Autoantibody against activating transcription factor-2 in patients with systemic sclerosis

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Abstract

Objective

To determine the prevalence and clinical correlation of autoantibody to activating transcription factor (ATF)-2, a transcription factor of ATF/CREB family, in patients with systemic sclerosis (SSc).

Methods

Anti-ATF-2 Ab was examined by ELISA and immunoblotting using human recombinant ATF-2. ATF-2 activity to bind target DNA was evaluated by ELISA using a plate coated with oligonucleotide containing the consensus binding site for ATF-2.

Results

IgG anti-ATF-2 Ab levels in SSc patients (n=69) were significantly higher than those in normal controls (n=26). SSc patients positive for IgG anti-ATF-2 Ab had significantly longer disease duration, more frequent presence of decreased %VC and %DLco, and elevated levels of serum IgG, serum IgA, and erythrocyte sedimentation rates than those negative. More-over, IgG anti-ATF-2 Ab levels correlated inversely with %VC or %DLco. The presence of anti-ATF-2 Ab in SSc patients was confirmed by immunoblotting analysis. IgG isolated from serum samples of SSc patients positive for IgG anti-ATF-2 Ab by ELISA slightly but significantly inhibited ATF-2 activity compared with normal controls.

Conclusions

These results suggest that anti-ATF-2 Ab is a new autoantibody in SSc and that it serves as a novel serological marker for inflammation and lung involvement in SSc.

Key words

Systemic sclerosis, autoantibody, ATF-2, lung fibrosis, inflammation.

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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 the lungs, kidneys, esophagus, and heart. In particular, lung involvement is the most important complication in SSc (1-3). 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 (4). These autoantibodies react to various intracellular components, including DNA topoisomerase I, centromere, and RNA polymerases (4), and also to several intracellular and extracellular enzymes, such as antioxidant enzyme peroxiredoxin and matrix matelloproteinases (5-7). However, it remains controversial whether these SSc-specific autoantibodies directly contribute to the disease manifestations of SSc.

A transcription factor (TF) is one of major autoantigens in connective tissue disorders. Many studies have identified autoantibodies against various transcription factors, including nucleolar organizing region 90/human upstream binding factor, TFIIB, the RAP74 subunit of TFIIF, TFIIIA, transcriptional repressor ZF5, DNA binding protein B (dbpB), the p53 tumor suppressor gene, and Sp1 (8-14). Among these autoantibodies, Ab to nucleolar organizing region 90/human upstream binding factor, dbpB, and the p53 tumor suppressor gene are detected in SSc patients (8, 10, 13). Furthermore, anti-dbpB Ab is specific for SSc and is closely related to diffuse cutaneous SSc (dSSc) (8). Thus, autoimmune responses to TFs also occur in SSc patients and some of autoantibodies against TFs correlate with disease manifestations in SSc.

Activating transcription factor (ATF)-2 (also called CRE-BP1) belongs to the ATF/CREB family of TFs that contains a DNA-binding domain consisting of a cluster of basic amino acids and a leucine zipper region (15). DNA target sequence of ATF-2 is the widely distributed cAMP response element. ATF-2 is activated by the stress-activated protein kinases, such as the Jun amino-terminal kinase and p38 (15). The stress-activated protein kinases phosphorylate ATF-2 at sites close to the NH_2-terminal transcriptional activation domain and thereby stimulate their *trans*-activating capacity (15). A recent study has revealed that autoantibody against ATF-2 is detected in patients with lymphoma, especially Burkitt's lymphoma (16). However, it remained unknown whether anti-ATF-2 Ab was also detected in autoimmune diseases and whether it was related to some clinical manifestations. Therefore, we investigated the presence or levels of anti-ATF-2 Ab, its clinical correlation, and its functional significance in SSc patients.

Materials and methods

Serum samples

Serum samples were obtained from 69 Japanese patients with SSc (60 women and 9 men). All patients fulfilled the criteria proposed by the American College of Rheumatology (17). Patients were grouped according to the classification system proposed by LeRoy *et al.* (18): 29 patients (27 women and 2 men) had limited cutaneous SSc (lcSSc) and 40 patients (33 women and 7 men) had dcSSc. The age of patients (mean ± S.D.) was 45±16 years. Patients with dcSSc were aged 49±18, while those with lcSSc were 52±14 years old. The disease duration of lcSSc and dcSSc patients was 9±9 and 3±3 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. Anti-topoisomerase I Ab was positive for 30 patients (25 dcSSc and 5 lcSSc), anticentromere Ab for 24 (2 dcSSc and 22 lcSSc), anti-U1RNP Ab for 2 (1 dcSSc and 1 lcSSc), anti-U3RNP Ab for 1 (dcSSc), anti-RNA polymerases I and III Ab for 7 (all dcSSc), and Th/To Ab for 1 (lcSSc). The remaining 4 patients were negative for autoantibodies (all dcSSc). Patients with malignancy were excluded in this study. Twenty-six age- and sex-matched healthy Japanese individuals were used as normal controls. 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 tests, including vital capacity (VC) and diffusion capacity for carbon monoxide (DLco), were conducted for all patients within 3 to 5 weeks after serum collection. When the DLco and VC were <70% and <80%, respectively, of the predicted normal values, they were considered to be abnormal. Skin score was measured by scoring technique of the modified Rodnan total skin thickness score (modified Rodnan TSS) as previously described (19). The anatomical areas were rated as 0 (normal skin thickness), 1+ (mild but definite thickening), 2+ (moderate skin thickening), and 3+ (severe skin thickening) and the modified Rodnan TSS was derived by summation of the score from all 17 areas (range 0-51). Organ involvement was defined as described previously with some modifications (20): pulmonary fibrosis = bibasilar fibrosis on chest radiography and high resolution computed tomography; esophagus = hypomotility shown by barium radiography; joints = inflammatory polyarthralgias or arthritis; heart = pericarditis, congestive heart failure, arrhythmias requiring treatment; kidney = malignant hypertension and rapidly progressive renal failure with no other explanation; and muscle = proximal muscle weakness and elevated serum creatine kinase. The protocol was approved by Kanazawa University Graduate School of Medical Science and Kanazawa University Hospital and informed consent was obtained from all patients.

ELISA for anti-ATF-2 Ab

ELISA was performed as previously described (21). Briefly, 96-well plates were coated with human recombinant ATF-2 (1 μg/ml in 50 mM Hepes buffer, pH 7.0, and 5% trehalose; BioSource International, Inc., Camarillo, CA, USA) at 4ºC overnight. Wells were blocked with 2% bovine serum albumin and 1% gelatin in Tris-buffered saline (TBS) for 1 hour at 37ºC. After washing twice with TBS, serum samples diluted to 1:100 in TBS containing 1% bovine serum albumin were added to triplicate wells and incubated for 90 minutes at 20ºC. After washing 4 times with TBS containing 0.05% Tween-20, plates were incubated with alkaline phosphatase-conjugated goat anti-human IgG or IgM Abs (Cappel, Durham, NC, USA) for 1 hour at 20ºC. 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 (1 M diethanolamine, 0.5 M $MgCl_2$) was added and the optical density (OD) of the wells at 405 nm was subsequently determined. Absorbance values greater than the mean + 2 S.D. of normal controls were considered positive in this study.

Immunoblotting

Human recombinant ATF-2 (2 μg/lane; BioSource International) was subjected to electrophoresis and electrotransferred to nitrocellulose sheets. The nitrocellulose sheets were cut into strips and incubated overnight with serum samples diluted 1:100. Then, the strips were incubated for 1.5 hours with alkaline phosphatase-conjugated goat anti-human IgG Ab (Cappel). Color was developed using 5-bromo-4-chloro-3-indrolyl phosphate and nitro blue tetrazolium (Sigma-Aldrich). Eight SSc patients positive for IgG anti-ATF-2 Ab by ELISA, 4 SSc patients positive for anti-topoisomerase I Ab or anticentromere Ab but negative for IgG anti-ATF-2 Ab by ELISA, and 5 healthy individuals were evaluated.

ATF-2 activity 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, Pitscataway, NJ, USA). ATF-2 activity to bind target DNA was determined using a TransAM™ ATF-2 transcription factor assay kit (Active Motif North America, Carlsbad, CA, USA), according to the manufacturer's protocol. First, 1 μg of cell extract, which contained the active, phosphorylated form of ATF-2, was incubated with 30 μg of purified IgG for 30 minutes at 20˚C. Then, ATF-2 treated with IgG was added to each well of a 96-well plate, on which oligonucleotide containing the consensus binding site for ATF-2 was immobilized. After washing, wells were incubated with primary Abs that recognize an epitope on phosphorylated ATF-2 that is accessible only when ATF-2 is bound to the target DNA. Then, wells were incubated with peroxidase-conjugated anti-mouse IgG Ab, color was developed, and OD of the wells at 450 nm was subsequently determined. Five SSc patients positive for IgG anti-ATF-2 Ab by ELISA, 5 SSc patients positive for anti-topoisomerase I Ab or anticentromere Ab but not for IgG anti-ATF-2 Ab by ELISA, and 5 healthy individuals were assessed.

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. Spearman's rank correlation coefficient was used to examine the relationship between two continuous variables. A p-value<0.05 was considered statistically significant.

Results

Anti-ATF-2 Ab by ELISA

The presence and levels of anti-ATF-2 Ab in serum samples from SSc patients and normal controls were assessed by ELISA (Fig. 1). IgG anti-ATF-2 Ab levels in total SSc patients were significantly higher than those found in normal controls (p<0.0001). Regarding the disease subsets, IgG anti-ATF-2 Ab levels in lcSSc and dcSSc patients were significantly higher than those found in normal controls (p<0.0001 and p<0.001, respectively). In contrast, IgM anti-ATF-2 Ab levels in patients with dcSSc or lcSSc were not significantly elevated relative to controls. There was no significant difference in IgG or IgM anti-ATF-2 Ab levels among dcSSc and lcSSc patients.

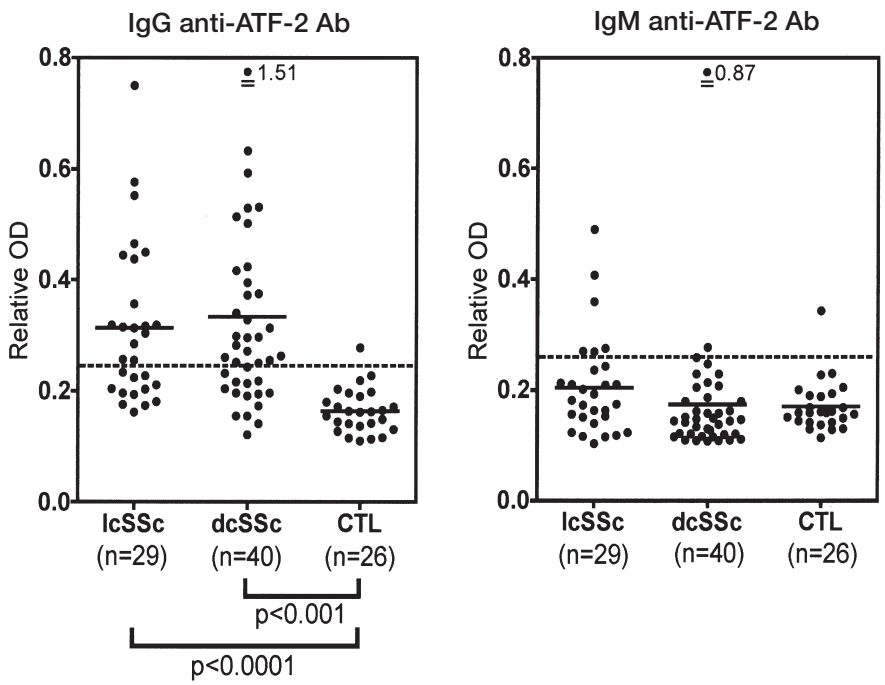

Fig. 1. IgG and IgM anti-ATF-2 Ab levels in serum samples from patients with lcSSc, dcSSc and healthy controls (CTL). Anti-ATF-2 Ab levels were determined by ELISA using human recombinant ATF-2. The short bar indicates the mean value in each group, while broken lines indicate the mean + 2 S.D. level of healthy controls.

OD values greater than the mean + 2 S.D. (0.244 for IgG anti-ATF-2 Ab and 0.262 for IgM anti-ATF-2 Ab) of normal controls were considered positive in this study (Fig. 1). In total patients with SSc, IgG or IgM anti-ATF-2 Ab was detected in 64% (Table I). IgG or IgM anti-ATF-2 Ab was detected in 59% of lcSSc patients and similar positivity was observed in dcSSc patients (68%). In general, IgG isotype of this autoantibody was dominant. In contrast, IgG or IgM anti-ATF-2 Ab was positive in only 2 healthy individuals (8%). Thus, IgG but not IgM anti-ATF-2 Ab levels were elevated in SSc.

Clinical correlation

Then, we assessed the clinical correlation of anti-ATF-2 Ab in the SSc patients. Those positive for IgG anti-ATF-2 Ab had significantly longer disease duration ($p<0.05$), more frequent presence of decreased %VC ($p<0.05$) and %DLco ($p<0.01$) than those negative (Table II). In addition, %VC and %DLco values significantly decreased in SSc patients with IgG anti-ATF-2 Ab relative to those without ($p<0.05$ and $p<0.01$, respectively). Consistently, IgG anti-ATF-2 Ab levels also correlated inversely with %VC (r=-0.256, $p<0.05$; Fig. 2A) or %DLco (r=-0.352, $p<0.01$; Fig. 2B). SSc patients positive for IgG anti-ATF-2 Ab had significantly elevated levels of serum IgG, serum IgA, and erythrocyte sedimentation rates (ESR) compared with those negative ($p<0.05$, respectively, Table II). However, IgG anti-ATF-2 Ab levels did not correlate with any other clinical parameters, including the modified Rodnan TSS. IgM anti-ATF-2 Ab positivity and levels did not correlate with any clinical parameters (data not shown). Thus, the presence of IgG anti-ATF-2 Ab was associated with longer disease duration, decreased %VC, decreased %DLco, increased Ig levels, and elevated ESR in SSc.

Table I. Frequency of anti-ATF-2 Ab positivity in SSc patients and normal controls.

	Anti-ATF-2 Ab		
	IgG	IgM	IgG or IgM
SSc (n=69)	42 (61)	8 (12)	44 (64)
lcSSc (n=29)	17 (59)	6 (21)	17 (59)
dcSSc (n=40)	25 (63)	2 (5)	27 (68)
Normal (n=26)	1 (4)	1 (4)	2 (8)

Values are the number (%) of patients with anti-ATF-2 Ab that was determined by ELISA using human recombinant ATF-2.
Isotypes (IgG or IgM) of anti-ATF-2 Ab were determined using isotype-specific anti-human immunoglobulin Abs.

Immunoblotting analysis for anti-ATF-2 Ab

The presence of anti-ATF-2 Ab was further evaluated by immunoblotting analysis using human recombinant ATF-2. Serum samples from SSc patients positive for IgG anti-ATF-2 Ab by ELISA exhibited reactivity with ATF-2 by immunoblotting (Fig. 3, lanes 2-5). By contrast, no reactivity with ATF-2 was observed using serum samples with either anti-topoisomerase I Ab or anticentromere Ab, but without IgG anti-ATF-2 Ab by ELISA (lane 6 and data not shown). Moreover, serum samples from healthy individuals did not react with ATF-2 (lane 7). Thus, the presence of anti-ATF-2 Ab in patients with SSc was confirmed by immunoblotting analysis.

Inhibition of ATF-2 activity by IgG isolated from serum samples of SSc patients which contained IgG anti-ATF-2 Ab

To determine the functional significance of anti-ATF-2 Ab, we investigated whether anti-ATF-2 Ab was able to inhibit ATF-2 binding to target DNA sequence. ATF-2 activity was determined with ELISA using a 96-well plate coated with oligonucleotide containing the consensus binding site for ATF-2. Bound ATF-2 was detected by primary Abs that recognize an epitope on phosphorylated ATF-2 that is accessible only when ATF-2 is bound to the target DNA. IgG isolated from serum samples of SSc patients positive for IgG anti-ATF-2 Ab by ELISA slightly but significantly inhibited ATF-2 activity by 21% compared with

Table II. Clinical and laboratory features of SSc patients with IgG anti-ATF-2 Ab.

	anti-ATF-2 Ab (+) n=42	anti-ATF-2 Ab (-) n=27
Sex, number of males/females	4/38	5/22
Age at onset, mean ± S.D. yrs	45 ± 16	46 ± 17
Disease duration, mean ± S.D. yrs	6.8 ± 7.9*	2.6 ± 3.4
Disease pattern, number with dcSSc/lcSSc	25/17	15/12
Clinical features		
Modified Rodnan TSS, mean ± S.D. points	14.5 ± 9.8	13.1 ± 10.4
Pitting scars	40	37
Diffuse pigmentation	52	56
Contracture of phalanges	45	44
Organ involvement		
Lungs		
Pulmonary fibrosis	45	37
%VC	87.7 ± 25.6*	104.5 ± 22.3
Decreased %VC	44*	17
%DLco	54.2 ± 15.9**	66.4 ± 18.5
Decreased %DLco	87**	48
Esophagus	54	52
Heart	19	11
Kidneys	5	0
Joints	19	22
Muscles	21	15
Laboratory findings		
Positive for anti-topoisomerase I Ab	45	41
Positive for anticentromere Ab	36	33
Serum IgG, mean ± S.D. mg/dl	1792 ± 587*	1478 ± 356
Serum IgA, mean ± S.D. mg/dl	345 ± 158*	270 ± 102
Serum IgM, mean ± S.D. mg/dl	209 ± 136	170 ± 82
ESR, mean ± S.D. mm/h	20.8 ± 15.9*	12.2 ± 8.4

Unless noted otherwise, values are percentages.
*p<0.05, **p<0.01 vs. SSc patients without IgG anti-ATF-2 Ab.

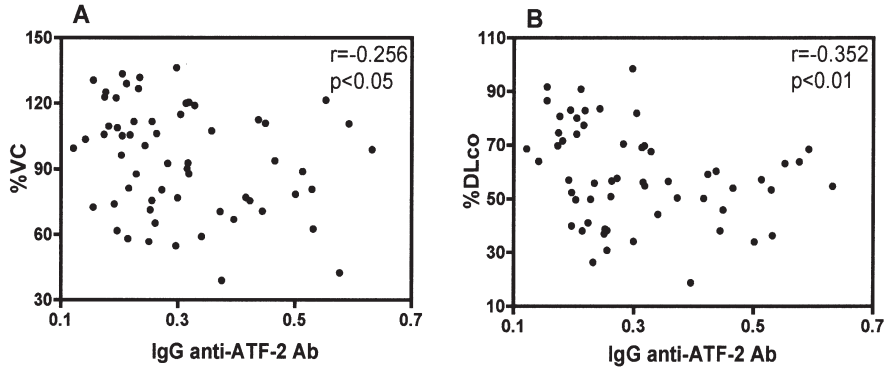

Fig. 2. The correlation of IgG anti-ATF-2 Ab levels against %VC (A) and %DLco (B) in SSc patients. Anti-ATF-2 Ab levels were determined by ELISA using human recombinant ATF-2.

normal controls (p<0.005; Fig. 4). ATF-2 activity was not inhibited by IgG isolated from serum samples that contained autoantibodies against topoisomerase I or centromere, but not IgG anti-ATF-2 Ab by ELISA. Thus, IgG anti-ATF-2 Ab from SSc patients was able to inhibit ATF-2 activity, although its inhibition was modest.

Discussion

The present study is the first to reveal that autoantibody against ATF-2 was detected in SSc patients by ELISA. Moreover, the presence of anti-ATF-2 Ab was confirmed by immunoblotting analysis. Thus, autoimmune response to ATF-2 appeared to be common in SSc. Furthermore, a recent study has demonstrated that anti-ATF-2 Ab is detected in 31% (22/71) of patients with lymphoma, including Burkitt's lymphoma, while it is positive in only 2% (1/50) of healthy individuals (16). Therefore, these findings suggest that autoimmune response to ATF-2 occurs in SSc as well as lymphoma.

In Burkitt's lymphoma, most malignant cells express high levels of active, phosphorylated ATF-2, whereas total ATF-2 protein level is similar between malignant cells and normal germinal center B cells (16). Therefore, it is speculated that this high level of ATF-2 phosphorylation itself may create a novel epitope that may enhance immunogenicity of the protein, leading to the generation of anti-ATF-2 autoantibody (16). Indeed, ATF-2 phosphorylation may occur in response to various stimuli related to SSc disease process. Transforming growth factor (TGF)-β is a major fibrogenic growth factor, since it not only stimulates matrix synthesis, but also controls virtually all fibroblast function relevant to fibrosis including proliferation, chemotaxis, and differentiation (22). Many studies have suggested an important role of TGF-β in the development of fibrosis in SSc (22). TGF-β signaling induces phosphorylation of ATF-2 via Smad and TGF-β activated kinase-1 (23). Oxidative stress, which is enhanced and may be related to fibrosis and vascular damage in SSc (24), also induces phosphorylation of ATF-2 in lung fibroblasts by the Jun amino-terminal kinase and p38 (25). Furthermore, bleomycin, a chemical inducer of fibrosis, causes phosphorylation of ATF-2 through P38 activation and decrease in ATF-2 phosphorylation is associated with amelioration of bleomycin-induced lung fibrosis (26). Collectively, anti-ATF-2 Ab may be secondarily produced by ATF-2 activation and phosphorylation that occurs during disease process, such as fibrosis and oxidative stress in SSc. Consistent with this notion, SSc patients with anti-ATF-2 Ab showed longer disease dura-

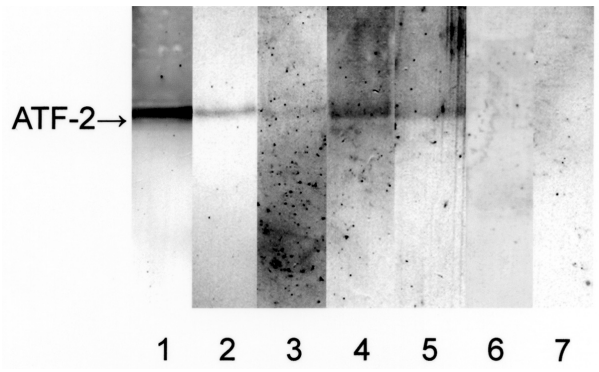

Fig. 3. Immunoblotting analysis of IgG anti-ATF-2 Ab in serum samples from SSc patients. Lane 1: colloidal gold-stained ATF-2. Lanes 2–5: serum samples from SSc patients positive for IgG anti-ATF-2 Ab by ELISA. Lane 6: a serum sample from a SSc patient positive for anti-topoisomerase I Ab, but not for IgG anti-ATF-2 Ab by ELISA. Lane 7: a serum sample from a healthy individual.

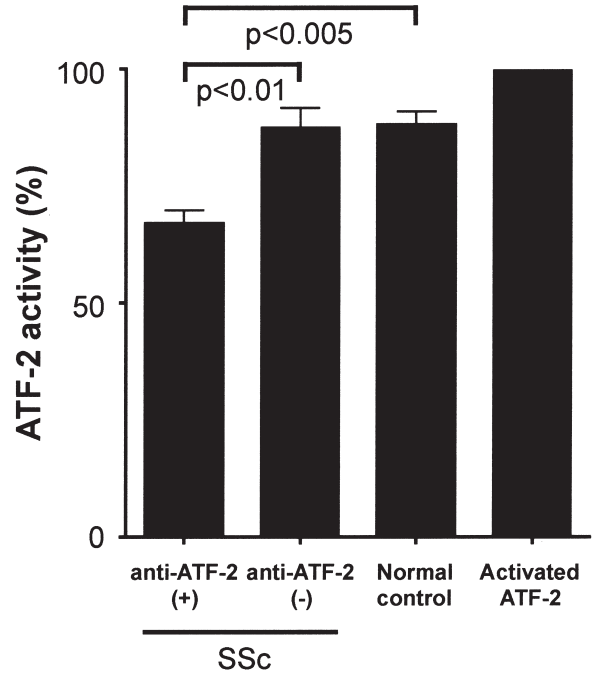

Fig. 4. Inhibition of ATF-2 activity by IgG isolated from serum samples that contained anti-ATF-2 Ab. IgG was purified from serum samples of SSc patients positive for IgG anti-ATF-2 Ab by ELISA [anti-ATF-2 (+)], those positive for either anti-topoisomerase I Ab or anticentromere Ab but not for IgG anti-ATF-2 Ab [anti-ATF-2 (-)], and normal control. ATF-2 activity is shown as percentage of activated ATF-2 that was defined as 100%. Each histogram shows the mean (+ S.D.) values obtained from 5 subjects of each group.

tion relative to those without. However, ATF-2 activity assay showed that autoantibody only weakly inhibited binding of phosphorylated ATF-2 to target DNA, suggesting that epitopes created by phosphorylation may not be a major factor for inducing autoantibody production. Further studies will be required for mechanisms of anti-ATF-2 Ab production in SSc.

In this study, SSc patients positive for anti-ATF-2 Ab exhibited elevated levels of serum IgG, serum IgA, and ESR compared to those negative, suggesting that anti-ATF-2 may be related to inflammation. A previous study using mice expressing small amount of a mutant ATF-2 protein has shown that ATF-2 is essential for immediate induction of inflammation, while in prolonged inflammation, ATF-2 may protect against overactive immune response (27). The finding that anti-ATF-2 Ab inhibited ATF-2 binding activity suggests that anti-ATF-2 Ab might enhance chronic inflammation by inhibiting ATF-2 function. Furthermore, the current study showed that anti-ATF-2 Ab was associated with the lung involvement in SSc. The promoter activity of interferon-γ, a potent anti-fibrotic cytokine (28), is enhanced by ATF-2 (29), suggesting that decreased expression of interferon-γ by anti-ATF-2 Ab might be related to the development of lung fibrosis. However, it should be noted that it remained unknown in this study whether anti-ATF-2 Ab could indeed inhibit the activity of an intranuclear molecule ATF-2 *in vivo*. Alternatively, it is possible that the correlation of anti-ATF-2 Ab with inflammation and lung involvement may be due to its association with longer disease duration. Nonetheless, the results of this study indicate that anti-ATF-2 Ab is a new serologic marker for inflammation and lung involvement in SSc.

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