

**Autoantibody against Caspase-3, an Executioner of Apoptosis,
in Patients with Systemic Sclerosis**

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ABSTRACT

Objective: To determine the presence or levels of antibodies (Abs) against caspase-3 and their clinical relevance in systemic sclerosis (SSc).

Methods: Anti-caspase-3 Ab was examined by enzyme-linked immunosorbent assay and immunoblotting.

Results: IgG anti-caspase-3 Ab levels in SSc patients were higher than in normal controls. SSc patients positive for IgG anti-caspase-3 Ab had significantly longer disease duration, more frequent presence of decreased %VC and %DLco, and elevated levels of serum immunoglobulin and erythrocyte sedimentation rates. IgG anti-caspase-3 Ab levels correlated positively with serum IgG levels, renal vascular resistance, and serum levels of 8-isoprostane. Immunoblotting analysis confirmed the presence of anti-caspase-3 Ab in sera from SSc patients. Caspase-3 enzymatic activity was inhibited by IgG isolated from SSc sera containing IgG anti-caspase-3 Ab.

Conclusion: These results suggest that autoantibody against caspase-3 is generated in SSc and that this Ab is related to the severity of pulmonary fibrosis, vascular damage, and inflammation.

Key Indexing Terms: systemic sclerosis, autoantibody, caspase-3, renal vascular damage, pulmonary fibrosis.

A Short Running Title: Anti-caspase-3 autoantibody in SSc

INTRODUCTION

Systemic sclerosis (SSc) is a multi-system disorder of connective tissue characterized by excessive fibrosis in the skin and various internal organs, such as 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 [1-4]. SSc patients have autoantibodies that react to various intracellular components, such as DNA topoisomerase I, centromere, RNA polymerases, U1RNP, U3RNP, Th/To, and histones [1-4]. Although it remains controversial whether these SSc-specific autoantibodies directly contribute to the clinical manifestations of SSc, autoantibodies against several intracellular and extracellular enzymes, including one of the antioxidant enzymes peroxiredoxin and matrix metalloproteinases, may play a pathogenetic role [5-7].

Ischemic/reperfusion injury following Raynaud's phenomenon, the most frequent first clinical manifestation in SSc, can generate reactive oxygen species that may result in vascular endothelial damage by inducing apoptosis [8], suggesting that endothelial cell apoptosis is a one of the preceding events in the development of SSc [9]. Caspases play an essential role in apoptosis that is critical for embryonic development and the pathogenesis of various diseases [10]. 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 [10]. Caspases are classified into initiators (caspases-2, -8, 9, and -10) and executioners (caspases-3, -6, and -7) of apoptosis [11]. The molecule of caspase-3 consists of two identical heterodimers composed of large (17 kDa) and small (11 kDa) subunits derived from a 32 kDa proenzyme by cleavage at multiple aspartic acid sites [12-14]. Caspase-3 is considered to be a key executor of apoptosis and is activated via the mitochondrial death receptor, such as Fas/Fas ligand and tumor necrosis factor receptor, or endoplasmic reticulum

routes [15]. Moreover, caspase-3 is the dominant and primary executioner caspase relative to the other executioner caspases, caspases-6 and -7, in proteolysis of caspase substrates as well as nuclear condensation and DNA fragmentation [16]. Caspase-3 has been shown to have additional functions including T cell activation and homeostasis [17]. Furthermore, recent studies suggest that caspase-3 activation via a Fas/Fas ligand-mediated pathway plays important roles in pathologic injury through abnormal immune functions in systemic lupus erythematosus and SSc [15, 18, 19]. In addition, autoantibodies against caspase-8, which is a initiator caspase and is also activated via a Fas/Fas ligand pathway, are detected in patients with SSc, systemic lupus erythematosus, or silicosis [20].

Therefore, we hypothesized that autoantibody against the major executioner caspase, caspase-3, could be detected in patients with SSc and that anti-caspase-3 Ab might contribute to dysregulation of apoptosis by inhibiting caspase-3 activation. To investigate this possibility, the presence or levels of autoantibodies against caspase-3, their clinical relevance, and their functional significance were investigated in SSc patients.

MATERIALS AND METHODS

Serum samples

Blood samples were obtained from 60 Japanese SSc patients (51 women and 9 men). All patients fulfilled the criteria proposed by the American College of Rheumatology [21]. These patients were grouped according to the classification system proposed by LeRoy et al. [22]: 23 patients (21 women and 2 men) had limited cutaneous SSc (lSSc) and 37 patients (30 women and 7 men) had diffuse cutaneous SSc (dSSc). The age of patients (mean \pm SD) was 45 ± 17 years. Patients with dSSc were aged 46 ± 18 , while those with lSSc were 44 ± 17 years old. The disease duration of patients with lSSc and dSSc was 10.1 ± 10.0 and 3.1 ± 3.0 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 enzyme-linked immunosorbent assay (ELISA) and immunoprecipitation. Anticentromere Ab was positive for 21 patients (4 dSSc and 17 lSSc), anti-topoisomerase I Ab for 28 (24 dSSc and 4 lSSc), anti-U1RNP Ab for 2 (1 dSSc and 1 lSSc), anti-U3RNP Ab for 1 (dSSc), anti-RNA polymerases I and III Ab for 5 (all dSSc), and Th/To Ab for 1 (lSSc). The remaining 2 patients were negative for autoantibodies. Thirty-four 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) [23].

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 [24]: pulmonary fibrosis = bibasilar fibrosis on chest radiography and high-resolution computed tomography; isolated pulmonary hypertension (PH) = clinical evidence of PH and increased systolic pulmonary arterial pressure (>35 mmHg) by Doppler 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 = 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. Renal vascular resistance was determined as pulsatility index (PI) by color-flow Doppler ultrasonography of the renal interlobar arteries of both kidneys [25]. The PI was calculated as an average value obtained with 8 waveforms on the renal interlobar arteries of both kidneys. 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.

ELISA for anti-caspase-3 Ab

ELISA was performed as described elsewhere [26]. Briefly, 96-well plates were coated with recombinant human caspase-3 (5 µg/ml; R&D Systems, Minneapolis, MN, USA) at 4°C overnight. Recombinant human caspase-3 sequences corresponding to amino acids 29-175 and 182-277 of human caspase-3 were expressed in *E. coli* and were migrated as two

polypeptides of 18 kD (p18) and 10 kD (p10) on polyacrylamide gels. 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 µl) 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 µg/µl *p*-nitrophenyl phosphate (Sigma-Aldrich Co., St. Louis, MO, USA) in diethanolamine buffer (1M diethanolamine, 0.5M MgCl₂) was added and the optical density (OD) of the wells at 405 nm was subsequently determined.

ELISA for serum levels of 8-isoprostane

ELISA for serum 8-isoprostane levels was performed as previously described using specific ELISA kit (Cayman, Ann Arbor, MI, USA), according to the manufacturer's protocol [27]. Each sample was tested in duplicate.

Immunoblotting

Immunoblotting was performed using recombinant human caspase-3 (R&D Systems) as previously described [28]. Recombinant human caspase-3 (1µg/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.).

Caspase-3 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-3 activity was determined by a caspase-3 fluorometric assay kit (ALEXIS biochemicals, Lausen, Switzerland), according to the manufacturer's protocol. This assay is based on fluorometric detection of 7-amino-4-trifluoromethyl coumarin (AFC) after cleavage from the AFC-labeled Asp-Glu-Val-Asp (DEVD) that is the sequence recognized by caspase-3. Caspase-3 (0.5 ng in 90 μ l of reaction buffer) was incubated with 20 μ g of purified IgG (20 μ l) for 20 minutes at 20°C, followed by addition of 10 μ l of 50 mM AFC-labeled DEVD substrate. Fluorescence was immediately determined at excitation of 400 nm and emission of 505 nm. Ten SSc patients positive for IgG anti-caspase-3 Ab by ELISA, 10 SSc patients negative for IgG anti-caspase-3 Ab by ELISA, and 10 healthy individuals were assessed. Ten serum samples selected from SSc patients positive for IgG anti-caspase-3 Ab by ELISA included patients with high titer of IgG anti-caspase-3 Ab (1.797, 1.045, 1.040, and 1.026), those with intermediate titer (0.766, 0.762, and 0.746), and those with low titer (0.577, 0.577, and 0.571).

Statistical analysis

Statistical analysis was performed using the Mann-Whitney U-test for determining the level of significance of differences between sample means, Fisher's exact probability test for comparison of frequencies, and Bonferroni's test for multiple comparisons. A p-value < 0.05 was considered statistically significant.

RESULTS

Autoantibodies to caspase-3 by ELISA

IgG but not IgM anti-caspase-3 Ab levels in lSSc and dSSc patients were significantly higher than those in normal controls ($p < 0.0001$, respectively; Figure 1). IgG or IgM anti-caspase-3 Ab levels were similar in lSSc and dSSc patients. OD values greater than the mean + 2SD (0.571 for IgG anti-caspase-3 Ab) of normal controls were considered positive in this study. In total SSc patients, IgG anti-caspase-3 Ab was detected in 48% (29/60). IgG anti-caspase-3 Ab was positive in 48% (11/23) of lSSc patients and in 49% (18/37) of dSSc patients, while it was detected in only three healthy individuals (9%, 3/34). Thus, IgG but not IgM anti-caspase-3 Ab levels were elevated in SSc.

Clinical correlation of anti-caspase-3 Ab positivity

To investigate the clinical association of anti-caspase-3 Ab, physical and laboratory findings were compared between SSc patients positive for IgG anti-caspase-3 Ab and those negative (Table 1). SSc patients positive for IgG anti-caspase-3 Ab had significantly longer disease duration ($p < 0.05$) and more frequent presence of decreased %VC ($p < 0.05$) and %DLco ($p < 0.05$) than those negative. In addition, SSc patients positive for IgG anti-caspase-3 Ab had significantly elevated levels of serum IgG, IgA, IgM, and erythrocyte sedimentation rates compared with those negative ($p < 0.05$, respectively). Regarding correlation of IgG anti-caspase-3 Ab levels with clinical parameters, IgG anti-caspase-3 Ab levels also correlated inversely with %VC ($r = -0.31$, $p < 0.05$; Figure 2A). Furthermore, IgG anti-caspase-3 Ab levels correlated positively with renal vascular resistance, which was determined as the PI value in the renal interlobar arteries by color-flow Doppler scans ($r = 0.35$, $p < 0.05$; Figure 2B), serum IgG levels ($r = 0.38$, $p < 0.05$; Figure 2C), and serum levels of 8-isoprostane, a reliable marker of oxidative stress ($r = 0.37$, $p < 0.01$; Figure 2D). Thus, the

presence of IgG anti-caspase-3 Ab was associated with longer disease duration, severity of pulmonary fibrosis, renal vascular damage, hyper- γ -globulinemia, and oxidative stress in SSc.

Immunoblotting analysis

The presence of IgG anti-caspase-3 Ab was further evaluated by immunoblotting analysis using recombinant human caspase-3 that consists of two subunits, p18 and p10. IgG anti-caspase-3 Ab was detected in 90% (26/29) of patients who were positive for this autoantibody by ELISA (Table 2 and Figure 3). The reactivity with both p18 and p10 was observed in 88% (23/26) of patients who had anti-caspase-3 Ab by immunoblotting. On the other hand, anti-caspase-3 Ab was not detected by immunoblotting in any patients who were negative for this autoantibody by ELISA. Thus, the presence of anti-caspase-3 Ab in patients with SSc was confirmed by immunoblotting analysis.

Inhibition of caspase-3 activity by IgG isolated from serum samples of SSc patients

To determine the functional significance of anti-caspase-3 Ab, we assessed whether anti-caspase-3 Ab was able to inhibit caspase-3 activity. Caspase-3 activity assay is based on fluorometric detection of AFC after cleavage from the AFC-labeled DEVD that is the sequence recognized by caspase-3. IgG isolated from SSc patients positive for IgG anti-caspase-3 Ab by ELISA slightly but significantly inhibited the caspase-3 activity relative to the untreated caspase-3 activity ($p < 0.001$; Figure 4). However, IgG isolated from healthy individuals did not inhibit caspase-3 activity. IgG isolated from SSc patients suppressed the caspase-3 activity more strongly than that from healthy individuals ($p < 0.01$). Furthermore, SSc patients negative for IgG anti-caspase-3 Ab by ELISA did not inhibit caspase-3 activity. Thus, anti-caspase-3 Ab was able to inhibit caspase-3 activity in vitro, although its inhibitory

effect was modest.

DISCUSSION

This study is the first to reveal that IgG anti-caspase-3 Ab levels were significantly elevated in serum samples from SSc patients relative to normal controls by ELISA. The presence of anti-caspase-3 Ab in SSc patients was further confirmed by immunoblotting analysis. The presence of IgG anti-caspase-3 Ab was associated with longer disease duration, decreased %VC and %DLco, and increased levels of serum immunoglobulin and erythrocyte sedimentation rates. In addition, IgG anti-caspase-3 Ab levels correlated inversely with %VC and positively with renal vascular damage, serum IgG levels, and serum levels of 8-isoprostane, which is produced by random oxidation of tissue phospholipids and is a reliable and stable marker for oxidative stress [29]. These results suggest that autoimmune responses to caspase-3, the dominant executioner of apoptosis, are generated in SSc and that this autoantibody is related to the severity of pulmonary fibrosis, vascular damage, and inflammation.

Apoptosis is detected in endothelial cells of early stage in SSc [30]. It has been shown that cleavage of certain autoantigens during apoptosis reveals immunocryptic epitopes that could induce autoantibody immune response in systemic autoimmune diseases [31]. Many studies have shown that SSc is characterized by increased oxidative stress [32]. Indeed, ischemia and reperfusion injury following Raynaud's phenomenon can generate reactive oxygen species that may result in vascular endothelial apoptosis [33, 34]. Furthermore, it is well known that Raynaud's phenomenon is observed in SSc patients from an early stage. Since caspase-3 is one of important components of apoptosis, these results suggest that anti-caspase-3 Ab may be produced during apoptosis that is induced in part by oxidative stress. Consistent with this, in the present study, anti-caspase-3 Ab levels correlated positively with serum levels of 8-isoprostane in SSc. Furthermore, our finding that the presence of anti-caspase-3 Ab was associated with longer disease duration in SSc may also support this

hypothesis.

In this study, anti-caspase-3 Ab was associated with renal vascular damage, the severity of pulmonary fibrosis, and inflammation in SSc. Previous reports show that proapoptotic Fas/Fas-ligand signaling and caspase-3 activation play an important role in inflammatory lung diseases including pulmonary fibrosis [35-37]. Although SSc sera containing anti-caspase-3 Ab could inhibit caspase-3 activity in vitro, its inhibitory effect was modest. Therefore, it is unlikely that anti-caspase-3 Ab itself could directly induce clinical manifestations, especially vascular damage, pulmonary fibrosis, oxidative stress, and inflammation. A previous study has demonstrated that serum 8-isoprostane levels are elevated in SSc and correlate positively with renal vascular damage, suggesting that enhanced oxidative stress may contribute to renal vascular damage [27]. Therefore, the enhanced oxidative stress may be related to the production of anti-caspase-3 Ab and vascular damage through induction of apoptosis in SSc. In this regard, the production of anti-caspase-3 Ab may be secondary and even protective for excessive apoptosis if anti-caspase-3 Ab could inhibit caspase-3 activity in SSc. However, it should be noted that it remained unknown in this study whether or not anti-caspase-3 Ab could indeed inhibit the activity of an intracellular enzyme caspase-3 in vivo. The further study will be needed to clarify in vivo functional relevance of anti-caspase-3 Ab.

In normal immune circumstances, activity against self-antigens is prevented by several mechanisms including the Fas pathway of apoptosis, which is involved in the process of immune tolerance by deletion of autoreactive T and B cells [38]. A recent study has shown that T cells in SSc patients are less susceptible to undergoing apoptosis after anti-Fas Ab stimulation and exhibit decreased caspase-3 activity [19]. Furthermore, in the present study, anti-caspase-3 Ab was associated with hyper- γ -globulinemia, suggesting that anti-caspase-3 Ab may be related to hyperactivity of B cells in SSc. Therefore, the presence of

anti-caspase-3 Ab might be involved in immunological abnormalities and autoimmune induction by inhibiting apoptosis of autoreactive T and B cells. However, this possibility also remained unproven in this study. Nonetheless, the results of this study suggest that anti-caspase-3 Ab would be a clue for understanding the pathogenesis in SSc.

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FIGURE LEGENDS

Figure 1. IgG and IgM anti-caspase-3 Ab levels in serum samples from patients with ISSc, those with dSSc, and healthy controls (CTL). Anti-caspase-3 Ab levels were determined by ELISA using recombinant human caspase-3. The short bar indicates the mean value in each group, while the broken line indicates the mean + 2SD of CTL.

Figure 2. The correlation of IgG anti-caspase-3 Ab levels against %VC (A), the PI value (B), serum IgG levels (C), and serum 8-isoprostane levels (D) in SSc patients. Anti-caspase-3 Ab levels and serum 8-isoprostane levels were determined by ELISA. The PI is a parameter for renal vascular resistance determined by color-flow Doppler ultrasonography of the renal interlobar arteries of both kidneys.

Figure 3. Representative immunoblotting of IgG anti-caspase-3 Ab in serum samples from SSc patients and healthy individuals, using recombinant human caspase-3. Lane 1: colloidal gold staining. Lanes 2 and 3: serum samples from SSc patients positive for IgG anti-caspase-3 Ab with high titer by ELISA (OD=1.797 and 1.045, respectively). Lanes 4 and 5: serum samples from SSc patients positive for IgG anti-caspase-3 Ab with intermediate titer by ELISA (OD=0.766 and 0.762, respectively). Lanes 6 and 7: serum samples from SSc patients positive for IgG anti-caspase-3 Ab with low titer by ELISA (OD=0.609 and 0.583, respectively). Lanes 8 and 9: serum samples from healthy individuals.

Figure 4. Inhibition of caspase-3 activity by IgG isolated from SSc patients and healthy individuals (CTL). IgG was purified from serum samples of 10 SSc patients positive for IgG anti-caspase-3 Ab by ELISA, 10 SSc patients negative for IgG anti-caspase-3 Ab by ELISA, and 10 normal individuals. Caspase-3 activity assay is based on fluorometric detection of

AFC after cleavage from the AFC-labeled DEVD that is the sequence recognized by caspase-3. Caspase-3 activity is shown as percentage of untreated caspase-3 (Untreated) that was defined as 100%. Each histogram shows the mean + SD values obtained from subjects of each group.