Autoantibody against a protease domain of caspase-8 in patients with systemic sclerosis

T. Yamaoka, F. Ogawa, E. Muroi, T. Hara, K. Komura, Y. Iwata, M. Takenaka, K. Shimizu, M Hasegawa[1], M. Fujimoto[1], S. Sato

Department of Dermatology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan and [1]Department of Dermatology, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan.

Abstract

Background

Systemic sclerosis (SSc) is characterized by autoantibodies against various cellular components.

Objective

To determine the presence or levels of antibodies (Abs) against a protease domain (PD) of caspase-8 and their clinical relevance in SSc.

Methods

Anti-caspase-8 PD Ab was examined by enzyme-linked immunosorbent assay and immunoblotting using human recombinant caspase-8 PD. Caspase-8 activity was evaluated by spectrophotometric detection of cleavage from p-nitroanilide-labeled IETD, a substrate of caspase-8.

Results

IgG anti-caspase-8 PD Ab levels in patients with SSc, systemic lupus erythematosus, or dermatomyositis were higher than in normal controls (CTL). Furthermore, anti-caspase-8 PD Ab levels in limited cutaneous SSc (lSSc) patients were elevated compared to diffuse cutameous SSc (dSSc) patients. To investigate the clinical correlation, laboratory findings were compared between SSc patients with high levels (>the mean+2SD of CTL) of anti-caspase-8 PD Ab and those with low levels. SSc patients with high levels exhibited lower frequency of male and decreased C-reactive protein levels relative to those with low levels. Immunoblotting showed that anti-caspase-8 PD Ab was present in all SSc patients examined, while it was also detected in 75% of CTL. Caspase-8 activity was inhibited by IgG isolated from sera of SSc patients and CTL, although inhibitory effect was greater in SSc patients than CTL.

Conclusion

These results suggest that immune response to caspase-8 occurs in healthy individuals, although it is greater in patients with systemic autoimmune diseases including SSc. Furthermore, high levels of anti-caspase-8 PD Ab may be a serological indicator for a milder SSc subset.

Key words

Systemic sclerosis, caspase-8, autoantibody.

Toshifumi Yamaoka, MD
Fumihide Ogawa, MD, PhD
Eiji Muroi, MD
Toshihide Hara, MD
Kazuhiro Komura, MD, PhD
Yohei Iwata, MD
Motoi Takenaka, MD, PhD
Kazuhiro Shimizu, MD, PhD
Minoru Hasegawa, MD, PhD
Manabu Fujimoto, MD, PhD
Shinichi Sato, MD, PhD

Please address correspondence and reprint requests to:
Shinichi Sato, MD, PhD,
Department of Dermatology,
Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki, 852-8501 Japan.
E-mail: s-sato@nagasaki-u.ac.jp

Received on November 2, 2007; accepted in revised form on May 16, 2008.

Competing interests: none declared.

Introduction

Systemic sclerosis (SSc) is a multisystem disorder of connective tissue characterized by excessive fibrosis in the skin and various internal organs, such as the lungs, kidneys, esophagus, and heart. Although the pathogenesis of SSc remains unknown, systemic autoimmunity is one of the central features of SSc, since antinuclear antibodies (Abs) are detected in more than 90% of SSc patients (1). SSc patients have autoantibodies that react to various intracellular components, such as DNA topoisomerase I, centromere, RNA polymerases, U1RNP, U3RNP, Th/To, and histones (1). Although it remains controversial whether these SSc-specific autoantibodies directly contribute to the clinical manifestations of SSc, autoantibodies against several intracellular and extracellular enzymes, such as antioxidant enzyme peroxiredoxin and matrix matelloproteinases, may play a pathogenetic role (2-4).

The caspase family consists of proteases that use cysteine residues as the catalytic nucleophile and shares a specificity for cleaving target proteins at sites next to aspartic acid residues (5). Caspases play an essential role in apoptosis that is critical for embryonic development and the pathogenesis of various diseases (5). The cell death caspases are classified into initiators (caspases-2, -8, 9, and -10) and executioners (caspases-3, -6, and -7) of apoptosis (6). The initiator caspases sense death signals, and activate more downstream executioner caspases, which cleave cellular substrates, mediating the changes associated with apoptosis (6). Caspase-8 is one of apoptosis initiators that mediate cell death through death receptors, such as CD95 (Fas/APO-1) (5). Procaspase-8 consists of two death effector domains and a protease domain comprising two subunits, p18 and p12. Procaspase-8 possesses autoprocessing capacity, leading to the formation of hetero-tetramer consisting of 2 each of p18 and p12 as active caspase-8 (7). Caspase-8 has been shown to have additional functions unrelated to cell death, including T cell activation, cell motility, tumor metastasis, and pancreatic β cell function (6, 8-10)

A recent study has shown that autoantibodies against caspase-8 are detected in healthy individuals and patients with SSc, systemic lupus erythematosus (SLE), or silicosis (11). Since the number of SSc patients examined was limited in that study (n=10), prevalence of high levels of anti-caspase-8 Ab and clinical significance remained unknown in SSc. Furthermore, it also remained unknown whether autoantibodies against caspase-8 could inhibit caspase-8 activity. Therefore, in the present study, the presence or levels of autoantibodies against a protease domain (PD) of caspase-8, their clinical relevance, and their functional significance were investigated.

Materials and methods

Patients and controls

Blood samples were obtained from 70 Japanese SSc patients (61 women and 9 men). All patients fulfilled the criteria proposed by the American College of Rheumatology (12). These patients were grouped according to the classification system proposed by LeRoy *et al*. (13): 30 patients (28 women and 2 men) had limited cutaneous SSc (lSSc) and 40 patients (33 women and 7 men) had diffuse cutaneous SSc (dSSc). The age of patients (mean±SD) was 50±16 years. Patients with dSSc were aged 49±18, while those with lSSc were 53±14 years old. The disease duration of patients with lSSc and dSSc was 8.3±9.3 and 3.0±2.9 years, respectively. None of SSc patients was treated with oral corticosteroid, D-penicillamine, or other immunosuppressive therapy at the evaluation. Antinuclear Ab was determined by indirect immunofluorescence using HEp-2 cells as the substrate, and specificities of autoantibody were further assessed by ELISA and immunoprecipitation. Anticentromere Ab was positive for 26 patients (4 dSSc and 22 lSSc), anti-topoisomerase I Ab for 31 (25 dSSc and 6 lSSc), anti-U1RNP Ab for 2 (all lSSc), anti-U3RNP Ab for 1 (dSSc), anti-RNA polymerases I and III Ab for 7 (all dSSc), and Th/To Ab for 1 (lSSc). The remaining 4 patients were negative for autoantibodies. Twenty patients with SLE, who fulfilled the American College of Rheumatology criteria

(14), were also examined as disease control in this study. In addition, 20 patients with dermatomyositis (DM) that fulfilled Bohan and Peter criteria (15, 16) were included. Twenty-two age- and sex-matched healthy Japanese individuals were used as normal control. Fresh venous blood samples were centrifuged shortly after clot formation. All samples were stored at -70˚C prior to use.

Clinical assessment

Complete medical histories, physical examinations, and laboratory test, including vital capacity (VC) and diffusion capacity for carbon monoxide (DLco), were conducted for all patients within 3 to 5 weeks after serum collection. Skin score was measured by scoring technique of the modified Rodnan total skin thickness score (modified Rodnan TSS) (17). The 17 anatomical areas were rated as 0 (normal skin thickness), 1+ (mild but definite skin thickening), 2+ (moderate skin thickening), and 3+ (severe skin thickening) and the modified Rodnan TSS was derived by summation of the scores from all 17 areas (range 0-51). Organ involvement was defined as described previously with some modifications (18): pulmonary fibrosis=bibasilar fibrosis on chest radiography and high-resolution computed tomography; esophagus=hypomotility shown by barium radiography; joint=inflammatory polyarthralgias or arthritis; heart=pericarditis, congestive heart failure or arrhythmias requiring treatment; kidney=malignant hypertension and rapidly progressive renal failure without any other explanation; and muscle=proximal muscle weakness and elevated serum creatine kinase. When the DLco and VC were <75% and <80%, respectively, of the predicted normal values, they were considered to be abnormal. The protocol for the study was approved by local ethical committee of Kanazawa University School of medicine and Kanazawa University Hospital, and informed consents were obtained from all patients according to the declaration of Helsinki.

Enzyme-linked immunosorbent assay (ELISA) for anti-caspase-8 PD Ab

Ninety six-well plates were coated with a PD (amino acids 217-479) of recombinant human caspase-8 (1 mg/ml; R&D Systems, Minneapolis, MN, USA) at 4˚C overnight. The wells were blocked with 2% bovine serum albumin and 1% gelatin in Tris-buffered saline (TBS) for 1 hour at 37˚C. After washing twice with TBS, the serum samples (100 ml) diluted to 1:100 in TBS containing 1% bovine serum albumin were added to triplicate wells and incubated for 90 minutes at 20˚C. After washing 4 times with TBS containing 0.05% Tween-20, the plates were incubated with alkaline phosphatase-conjugated goat anti-human IgG or IgM Abs (Cappel, Durham, NC, USA) for 1 hour at 20˚C. After washing 4 times with TBS containing 0.05% Tween-20, substrate solution containing 0.91 mg/ml *p*-nitrophenyl phosphate (Sigma-Aldrich Co., St. Louis, MO, USA) in diethanolamine buffer (1M diethanolamine, 0.5M $MgCl_2$) was added and the optical density (OD) of the wells at 405 nm was subsequently determined.

Immunoblotting

Immunoblotting was performed using a PD (amino acids 217-479) of recombinant human caspase-8 as described (19). A PD of recombinant human caspase-8 (1mg/lane) was subjected to electrophoresis and electrotransferred to nitrocellulose sheets. The nitrocellulose sheets were cut into strips and incubated overnight at 4˚C with serum samples diluted 1:100. Then, the strips were incubated for 1.5 hours with alkaline phosphatase-conjugated goat anti-human IgG Ab. Color was developed using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Sigma-Aldrich Co.). Ten SSc patients, five SLE patients, and five DM patients with high levels of IgG anti-caspase-8 Ab by ELISA and 8 healthy individuals were evaluated. Furthermore, polyclonal anti-caspase-8 Ab that recognized p12 and p18 (R&D Systems) was used as a positive control.

Caspase-8 activity inhibition assay

IgG was purified from serum samples using magnetic beads coated with recombinant protein G covalently coupled to the surface (Dynal Lake Success, NY, USA). Final IgG concentration was measured by spectrophotometer (Gene Quant II, Amersham Biosciences, Piscataway, NY, USA). Caspase-8 activity was determined by a caspase-8 colorimetric assay kit (ALEXIS biochemicals, Lausen, Switzerland), according to the manufacturer's protocol. This assay is based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the

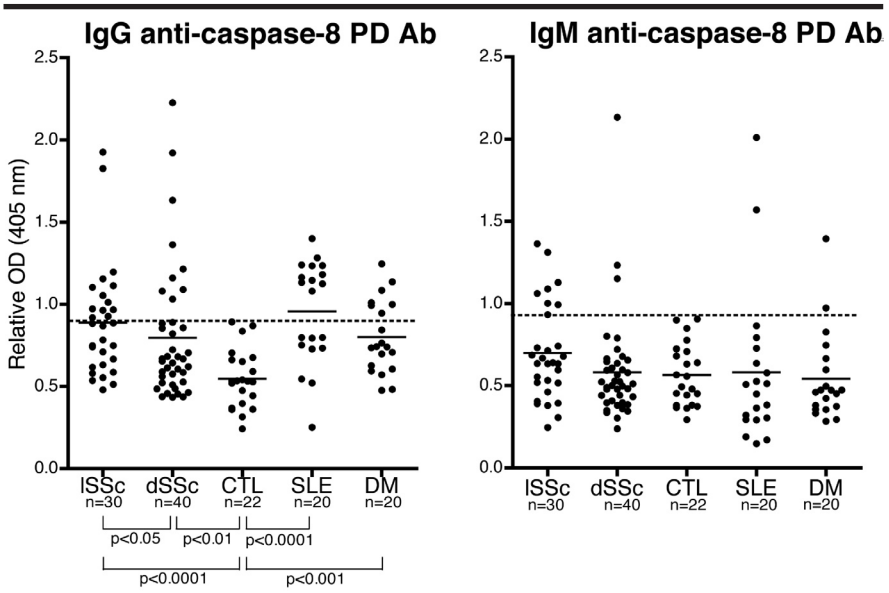

Fig. 1. IgG and IgM anti-caspase-8 PD Ab levels in serum samples from patients with lSSc, dSSc, SLE, or DM and healthy controls (CTL). Anti-caspase-8 PD Ab levels were determined by ELISA using recombinant human caspase-8 PD. The short bar indicates the mean value in each group, while the broken line indicates the mean+2SD of CTL.

pNA-labeled IETD that is the sequence recognized by caspase-8. IETD stands for isoleucine, glutamic acid, threonine, and aspartic acid, respectively. First, a pre-reaction cocktail, which contained 25 mM Hepes-KOH buffer (pH 7.5), 0.1% Chaps, and 10 mM dithiothreitol in 170 ml total volume, was prepared. Then, 0.04 mg of caspase-8 (10 ml) was incubated with 60 mg of purified IgG (60 ml) for 20 minutes at 20˚C. Then, caspase-8 treated with IgG and pre-reaction cocktail were added to each well in the ELISA plate, followed immediately by addition of 60 ml of reaction buffer and 5 ml of the 4 mM IETD-pNA substrate. After wells were incubated at 37˚C for 1 hour, OD at 400 nm was determined, excitation 505 nm. Six SSc patients with high levels of IgG anti-caspase-8 Ab and 6 healthy individuals were assessed.

Statistical analysis

Comparisons between two groups of data were performed using a Mann-Whitney U-test. Comparisons among three or more groups were performed using a one-way ANOVA followed by a Bonferroni's test. Fisher's exact probability test was used for comparison of frequencies. A p-value <0.05 was considered statistically significant.

Table I. Clinical and laboratory features of SSc patients with high levels of IgG anti-caspase-8 PD Ab.

	High levels of IgG anti-caspase-8 PD Ab (n=22)	Low levels of IgG anti-caspase-8 PD Ab (n=48)
Sex, males/females	0/22*	9/39
Age at onset, mean±SD, years	44 ± 16	47 ± 17
Disease duration, mean±SD, years	4.9 ± 6.4	5.3 ± 7.1
Disease pattern, dSSc/lSSc	9/13	31/17
Clinical feature		
Modified Rodnan TSS, mean±SD	12.7 ± 10.1	14.4 ± 9.9
Pitting scars	27	44
Diffuse pigmentation	45	58
Contracture of phalanges	45	45
Organ involvement		
Lungs		
Pulmonary fibrosis	45	42
Decreased %VC	24	40
Decreased %DLco	67	74
Esophagus	45	55
Heart	14	17
Kidneys	9	0
Joints	18	21
Muscle	9	23
Laboratory findings		
Positive for anti-topoisomerase I Ab	23	40
Positive for anticentromere Ab	41	35
Serum IgG, mean±SD, mg/dl	1710 ± 487	1657 ± 549
ESR, mean±SD, mm/h	15.5 ± 9.0	18.7 ± 15.8
CRP, mean±SD, mg/dl	0.135 ± 0.24*	0.385 ± 0.69

Unless noted otherwise, values are percentages. Anti-caspase-8 PD Ab levels were determined by ELISA using human recombinant caspase-8 PD. OD values greater than the mean+2 SD of normal controls were considered "high levels" in this study.
TSS: total skin thickness score and ESR: erythrocyte sedimentation rates.
*p< 0.05 *vs*. SSc patients with low levels of IgG anti-caspase-8 PD Ab.

Results

Autoantibodies to human caspase-8 PD by ELISA

IgG anti-caspase-8 PD Ab levels in total SSc patients were significantly higher than those found in normal controls (p<0.001; Fig. 1). Regarding the disease subsets, IgG anti-caspase-8 PD Ab levels in lSSc patients were significantly elevated compared to those in dSSc (p<0.05) as well as normal controls (p<0.0001). Likewise, dSSc patients exhibited significantly increased IgG anti-caspase-8 PD Ab levels relative to normal controls (p<0.01). In contrast, IgM anti-caspase-8 PD Ab levels in patients with dSSc or lSSc were similar to normal controls. IgG but not IgM anti-caspase-8 PD Ab levels were significantly elevated in SLE patients and DM patients than CTL (p<0.0001 and p<0.001, respectively). However, there was no significant difference in IgG or IgM anti-caspase-8 PD Ab levels among SLE, DM, lSSc, and dSSc patients.

OD values greater than the mean+2SD (0.903 for IgG anti-caspase-8 PD Ab and 0.939 for IgM anti-caspase-8 PD Ab) of normal controls were considered "high levels" in this study (Fig. 1). In total SSc patients, high levels of IgG or IgM anti-caspase-8 Ab were observed in 39%. High levels of IgG or IgM anti-caspase-8 PD Ab were detected in 57% of lSSc patients, while only 25% of dSSc patients had high levels. Similarly, 55% of SLE patients and 35% of DM patients exhibited high levels of IgG or IgM anti-caspase-8 PD Ab. In contrast, high levels of IgG or IgM anti-caspase-8 PD Ab levels were not present in healthy individuals. Thus, IgG but not IgM anti-caspase-8 PD Ab levels were elevated in SSc, especially lSSc, although they were also increased in SLE and DM.

Clinical correlation of high anti-caspase-8 PD Ab levels

To investigate the clinical association of high anti-caspase-8 PD Ab levels, physical and laboratory findings were compared between SSc patients with high levels of IgG anti-caspase-8 PD Ab and those with low levels of this Ab (Table I). Prevalence of lSSc tended to be higher in SSc patients with high Ab levels than that found in those with low levels; however, the difference did not reach statistical significance (59% vs. 35%; p=0.07). SSc patients with high Ab levels exhibited significantly lower frequency of male and lower levels of C-reactive protein (CRP) than those with low levels (p<0.05, respectively). However, any significant association of high levels of IgM anti-caspase-8 PD Ab levels with clinical parameters was not observed (data not shown). Thus, high levels of IgG anti-caspase-8 PD Ab were associated with less

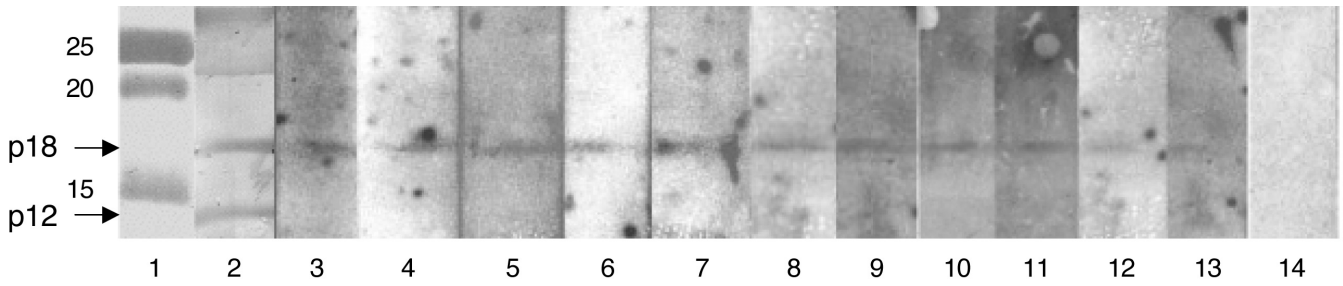

Fig. 2. Representative Immunoblotting of IgG anti-caspase-8 PD Ab in serum samples from patients with SSc, SLE, or DM and healthy individuals, using recombinant human caspase-8 PD. By autoprocessing, this PD is cleaved into two subunits, p18 and p12, after the aspartate corresponding to aspartate 384. Lane 1: markers for molecular weights (kDa). Lane 2: polyclonal anti-caspase-8 Ab that recognized p12 and p18. Lanes 3-7: serum samples from SSc patients. Lanes 8-9: serum samples from SLE patient. Lanes 10-11: serum samples from DM patients. Lanes 12-14: serum samples from healthy individuals who did not exhibit high levels of IgG anti-caspase-8 PD Ab by ELISA. Serum samples from SSc, SLE, or DM patients contained high levels of IgG anti-caspase-8 PD Ab by ELISA. The results represent those obtained with 10 SSc patients, 5 SLE patients, 5 DM patients, and 8 healthy individuals.

frequency of male and decreased CRP levels.

Immunoblotting analysis

The presence of anti-caspase-8 PD Ab was evaluated by immunoblotting analysis using a PD (amino acids 217-479) of recombinant human caspase-8. By autoprocessing, this PD is cleaved into two subunits, p18 and p12, after the aspartate corresponding to aspartate 384, as shown by immunoblotting analysis using polyclonal anti-caspase-8 Ab that recognized p12 and p18 (Fig. 2, lane 2). Serum samples from all examined patients including 10 SSc patients, 5 SLE patients, and 5 DM patients, all of whom had high levels of IgG anti-caspase-8 PD Ab by ELISA, exhibited reactivity with p18 but not p12 by immunoblotting (lanes 3-11). As expected by the ELISA assay, in which reactivity with caspase-8 PD generally overlapped among SSc patients, SLE patients, DM patients, and normal controls (Fig. 1), 6 serum samples from 8 healthy individuals who did not have high levels of IgG anti-caspase-8 PD Ab by ELISA reacted with p18 but not p12 (lanes 12-13), although the band intensity was generally lower than that found in SSc, SLE, or DM patients. The remaining 2 samples were negative for anti-caspase-8 PD Ab (lane 14). Thus, anti-caspase-8 PD Ab was present in patients with SSc, SLE, or DM and some normal individuals by immunoblotting analysis.

Inhibition of caspase-8 activity by IgG isolated from serum samples of SSc patients and healthy individuals

To determine the functional significance of anti-caspase-8 PD Ab, we assessed whether anti-caspase-8 PD Ab was able to inhibit caspase-8 activity. The caspase-8 activity assay is based on spectrophotometric detection of the chromophore pNA after cleavage from the pNA-labeled IETD that is the sequence recognized by caspase-8. IgG isolated from both SSc patients and healthy individuals significantly inhibited the caspase-8 activity by 40% and 23%, respectively, relative to the untreated caspase-8 activity ($p<0.05$). However, IgG isolated from SSc patients suppressed the caspase-8 activity more strongly than that from healthy individuals ($p<0.05$; Fig. 3).

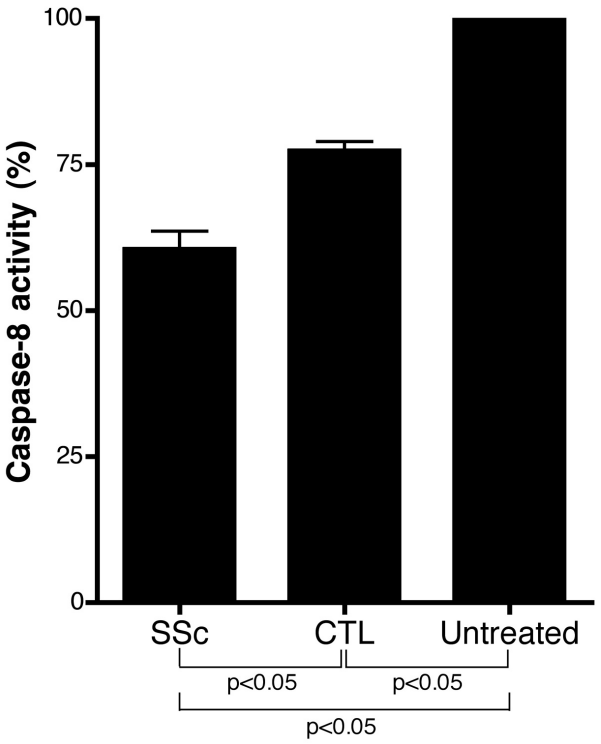

Fig. 3. Inhibition of caspase-8 activity by IgG isolated from SSc patients and healthy individuals. IgG was purified from serum samples of SSc patients with high levels of IgG anti-caspase-8 PD Ab by ELISA and normal individuals who did not exhibit high levels of IgG anti-caspase-8 PD Ab by ELISA. The caspase-8 activity assay is based on spectrophotometric detection of the chromophore pNA after cleavage from the pNA-labeled IETD that is the sequence recognized by caspase-8. Caspase-8 activity is shown as percentage of untreated caspase-8 (Untreated) that was defined as 100%. Each histogram shows the mean (+SD) values obtained from 6 subjects of each group.

Discussion

In this study, IgG anti-caspase-8 PD Ab levels were significantly elevated in serum samples from SSc patients relative to normal controls by ELISA. Similarly, patients with SLE or DM exhibited significantly higher IgG anti-caspase-8 PD Ab levels than normal controls. Consistent with this finding, a previous study has shown that IgG (mainly IgG1) anti-caspase-8 Ab levels in patients with SSc or SLE were generally higher than those found in healthy individuals, although the number of each group was small (n=3–6) (11). Therefore, IgG anti-caspase-8 PD Ab was not specific to SSc and appeared

to be commonly detected in connective tissue diseases. Furthermore, there was overlap in IgG anti-caspase-8 PD Ab levels by ELISA between SSc patients and normal controls. In addition, immunoblotting analysis showed that anti-caspase-8 PD Ab was detected in 75% of healthy individuals as well as SSc patients. A previous study has also revealed that anti-caspase-8 PD Ab is detected in 70% of healthy individuals (11). Therefore, these results suggest that immune response to caspase-8 occurs in healthy individuals, although immune response to caspase-8 is generally greater in patients with SSc, SLE, or DM. Furthermore, anti-caspase-8 PD Ab levels were significantly higher in patients with a milder form of SSc, lSSc, than dSSc patients. In addition, SSc patients with high levels of anti-caspase-8 PD Ab exhibited decreased CRP levels. These results suggest that anti-caspase-8 PD Ab is a serological indicator for a milder SSc subset.

It has been shown that cleavage of certain autoantigens during apoptosis reveals immunocryptic epitopes that could potentially induce autoantibody immune response in systemic autoimmune diseases (20). Apoptosis is detected in endothelial cells of early inflammatory disease stage of SSc (21). Ischemia and reperfusion injury following Raynaud's phenomenon can generate reactive oxygen species that may result in vascular endothelial damage and apoptosis (22, 23). Furthermore, topoisomerase I, an autoantigen specific for SSc, is one of major targets of proteases during CD95-mediated apoptosis (20). Since caspase-8, an initiator of apoptosis, is first activated following CD95 ligation (5) and some autoantigens are actually cleaved by caspases (24), this apoptosis-specific proteolysis of topoisomerase I may be catalyzed by caspases, especially caspase-8. These results suggest that anti-caspase-8 Ab in SSc is produced during apoptosis. As apoptosis is a physiological phenomenon (5), low levels of anti-caspase-8 Ab may also be generated in healthy individuals during such apoptosis. Collectively, these results suggest that anti-caspase-8 Ab does not induce clinical manifestations of connective tissue disorders, but rather is secondarily generated during apoptosis following tissue damage. A previous study has shown that more epitopes of caspase-8 are detected in SSc or SLE patients compared to healthy individuals, suggesting the intramolecular epitope spreading (5). Therefore, increased apoptosis accompanied by tissue destruction may be related to higher levels of anti-caspase-8 Ab in systemic autoimmune disorders, such as SSc and SLE.

In this study, caspase-8 activity was inhibited by IgG isolated from sera of both SSc patients and normal controls, although the inhibitory effect was significantly greater in SSc patients than normal controls. Two important catalytic cysteine residues (Cys287 and Cys360) are identified in a p18 subunit of the caspase-8 PD (25, 26). In the present study, immunoblotting analysis showed that autoantibody to p18 but not p12 was detected in sera from SSc patients and normal controls, which was also observed by a previous study (11). In addition, the same study has demonstrated that these 2 cysteine residues are included in the epitopes of p18 (11), suggesting that anti-p18 Ab may interfere with activity of the 2 cysteine residues. Furthermore, both Cys287 and Cys360 are recognized by Abs from SSc patients, while only Cys287 is recognized by Abs from healthy individuals (11). This finding may explain why SSc patients exhibited more strong inhibitory effect for caspase-8 activity. However, it should be noted that it remained unknown in this study whether anti-caspase-8 Ab could indeed inhibit the activity of an intracellular enzyme caspase-8 *in vivo*. Further study will be needed to clarify *in vivo* functional relevance of anti-caspase-8 Ab.

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