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ORIGINAL PAPER

Autoantibody against one of the antioxidant repair enzymes, methionine sulfoxide reductase A, in systemic sclerosis: association with pulmonary fibrosis and vascular damage

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Abstract Systemic sclerosis (SSc) is a connective tissue disease characterized by fibrosis and vascular changes in the skin and internal organs with autoimmune background. It has been suggested that oxidative stress plays an important role in the development of SSc. To determine the prevalence and clinical correlation of autoantibody to methionine sulfoxide reductase A (MSRA), one of the antioxidant repair enzymes, in SSc, serum anti-MSRA autoantibody levels were examined in patients with SSc by enzyme-linked immunosorbent assay using recombinant MSRA. The presence of anti-MSRA antibody was evaluated by immunoblotting. To determine the functional relevance of anti-MSRA antibody in vivo, we assessed whether anti-MSRA antibody was able to inhibit MSRA enzymatic activity. Serum anti-MSRA antibody levels in SSc patients were significantly higher compared to controls and this autoantibody was detected in 33% of SSc patients. Serum anti-MSRA levels were significantly elevated in SSc patients with pulmonary fibrosis, cardiac involvement, or decreased total antioxidant power compared with those without them. Anti-MSRA antibodies also correlated positively with renal vascular damage determined as pulsatility index by color-flow Doppler ultrasonography of the renal interlobar arteries and negatively with pulmonary function tests. Furthermore, anti-MSRA antibody levels correlated positively with serum levels of 8-isoprostane and heat shock protein 70 that are markers of oxidative and cellular stresses. Remarkably, MSRA activity was inhibited by IgG isolated from SSc sera containing IgG anti-MSRA antibody. These results suggest that elevated anti-MSRA autoantibody is associated with the disease severity of SSc and may enhance the oxidative stress by inhibiting MSRA enzymatic activity.

Keywords Systemic sclerosis · Oxidative stress · Methionine sulfoxide reductase A · Pulmonary fibrosis · Vascular damage

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Introduction

Although the pathogenesis of systemic sclerosis (SSc) remains unknown, oxidative stress has been suggested to contribute to clinical manifestations associated with SSc, such as vascular damage, fibrosis, and production of autoantibodies [6, 9, 22, 31]. Indeed, ischemia/reperfusion injury following Raynaud's phenomenon can generate reactive oxygen species (ROS) that may result in vascular endothelial damages [4, 39]. ROS are also released from skin fibroblasts of SSc patients in vitro [31] and have been shown to stimulate fibroblast proliferation, which may cause fibrosis [23]. Furthermore, 8-isoprostane, which is a reliable oxidative stress marker, increases in urine, bronchoalveolar lavage, and serum samples in patients with SSc [17, 27, 37]. In addition, 8-isoprostane levels correlate with the disease severity of SSc [27]. Increased levels of serum heat shock protein (Hsp) 70, which is a biomarker of cellular stress, are observed in SSc patients and correlate with disease severity, especially fibrosis and vascular damage [26].

To counteract oxidative damages to lipid, proteins, and DNA, organisms are equipped with antioxidant defense mechanism [42, 43]. The peptide methionine sulfoxide reductases (MSRs) belong to a group of repair enzymes that has received considerable attention recently [25]. These enzymes reduce oxidized methionines (methionine sulfoxide; MetO) in an epimer-specific manner, with methionine sulfoxide reductase A (MSRA) reducing methionine-*S*-sulfoxide and MSRB reducing methionine-*R*-sulfoxide [46]. MSRs have been described in a large variety of living organisms. MSRA was first identified in *Escherichia coli*, and later found to be expressed in a number of human tissues with varying expression levels [15]. On a cellular level, MSRA is present in the cytosol and in the mitochondrial matrix [8, 45]. Recently, MSRA activity is shown in serum samples [18]. MSRA reverses the inactivation of many proteins due to oxidation of critical methionine residues by reducing MetO to methionine. Unlike other antioxidant enzymes, its action is independent of metals or cofactors, but requires thioredoxin reducing system [10]. The oxidation of methionine to MetO has been shown to alter cellular signaling and is implicated in a variety of degenerative diseases, such as emphysema, cataractogenesis, Alzheimer's disease, and rheumatoid arthritis [35, 44]. In addition, it is shown that MSRA plays an important role for protecting against ischemia–reperfusion stress in cardiac myocytes [30].

Although the autoantibody production is one of the central features in SSc [28], it remains controversial whether SSc-specific antibodies (Abs) directly contribute to the clinical manifestations of SSc [13, 33]. It may be possible that several autoantibodies play a pathogenic role in SSc. Therefore, we hypothesized that anti-MSRA Ab could also be detected in patients with SSc and that anti-MSRA Ab might contribute to enhance oxidative stresses by inhibiting MSRA enzymatic activity, following tissue damage in SSc. To investigate this possibility, the presence or levels of anti-MSRA Ab, its clinical correlation, and its functional significance were investigated in the current study.

Patients and methods

Serum samples

Serum samples were obtained from 69 Japanese patients with SSc (61 female and 8 male). All patients fulfilled the criteria proposed by American College of Rheumatology [1]. Patients were grouped according to the classification system proposed by LeRoy et al. [16]: 30 patients (28 female and 2 male) had limited cutaneous SSc (lSSc) and 39 patients (33 female and 6 male) had diffuse cutaneous SSc (dSSc). The age of patients (mean ± SD) was 49.0 ± 17.1 years. Patients with dSSc were aged 48.5 ± 18.0, while those with lSSc were 52.6 ± 13.7 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 the SSc patients was treated with oral corticosteroids, D-penicillamine, or other immunosuppressive therapy at the evaluation. Antinuclear Ab was determined by indirect immunofluorescence using HEp-2 cells as the substrate, and specifies of autoantibody were further assessed by ELISA and immunoprecipitation. Anticentromere Ab was positive for 23 patients, anti-topoisomerase I Ab for 30, anti-U1RNP Ab for 3, anti-U3RNP for 1, anti-RNA polymerases I and III for 7, and Th/To for 1. The remaining four patients were negative for autoantibodies. Twenty-eight patients with systemic lupus erythematosus (SLE), who fulfilled the American College of Rheumatology criteria [41], were also examined as disease control in this study. In addition, 27 patients with dermatomyositis (DM) that fulfilled Bohan and Peter criteria were included [2, 3]. Twenty-three age- and sex- matched healthy Japanese individuals (21 females and 2 males, age 47.2 ± 18.2 years) were used as normal controls. Smokers and patients with cardiovascular therapy were excluded in this study. 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 concluded for all patients. When the DLco and VC were <75 and <80%, respectively, of the predicted normal values, they were considered to be abnormal. Skin score was measured by scoring skin technique of the modified Rodnan total skin thickness score (modified Rodnan TSS) as previously described [5]: 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 modified Rodnan TSS was derived by summation of the score from all 17 areas (range 0–51). Organ involvement was defined as previously described with some modification [34]: pulmonary fibrosis = bibasilar fibrosis on chest radiography and high-resolution computed tomography; isolated pulmonary hypertension = clinical evidence of pulmonary hypertension 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; joints = inflammatory polyarthralgias or arthritis; heart = pericarditis, congestive heart failure, and arrhythmias requiring treatment; kidney = malignant

hypertension and rapidly progressive renal failure with no other explanation; muscle = proximal muscle weakness and elevated serum creatine kinase. Renal vascular resistance was determined as pulsatility index (PI) by color-flow Doppler ultrasonography of the renal interlobar arteries of both kidneys [24]. The PI, which represents vascular impedance, was calculated as $A - B$/mean, where A is the peak systolic frequency, B the end diastolic frequency and the mean is the time-averaged frequency. The PI was calculated as an average value obtained with eight waveforms on the renal interlobar arteries of both kidneys. 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-MSRA Ab

ELISA was performed as previously described [32]. Briefly, 96-well plates were coated with human recombinant MSRA (0.5 μg/μl, Jena Bioscience, Jena, Germany) at 4°C overnight. The wells were blocked with 2% bovine serum albumin and 1% gelatin in Tris-buffered saline (TBS) for 1 h 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 min at 20°C. After washing four times with TBS containing 0.05% Tween-20, the plates were incubated with alkaline phosphate-conjugate goat anti-human IgG (Capel, Durham, NC, USA) for 1 h at 20°C. After washing four times with TBS containing 0.05% Tween-20, substrates solution containing 0.91 μg/μl *p*-nitrophenyl phosphate (Sigma-Aldrich, St. Luis, 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 + 2SD of normal controls were considered positive in this study.

Relative levels of autoantibodies were determined for each group of patients and normal control using pooled serum samples as previously described [32]. Among each disease or control group, the same amounts of all the serum samples were pooled into a single tube. Then, the pooled sera were diluted at log intervals (1:10–$1:10^5$) and were subjected to ELISA assays to obtain OD versus dilution (log scale). The dilutions of pooled sera giving half-maximal OD values were determined by linear regression analysis, thus generating mean arbitrary unit per milliliter values for comparison between sets of sera. We could not generate arbitrary unit in each sample because of a large amount of the serum required for this purpose.

ELISA for 8-isoprostane and Hsp70

ELISA for serum 8-isoprostane levels was performed as previously described using specific ELISA kit (Cayman, Ann Abor, MI, USA), according to the manufacturer's protocol [27]. Serum Hsp70 levels were examined by a specific ELISA kit (Stressgen, Victoria, Canada) according to the manufacturer's protocol. Each sample was tested in duplicate.

Total antioxidant power assay

Total antioxidant power (Oxford Biomedical Research, Oxford, MI, USA) levels were examined by colorimetric microplate assay according to the manufacturer's protocol. This assay measures the total abilities of antioxidants provided by samples for reducing Cu^{2+} to Cu^{+}. Each sample was tested in duplicate.

MSRA 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, Piscataway, NJ, USA). MSRA activity was determined using yeast thioredoxin reductase system as described with some modifications [21]: first, a pre-reaction cocktail, which contained 50 mM Tris–HCl buffer (pH 7.5), 200 μM NADPH (Sigma-Aldrich), 100 μM methionine sulfoxide (Sigma-Aldrich), 5 μM yeast thioredoxin (LabFrontier, Seoul, Korea), and 0.8 μM yeast thioredoxin reductase (LabFrontier) in 77.5 μl total volume, was prepared. Next, 1 μM of MSRA (2.5 μl) was incubated with IgG (20 μl) for 30 min at 20°C. Then, MSRA treated with IgG (22.5 μl in total volume) and 77.5 μl of pre-reaction cocktail was added to each well. Five minutes later, MSRA activity is defined as NADPH oxidation per minute, as determined by the change in absorbance at 340 nm. To determine the difference of MSRA activity for each group, absorbance value was converted into percentage MSRA activity by calculating the ratio of its absorbance value to controls. Dose-related inhibition of MSRA activity was confirmed using diluted IgG purified from pooled sera of SSc patients. Five SSc patients positive for IgG anti-MSRA Ab and five healthy individuals were assessed in the current assay.

Statistical analysis

Statistical analysis was performed using Mann–Whitney U test for determining the levels of significance of differences

between sample means 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 of <0.05 was considered statistically significant.

Results

Anti-MSRA autoantibody by ELISA

The levels and presence of anti-MSRA Ab in serum samples from SSc patients and healthy controls were assessed by ELISA (Fig. 1). IgG anti-MSRA Ab levels in SSc patients were significantly higher than in healthy controls ($P < 0.05$). Patients with dSSc had significantly higher anti-MSRA Ab levels than healthy controls ($P < 0.05$). Similarly, lSSc patients exhibited elevated anti-MSRA Ab levels relative to normal controls ($P < 0.05$). Serum anti-MSRA Ab levels were similar between dSSc and lSSc patients.

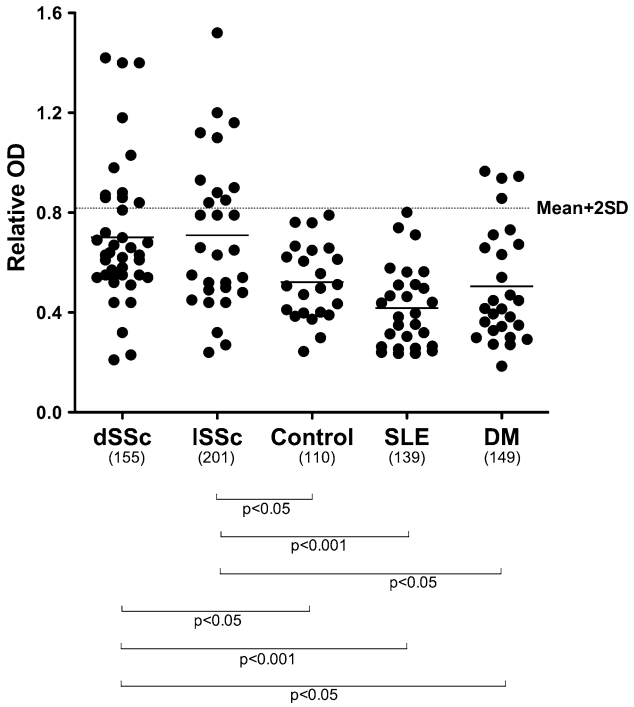

Fig. 1 IgG anti-MSRA Ab levels in serum samples from patients with dSSc, lSSc, SLE, DM, and healthy controls. Anti-MSRA Ab levels were determined by ELISA. The *horizontal line* indicates the median value in each group. The *broken line* indicates the cut-off value (mean + 2SD of healthy control samples). *Values* in *parentheses* represent the dilutions of pooled sera giving half-maximal optical density (*OD*) values in ELISA, which were determined by linear regression analysis to generate mean arbitrary units per milliliter that could be directly compared between each group

In addition, anti-MSRA Ab levels in dSSc patients significantly higher than in SLE and DM patients ($P < 0.001$ and $P < 0.05$, respectively). Similarly, lSSc patients exhibited elevated anti-MSRA Ab levels relative to SLE and DM patients ($P < 0.001$ and $P < 0.05$, respectively). Serum anti-MSRA Ab levels were similar between normal control and SLE or DM patients. Values higher than the mean + 2SD of the control serum samples were considered to be elevated in this study. In total patients with SSc, anti-MSRA Ab was detected in 32% (22/69). IgG anti-MSRA Ab was detected in 30% (12/40) of lSSc patients and similar positivity was observed in dSSc patients (34%, 10/29). In contrast, elevated IgG anti-MSRA Ab was not observed in SLE patients and healthy control samples. Furthermore, IgG anti-MSRA Ab was observed only in 14% (4/27) of DM patients. Thus, anti-MSRA Ab levels were elevated in SSc patients.

Clinical correlation

We assessed clinical correlation of anti-MSRA Ab in SSc patients. SSc patients positive for anti-MSRA Ab had significantly longer disease duration ($P < 0.05$) and decreased levels of %VC than those negative (Table 1). Regarding correlation of anti-MSRA Ab levels with clinical parameters, anti-MSRA Ab levels were significantly elevated in the SSc group with pulmonary fibrosis ($P < 0.05$), cardiac involvement ($P < 0.05$), or decreased levels of total antioxidant power ($P < 0.05$; Fig. 2) compared with those without each complication or parameter. Furthermore, serum anti-MSRA Ab levels loosely correlated with decreased %VC ($r = -0.38$, $P < 0.01$) and %DLco ($r = -0.31$, $P < 0.05$; Fig. 3). There was mild positive association between anti-MSRA Ab levels and renal vascular resistance ($r = 0.35$, $P < 0.05$), which was determined as the PI value in the renal interlobar arteries by color-flow Doppler scans. Serum anti-MSRA Ab levels correlated positively with serum levels of 8-isoprostane ($r = 0.52$, $P < 0.001$), which is one of the reliable markers that reflect free radical formation in vivo. In addition, anti-MSRA Ab levels have weak correlation with serum Hsp70 levels ($r = 0.31$, $P < 0.05$). However, anti-MSRA Ab levels did not correlate with any other clinical parameters, including modified Rodnan TSS.

SSc patients positive for anti-MSRA Ab had significantly higher frequency of elevated levels of serum IgG, IgA, and IgM ($P < 0.01$, $P < 0.05$, and $P < 0.01$, respectively; Table 1).

Thus, the presence of anti-MSRA Ab was associated with longer disease duration, pulmonary fibrosis, vascular damage, increased immunoglobulin, and increased oxidative and cellular stress.

Table 1 Clinical and laboratory data of patients with SSc showing elevated serum anti-MSRA Ab levels

	High anti-MSRA Ab ($n = 22$)	Low anti-MSRA Ab ($n = 47$)
Age at onset (years, mean ± SD)	43.9 ± 18.8	46.9 ± 15.4
Sex (F:M)	20:2	41:6
Duration (years, mean ± SD)	8.0 ± 10.1*	3.9 ± 4.0
Clinical features		
Pitting scars	9	30
Short sublingual frenulum	17	39
Contracture of phalanges	16	30
Diffuse pigmentation	17	38
Calcinosis	6	5
Modified Rodnan TSS	14.6 ± 8.6	13.7 ± 10.6
Organ involvement		
Lung		
Pulmonary fibrosis	19	25
Pulmonary hypertension	2	13
%VC	82.9 ± 26.3*	98.0 ± 23.5
%DLco	53.2 ± 18.4	61.0 ± 16.8
Esophagus	20	52
Heart	7	9
Kidney		
Increased vascular resistance	9	11
Renal crisis	1	1
Joint	3	17
Muscle	4	15
Laboratory findings		
Anti-topoisomerase I antibody	17	28
Anticentromere antibody	12	22
Anti-U1RNP antibody	1	4
Serum IgG (mean ± SD, mg/dl)	1,983 ± 510**	1,576 ± 544
Serum IgA (mean ± SD, mg/dl)	379 ± 161*	293 ± 123
Serum IgM (mean ± SD, mg/dl)	262 ± 168*	164 ± 68

Unless noted otherwise, values are percentages. Anti-MSRA Ab levels were determined by ELISA using human recombinant MSRA. OD values grater than the mean + 2SD of normal controls were considered "high levels" in this study

TSS total skin thickness score

* $P < 0.05$ versus SSc patients with low levels of anti-MSRA Ab

** $P < 0.01$ versus SSc patients with low levels of anti-MSRA Ab

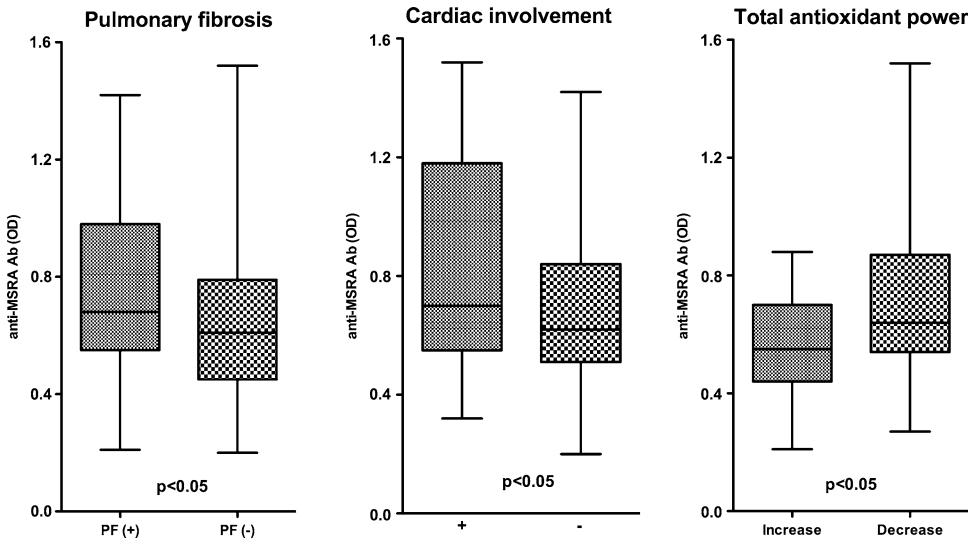

Fig. 2 Serum anti-MSRA Ab levels in SSc patients with pulmonary fibrosis, cardiac involvement, or elevated levels of total antioxidant power compared with those without each complication or parameter. The *horizontal line* indicates the median value

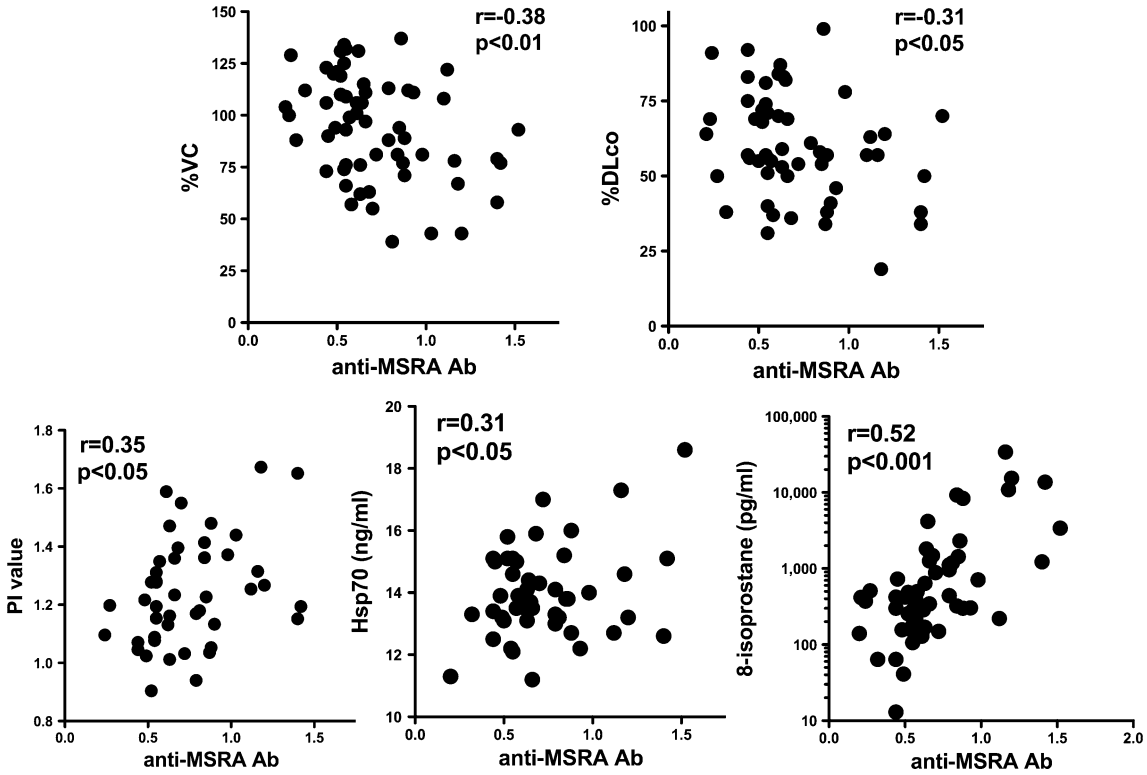

Fig. 3 The correlation of serum anti-MSRA Ab levels against %VC, %DLco, pulsatility index (PI), serum levels of Hsp70, and serum levels of 8-isoprostane in SSc patients. Serum Hsp70 levels and 8-isoprostane levels were determined by ELISA. The PI is a parameter for renal vascular resistance determined by color-flow Doppler ultrasonography of renal interlobar arteries of both kidneys

Inhibition of MSRA activity by IgG isolated from serum samples of SSc patients that contained IgG anti-MSRA Ab

To determine the functional relevance of anti-MSRA Ab in vivo, we assessed whether anti-MSRA Ab was able to inhibit MSRA activity. MSRA activity was determined using yeast thioredoxin reductase system. The activity is determined as MSRA-mediated NADPH oxidation per minute by the change in absorbance at 340 nm. The MSRA activity was not inhibited by IgG isolated from healthy individuals (Fig. 4). In contrast, IgG isolated from serum samples of SSc patients positive for IgG anti-MSRA Ab by ELISA significantly inhibited the MSRA enzymatic activity compared with healthy controls ($P < 0.05$). Thus, IgG isolated from serum samples of SSc patients, which contained anti-MSRA Ab, was able to inhibit the MSRA enzymatic activity.

Discussion

The present study is the first to reveal that serum anti-MSRA Ab levels were significantly elevated in SSc patients than in normal controls by ELISA. Interestingly, anti-MSRA Ab levels were significantly elevated in the SSc group with pulmonary fibrosis or cardiac involvement. Furthermore, anti-MSRA Ab levels significantly increased in the SSc group with decreased serum total antioxidant power levels, which reflect decreased antioxidant ability in SSc patients' sera. Serum anti-MSRA Ab levels were negatively correlated with %VC and %DLco and positively with renal vascular damage determined as PI value. In addition, anti-MSRA Ab levels in SSc significantly correlated with serum 8-isoprostane and Hsp70 levels. Anti-MSRA Ab from SSc patients inhibited MSRA enzymatic activity in vitro. These results suggest that anti-MSRA Ab in SSc patients plays a role in the development of SSc by enhancing oxidative stress.

In this study, serum anti-MSRA Ab levels significantly correlated with renal vascular damage, cardiac involvement, and %DLco in SSc. Vascular endothelial dysfunction is one of the central events in SSc, and cold- and stress-induced vasospasm (Raynaud's phenomenon) is the most characteristic sign that reflects this dysfunction. Ischemia and reperfusion injury following Raynaud's phenomenon may result in vascular endothelial damage [4, 39]. In this process, 8-isoprostane, which is one of the eicosanoid families, is produced by random oxidation of tissue phospholipids [20]. Serum 8-isoprostane levels correlate with renal vascular damage determined by color-flow Doppler

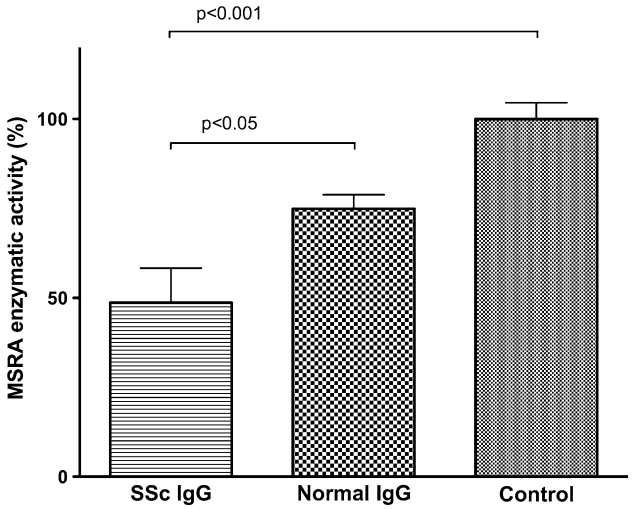

Fig. 4 Inhibition of MSRA enzymatic activity by IgG isolated from serum samples that contained anti-MSRA Ab. IgG was purified from serum samples of SSc patients positive for IgG anti-MSRA Ab (SSc IgG) by ELISA and healthy controls (normal IgG). MSRA enzymatic activity is shown as percentage of IgG-untreated MSRA that was defined as 100%. Each *histogram* shows the mean (+SEM) values obtained from five subjects of each group

ultrasonography of renal interlobar arteries in SSc [27]. Therefore, the finding that anti-MSRA Ab levels in SSc correlated with serum levels of 8-isoprostane in this study suggests that anti-MSRA Ab is related to vascular damage in SSc. In addition, it is showed that the enzyme MSRA inhibits apoptosis caused by ROS after hypoxia [47] and that MSRA protects cardiac myocytes against hypoxia/reoxygenation stress [30]. On the other hand, silencing MSRA enzyme leads to capase-3 activation that resulted in cell death [36]. Furthermore, it has been hypothesized that ischemia/reperfusion injury including apoptosis induces autoantigen fragmentation and cryptic epitope expression, leading to autoantibody production [29]. Autoantibody production is one of the central features in SSc, since more than 90% patients have antinuclear Abs [22]. These results suggest that serum anti-MSRA Ab contributes to the vascular damage and autoantibody production in SSc.

Oxidative stress induces several biomarker up-regulation in cells. Hsp is one of the reliable cellular stress markers and is highly conserved proteins found in all organisms cells [19]. The current study showed that serum anti-MSRA Ab levels positively correlated with serum Hsp70 levels. Hsp70 is known as a stress marker of lung injury [7, 12, 14]. It is shown that serum Hsp70 levels increase in SSc patients and correlate with pulmonary fibrosis [26]. Moreover, in this study, anti-MSRA Ab levels increased in the SSc group with pulmonary fibrosis and significantly correlated with %VC. Anti-MSRA Ab levels were also significantly higher in the SSc group with decreased serum levels of total antioxidant power, which is the marker of total antioxidant ability in samples. Collectively, anti-MSRA Ab may be involved in the regulation of oxidation–reduction (redox) balance. Therefore, the impairment of the balance may be associated with the disease severity, including lung fibrosis, in SSc.

In the current study, anti-MSRA Ab isolated from SSc patients sera inhibited MSRA enzymatic activity in vitro. It is known that thrombomodulin, calmodulin, and fibronectin are proteins whose biological activities are affected by oxidation of methionine residues [46]. Thrombomodulin is a vascular endothelial surface glycoprotein that is present on the luminal surface of endothelial cells. Soluble thrombomodulin reflects the injury to the endothelial cells, since the thrombomodulin fragmentation detected in blood is not secreted by endothelial cells under physiological condition [40]. Decreased levels of thrombomodulin are well-established risk factors for heart disease [38]. Interestingly, plasma levels of thrombomodulin are increased in SSc patients compared with normal controls [11]. In addition, oxidation of methionine 388 in thrombomodulin changes the thrombomodulin activity [38]. In this study, anti-MSRA Ab correlated with cardiac involvement. Since the MSRA enzyme reverses the MetO to methionine, the presence of anti-MSRA Ab may reduce the repairing of protein impairment due to oxidation and thereby may contribute to heart involvement by altering the coagulate state in SSc.

In conclusion, our study suggests that anti-MSRA Ab is related to enhanced oxidative stress and is a useful serological marker for the disease severity of SSc. In addition, anti-MSRA Ab in SSc may contribute to the redox regulation following vascular damage and related organ involvements by inhibiting MSRA enzymatic activity.

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