, anti-RNAP I and anti-RNAP III (anti-RNAP I/III) are highly specific for SSc and almost always coexist in most anti-RNAP III–positive SSc sera, while some of these sera contain anti-RNAP II as well (anti-RNAP I/II/III) (23,24).

The frequency of anti-RNAP III positivity varies in different ethnicities. In a US cohort, 61 (25%) of 247 patients with SSc were positive for anti-RNAP III, whereas in a French cohort 5 (4%) of 127 patients were

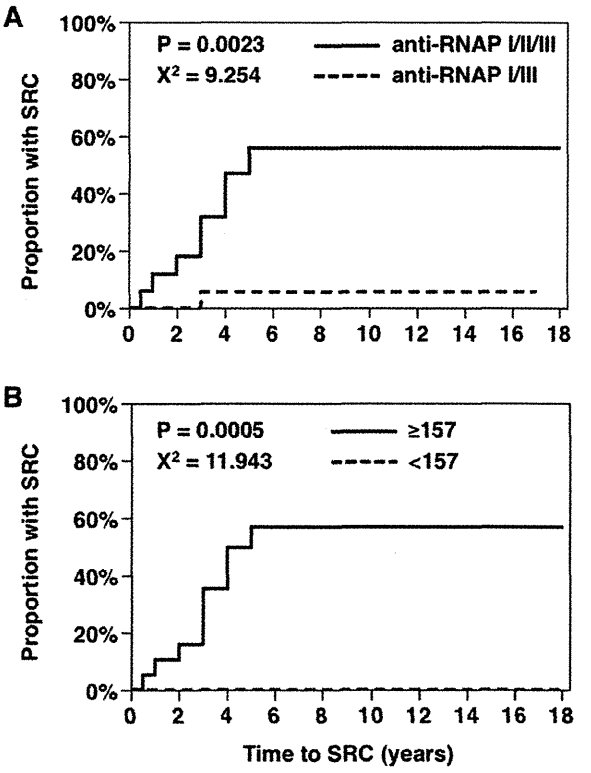

Figure 2. Kaplan-Meier plots of time to the development of scleroderma renal crisis (SRC). **A,** Significantly increased incidence of SRC in patients with anti-RNA polymerase I/II/III (anti-RNAP I/II/III) versus those with anti-RNAP I/III ($P = 0.0023$ by log rank test). **B,** Significantly increased incidence of SRC in patients with an enzyme-linked immunosorbent assay (ELISA) index for anti-RNAP III of ≥157 versus those with an ELISA index for anti-RNAP III of <157 ($P = 0.0005$ by log rank test).

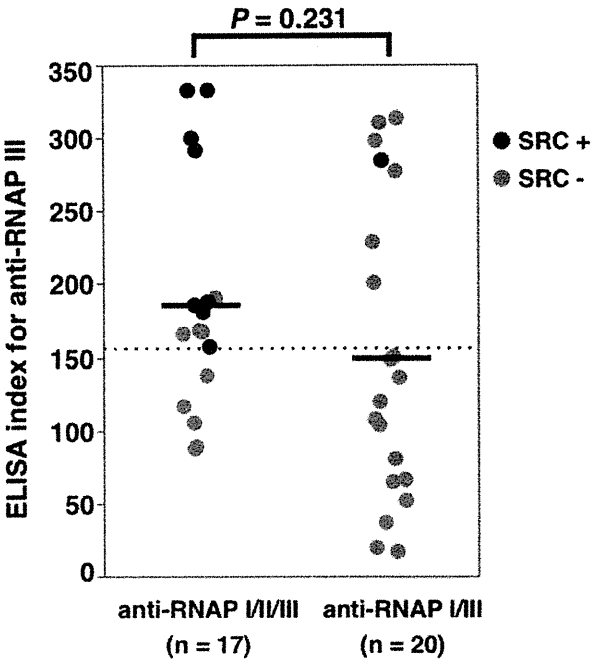

Figure 3. Enzyme-linked immunosorbent assay (ELISA) index for anti-RNA polymerase III (anti-RNAP III) in patients with anti-RNAP I/II/III with or without scleroderma renal crisis (SRC) and patients with anti-RNAP I/III with or without SRC. Circles represent individual patients; horizontal lines show the mean. The broken line indicates the cutoff value of 157.

positive (25). Nikpour et al reported that 69 (15.3%) of 451 SSc patients had anti–RNAP III in an Australian cohort (26). In a Japanese population, anti–RNAP III was detected in 38 (10.7%) of 354 patients with SSc (19). In the present study, the frequency of anti–RNAP III was 6% (37 of 583 SSc patients), confirming the previous finding that anti–RNAP III is more prevalent in North American than in Japanese SSc patients (14,15).

Several factors associated with SRC have been identified, including a disease duration of <4 years, diffuse and rapidly progressive skin thickening, new cardiac events, and the presence of anti–RNAP III (4,6,27). Steen (28) also demonstrated that reduction of renal blood flow caused by sepsis, dehydration, and cardiac dysfunction is a potential trigger for SRC. Regarding genetic factors, HLA–DRB1*0407 and *1304 are independent risk factors for the development of SRC (29).

Our study revealed that the presence of anti–RNAP II in combination with anti–RNAP I/III is a predictive factor for SRC. Harvey et al reported a detailed consideration of clinical associations with a subset of anti-RNAP (30). The incidence of renal involvement was associated with anti-RNAP. The highest rate of SRC was observed in patients with anti–RNAP I/II/III (40%), while the anti–RNAP I/III group had a lower frequency of SRC (14.3%). There was no statistically significant difference between these 2 groups, possibly due to the small numbers involved. Our results confirmed the findings of that previous study. Therefore, it is likely that coexistence of anti–RNAP II and anti–RNAP I/III (anti–RNAP I/II/III) is associated with an increased incidence of SRC.

In this study, all 9 patients with SRC had an ELISA index for anti–RNAP III of ≥157. There are several studies that describe the association of an ELISA index for anti–RNAP III with clinical features that include internal organ involvement. Kuwana et al (18) reported the association of anti–RNAP III levels with clinical findings and found that patients with a high level of anti–RNAP III had dcSSc more frequently than those with a lower level. The maximum total skin score and frequency of tendon friction rubs were also significantly increased in the high-level group compared with the low-level group. However, there were no significant differences in the frequencies of internal organ involvement, including SRC (18). Nihtyanova et al (31) also reported no correlation between peak anti–RNAP III ELISA index levels and the onset of SRC.

In contrast, our multivariate analyses revealed that a higher ELISA index for anti–RNAP III was another predictive factor for SRC. Several possibilities could account for this discrepancy. The study by Kuwana et al (18) analyzed a total of 90 patients (17 from Tokyo and 73 from Pittsburgh), a larger number of patients than our study, although with fewer Japanese patients. The study by Nihtyanova et al (31) was limited to Caucasian patients. In contrast, our study included all Japanese patients. Therefore, it cannot be ruled out that ethnicity can affect the results. Alternatively, the small number of patients in this study may explain this difference, since this study included only 9 anti–RNAP III–positive patients who developed SRC.

Exposure to corticosteroids has been considered a risk factor for SRC. In a case–control study, the use of corticosteroid therapy at a prednisone dosage of >15 mg/day was prescribed significantly more frequently in SRC patients (36%) than in controls (12%) (32). In addition, Helfrich et al observed an association of high-dose (>30 mg/day) corticosteroid therapy with normotensive SRC (33). In this study, we did not detect any influence of corticosteroid treatment in the development

of SRC. This may be explained in part by the fact that most patients with anti–RNAP III were treated with corticosteroids despite the presence or absence of SRC. However, the possibility cannot be ruled out that the use of corticosteroids increases the overall incidence of SRC. Whether there is an association of the use of corticosteroids with the incidence of SRC in Japanese SSc patients remains unclear. Further studies are needed to clarify the influence of corticosteroids as a trigger for developing SRC.

In conclusion, this study demonstrated the clinical importance of the presence of anti–RNAP I/II/III and/or an elevated ELISA index for anti–RNAP III as predictive factors for SRC. However, clinicians should watch all anti–RNAP III–positive patients for the development of SRC, since the identification of anti–RNAP II is limited to certain facilities. Establishing a system that could routinely screen for anti–RNAP II, such as ELISA, is needed. Our findings should be confirmed in a large-scale study of patients of different ethnicities in future studies.

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Study conception and design. Hamaguchi, Kodera, Matsushita, Hasegawa, Inaba, Usuda, Kuwana, Takehara, Fujimoto.

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Analysis and interpretation of data. Hamaguchi, Kodera, Matsushita, Hasegawa, Inaba, Usuda, Kuwana, Takehara, Fujimoto.

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Biomarkers in systemic sclerosis: their potential to predict clinical courses

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Abstract:	The concept of biomarker was defined as " a characteristic marker that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" by the National Institutes of Health Biomarkers Definitions Working group in 2001. Clinical features, disease progress, therapeutic response, and prognosis are heterogeneous among patients with systemic sclerosis (SSc). Therefore, biomarkers that can predict these matters are required for the progress of clinical practice. At present, SSc-specific autoantibodies are most useful biomarkers for diagnosis and predicting clinical features. Otherwise, biomarkers specific only for SSc have not been identified yet. The glycoprotein krebs von den Lungen-6, surfactant protein-D, and CCL18 are promising serum biomarkers of SSc-related interstitial lung diseases. Serum/plasma levels of brain natriuretic peptide and serum N-terminal pro-brain natriuretic peptide have been used as biomarkers for SSc-related pulmonary arterial hypertension. Other potential serum/plasma biomarkers for fibrosis and vascular involvement of SSc are connective tissue growth factor, interleukin 6, CCL2, CXCL4, intercellular adhesion molecule-1, P-selectin, vascular endothelial growth factor, Von Willebrand factor, endostatin, endoglin, and endothelin-1. In our multicentre prospective studies of Japanese early SSc, serum ICAM-1 levels were predictive for subsequent respiratory dysfunction and serum levels of CXCL8 and P-selectin were predictive for subsequent physical disability. Further large, multicentre, prospective longitudinal studies will be needed to identify and validate critical biomarkers of SSc.

Biomarkers in systemic sclerosis: their potential to predict clinical courses

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Abstract

The concept of biomarker was defined as " a characteristic marker that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" by the National Institutes of Health Biomarkers Definitions Working group in 2001. Clinical features, disease progress, therapeutic response, and prognosis are heterogeneous among patients with systemic sclerosis (SSc). Therefore, biomarkers that can predict these matters are required for the progress of clinical practice. At present, SSc-specific autoantibodies are most useful biomarkers for diagnosis and predicting clinical features. Otherwise, biomarkers specific only for SSc have not been identified yet. The glycoprotein krebs von den Lungen-6, surfactant protein-D, and CCL18 are promising serum biomarkers of SSc-related interstitial lung diseases. Serum/plasma levels of brain natriuretic peptide and serum N-terminal pro-brain natriuretic peptide have been used as biomarkers for SSc-related pulmonary arterial hypertension. Other potential serum/plasma biomarkers for fibrosis and vascular involvement of SSc are connective tissue growth factor, interleukin 6, CCL2, CXCL4, intercellular adhesion molecule-1, P-selectin, vascular endothelial growth factor, Von Willebrand factor, endostatin, endoglin, and endothelin-1. In our multicentre prospective studies of Japanese early SSc, serum ICAM-1 levels were predictive for subsequent respiratory dysfunction and serum levels of CXCL8 and P-selectin were predictive for subsequent physical disability. Further large, multicentre, prospective longitudinal studies will be needed to identify and validate critical biomarkers of SSc.

Key words: systemic sclerosis, biomarker, fibrosis, vascular injury, autoantibody