Autoantibody against peroxiredoxin I, an antioxidant enzyme, in patients with systemic sclerosis: possible association with oxidative stress

Y. Iwata¹²³, F. Ogawa¹, K. Komura¹, E. Muroi¹, T. Hara¹, K. Shimizu¹, M. Hasegawa², M. Fujimoto², Y. Tomita³ and S. Sato¹

Objectives. To determine the prevalence and clinical correlation of autoantibody to peroxiredoxin (Prx) I, an antioxidant enzyme, in patients with systemic sclerosis (SSc).

Methods. Serum samples from SSc patients (n = 70) and healthy controls (n = 23) were examined by ELISA using human recombinant Prx I. The presence of anti-Prx I antibody was further evaluated by immunoblotting analysis. To determine the functional relevance of anti-Prx I antibody in vivo, we assessed whether anti-Prx I antibody was able to inhibit Prx I enzymatic activity using yeast thioredoxin reductase system.

Results. IgG anti-Prx I antibody levels in SSc patients were significantly higher than healthy controls and this autoantibody was detected in 33% of SSc patients. The presence of IgG anti-Prx I antibody was associated with longer disease duration, more frequent presence of pulmonary fibrosis, heart involvement, and anti-topoisomerase I antibody and increased levels of serum immunoglobulin and erythrocyte sedimentation rates. IgG anti-Prx I antibody levels also correlated positively with renal vascular damage and negatively with pulmonary function tests. Furthermore, anti-Prx I antibody levels correlated positively with serum levels of 8-isoprostane, a marker of oxidative stress. Immunoblotting analysis confirmed the presence of anti-Prx I antibody. Remarkably, Prx I enzymatic activity was inhibited by IgG isolated from SSc sera containing IgG anti-Prx I antibody.

Conclusions. These results suggest that elevated IgG anti-Prx I autoantibody is associated with the disease severity of SSc and that anti-Prx I antibody may enhance the oxidative stress by inhibiting Prx I enzymatic activity.

Keywords: Systemic sclerosis, Oxidative stress, Peroxiredoxin, Pulmonary fibrosis, Autoantibody.

Introduction

Systemic sclerosis (SSc) is a connective tissue disease that is characterized by fibrosis and vascular changes in the skin and other internal organs with autoimmune background [1]. Although the pathogenesis of SSc remains unknown, it has been proposed that oxidative stress may play an important role in the development of SSc [2]. Ischaemia and reperfusion injury following Raynaud’s phenomenon can generate reactive oxygen species that may result in vascular endothelial damage [3, 4]. Reactive oxygen species are also released from skin fibroblasts of SSc patients in vitro [5] and have been shown to stimulate fibroblast proliferation, which may result in fibrosis [6, 7]. Furthermore, enhanced oxidative stress in SSc has been demonstrated, since 8-isoprostane, a reliable biomarker of oxidative stress, increases in urine [7, 8], bronchoalveolar lavage [9], and serum samples [10] in patients with SSc and serum 8-isoprostane levels correlate with the disease severity of SSc [10].

Exposure to reactive oxygen species from a variety of sources has led organisms to develop a series of defense mechanisms [11]. One of the important defense mechanisms against oxidative stress is defense by antioxidant enzymes, including peroxiredoxin (Prx), which is a recently discovered and characterized family of thiol-specific antioxidant enzyme [12–14]. Prxs have peroxidase activity (ROOH + 2e⁻ → ROH + H₂O) and thereby reduce hydrogen peroxide, peroxynitrate, and various organic hydroperoxides [15–17]. Prxs, which are present in a large variety of organisms, are produced at high levels in cells [15, 16, 18]. Six Prx isoforms (Prxs I–VI) have been identified [14] and Prx I is the most abundant and ubiquitously distributed member of the mammalian Prxs [16]. Recently, Karasawa et al. [19] have shown that 33% of patients with systemic autoimmune diseases, including systemic lupus erythematosus, primary vasculitis syndrome and rheumatoid arthritis, possess autoantibodies to Prx I. However, the prevalence and clinical correlation of anti-Prx I antibody (Ab) in patients with SSc and whether anti-Prx I Ab was able to inhibit the enzymatic activity of Prx I remained unclear in their study.

Autoantibody production is one of the central features in SSc, since more than 90% of patients have antinuclear Abs [20]. Although it remains controversial whether SSc-specific autoantibodies, such as anti-topoisomerase I, anticientromere and anti-RNA-polymerase Abs, directly contribute to the clinical manifestations of SSc, there may be possibilities that several autoantibodies play a pathogenetic role [1, 21]. Therefore, we hypothesized that anti-Prx I Ab could also be detected in patients with SSc and contribute to enhanced oxidative stress by inhibiting Prx I enzymatic activity, leading to tissue damage of SSc. To test this possibility, the presence or levels of anti-Prx I Ab, its clinical correlation and its functional significance were investigated in the current study.

Methods

Serum samples

Serum samples were obtained from 70 Japanese patients with SSc (61 women and 9 men). All patients fulfilled the criteria proposed by the American College of Rheumatology [22]. Patients were grouped according to the classification system proposed by...
LeRoy et al. [23]: 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±s.d.) was 50±16 yrs. Patients with dSSc were aged 49±18, while those with lSSc were 53±14-ys-old. The disease duration of patients with lSSc and dSSc was 8.3±9.3 and 3.0±2.9 yrs, respectively. None of SSc patients was treated with oral corticosteroid, with lSSc and dSSc was 8.3±9.3 years.

Frozen venous blood samples were centrifuged shortly after clot formation. All samples were stored at -80°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. 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 technique of the modified Rodnan total skin thickness score (modified Rodnan TSS) as previously described [24]: 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 [25]: 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; oesophagus = hypomotility shown by barium radiography; joints = inflammatory polyarthalgias or arthritis; heart = pericarditis, congestive heart failure, 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 by colour-flow Doppler ultrasonography of the renal interlobar arteries of both kidneys [26]. The pulsatility index, which represents vascular impedance, was calculated as A-B/mean, where A is the peak systolic frequency, B is the end diastolic frequency and the mean is the time-averaged frequency. The pulsatility index 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-Prx I Ab

ELISA was performed as previously described [27]. Briefly, 96-well plates were coated with human recombinant Prx I (0.3 μg/ml in 50 mM Hepes buffer, pH 7.0, and 5% trehalose; Sigma-Aldrich Co., St. Louis, MO, USA) at 4°C overnight. The wells were blocked with 2% bovine serum albumin (BSA) 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% BSA were added to triplicate wells and incubated for 90 minutes at 20°C. After washing four 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) in diethanolamine buffer (1M diethanolamine, 0.5 M MgCl2) 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.

Relative levels of autoantibodies were determined for each group of patients and normal controls using pooled serum samples as described previously [27]. 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 (1:10–1:105) and were subjected to ELISA assays to obtain OD vs 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 serum sample because of a large amount of the serum sample required for this purpose.

ELISA for 8-isoprostane

ELISA for serum 8-isoprostane levels was performed as previously described [10] using specific ELISA kits (Cayman, Ann Arbor, MI, USA), according to the manufacturer’s protocol. Each sample was tested in duplicate. The detection limit of this assay was 5 pg/ml.

Immunoblotting

Human recombinant Prx I (3 μg/lane; Sigma-Aldrich) 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). Colour was developed using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Sigma-Aldrich). Ten SSc patients positive for IgG anti-Prx I Ab by ELISA, 9 SSc patients positive for either anti-Prx I or anticientromere Ab, but not for IgG anti-Prx I Ab by ELISA, and 5 healthy individuals were evaluated.

Prx I 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). Prx I activity was determined using yeast thioredoxin reductase assay as described with some modifications [28]: first, a pre-reaction cocktail, which contained 50 mM Hepes-NaOH buffer (pH 7.0), 1 mM EDTA, 200 μM NADPH (Sigma-Aldrich), 1.5 μM yeast thioredoxin (LabFrontier, Seoul, Korea), and 0.8 μM yeast thioredoxin reductase (LabFrontier) in 180 μl total volume, was prepared. Next, 2 μg of Prx I (6.7 μl) was incubated with 60 μg of purified IgG (60 μl) for 30 minutes at 20°C. Then, Prx I treated with IgG (66.7 μl in total volume) and 20 μl of 100 μM H2O2 were added to each well in the ELISA plate, and immediately 180 μl of pre-reaction cocktail was added to each well. Five minutes later, absorbance values at 340 nm were determined. Similarly, absorbance values for 2 μg and 0 μg of Prx I untreated with IgG were determined in triplicate wells, respectively. The mean values for 2 μg and 0 μg of Prx I untreated with IgG were 0.572 and 0.643, respectively. Thus, absorbance reduction per 5 minutes at 340 nm
due to 2 μg of Prx I-mediated NADPH oxidation was calculated as 0.071. To clearly demonstrate the difference of Prx I activity for each group, absorbance value was converted into % Prx I activity by calculating the ratio of its absorbance value to controls. Ten SSc patients positive for IgG anti-Prx I Ab, 10 SSc patients positive for either anti-topoisomerase I Ab, anti-centromere Ab, or anti-U1RNP Ab but not for IgG anti-Prx I Ab, and 10 healthy individuals were assessed in the current assay.

Statistical analysis
Statistical analysis was performed using the Mann–Whitney U-test for determining the level of significance of differences between sample means and 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-Prx I autoantibody by ELISA
The levels and presence of anti-Prx I Ab in serum samples from SSc patients and normal controls were assessed by ELISA (Fig. 1). The dilution of pooled sera giving half-maximal optical density (OD) values in ELISAs, which were determined by linear regression analysis to generate mean arbitrary units per millilitre that could be directly compared between each group of SSc and healthy controls.

Clinical correlation
Then, we assessed clinical correlation of anti-Prx I Ab in SSc patients. SSc patients positive for IgG anti-Prx I Ab had significantly longer disease duration (P < 0.05), more frequent presence of pulmonary fibrosis (P < 0.05) and cardiac involvement (P < 0.01), and decreased %VC (P < 0.05) and %DLco (P < 0.01) than those negative (Table 1). Regarding correlation of IgG anti-Prx I Ab levels with clinical parameters, IgG anti-Prx I Ab levels also correlated inversely with %DLco (R = −0.37, P < 0.005; Fig. 2a) or %VC (R = −0.36, P < 0.005; Fig. 2b). Furthermore, anti-Prx I Ab levels correlated positively with disease duration (R = 0.284, P < 0.05), renal vascular resistance, which was determined as the pulsatility index value in the renal interlobular arteries by colour-flow Doppler scans (R = 0.33, P < 0.05; Fig. 2c) [26], and positively with serum levels of 8-isoprostane, a reliable marker of oxidative stress (R = 0.43, P < 0.001; Fig. 2d). However, IgG anti-Prx I Ab levels did not correlate with any other clinical parameters, including the modified Rodnan TSS. Frequency of IgM anti-Prx I Ab positivity did not correlate with the presence or absence of any organ involvement (data not shown). In addition, IgM anti-Prx I Ab levels did not correlate with any clinical parameters.

SSc patients positive for IgG anti-Prx I Ab had significantly higher frequency of elevated levels of serum IgG, IgA, and IgM and erythrocyte sedimentation rates (ESR) compared with those negative (P < 0.01, P < 0.05, P < 0.05 and P < 0.05, respectively; Table 1). Anti-topoisomerase I Ab was more frequently detected in SSc patients with anti-Prx I Ab than in those negative (P < 0.05). Regarding correlation of IgG anti-Prx I Ab levels with immunological parameters, IgG anti-Prx I Ab levels correlated positively with levels of serum IgG (R = 0.422, P < 0.001).
IgA ($R = 0.275, P < 0.05$) and IgM ($R = 0.322, P < 0.01$) and ESR ($R = 0.323, P < 0.01$). However, IgG anti-Prx I Ab levels did not correlate with any other immunological parameters. In addition, IgM anti-Prx I Ab levels did not correlate with any immunological parameters. Thus, the presence of IgG anti-Prx I Ab was associated with longer disease duration, the severity of pulmonary fibrosis, renal vascular damage, increased serum immunoglobulin, increased serum 8-isoprostane levels, elevated ESR and more frequent presence of anti-topoisomerase I Ab in SSc.

**Immunoblotting analysis for anti-Prx I Ab**

The presence of anti-Prx I Ab was evaluated by immunoblotting analysis using human recombinant Prx I. Serum samples from SSc patients positive for IgG anti-Prx I Ab by ELISA exhibited reactivity with Prx I (25 kDa) by immunoblotting (Fig. 3, lanes 2–5). By contrast, no reactivity with Prx I was observed using serum samples with either anti-topoisomerase I Ab, anticientromere Ab, or anti-U1RNP Ab, but without IgG anti-Prx I Ab by ELISA (lane 6 and data not shown). Furthermore, serum samples from healthy individuals did not react with Prx I (lane 7). Thus, the presence of anti-Prx I Ab in patients with SSc was confirmed by immunoblotting analysis.

**Inhibition of Prx I activity by IgG isolated from serum samples of SSc patients which contained IgG anti-Prx I Ab**

To determine the functional relevance of anti-Prx I Ab in vivo, we assessed whether anti-Prx I Ab was able to inhibit Prx I enzymatic activity. Prx I activity was determined using yeast thioredoxin reductase system. Absorbance reduction of Prx I-mediated NADPH oxidation was measured by ELISA. The Prx I 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-Prx I Ab by ELISA significantly inhibited the Prx I activity by 59% compared with healthy controls ($P < 0.01$). The Prx I activity was not inhibited by IgG isolated from serum samples that contained autoantibodies against topoisomerase I, centromere, or U1RNP, but not IgG anti-Prx I Ab. Thus, IgG isolated from serum samples of SSc patients, which contained anti-Prx I Abs, was able to inhibit the Prx I enzymatic activity.

**Discussion**

The present study is the first to reveal that IgG anti-Prx I Ab levels were significantly elevated in serum samples from SSc patients relative to normal controls by ELISA. The presence of anti-Prx I Ab was further confirmed by immunoblotting analysis. However, this autoantibody is not specific for SSc, since it is detected in patients with other autoimmune disorders [19]. Interestingly, IgG anti-Prx I Ab levels correlated with the severity of lung fibrosis, the extent of renal vascular damage, and the presence of heart involvement. Moreover, the presence of IgG anti-Prx I Ab was...
related to the immunological abnormalities, including hyperproduction of immunoglobulins and inflammation as shown by elevated ESR. Thus, the results of the present study indicate that elevated anti-Prx I Ab is generally associated with the disease severity in SSc.

There were no significant differences in anti-Prx I Ab levels between dSSc and iSSc patients. Furthermore, the presence and levels of anti-Prx I Ab did not correlate with skin involvements including modified Rodnan TSS, the presence of pitting scars, diffuse pigmentation, and contracture of phalanges. Similarly, when analysed in dSSc patients, there was no correlation between anti-Prx I Ab levels and modified Rodnan TSS (data not shown). Thus, although anti-Prx I Ab correlated with the severity of the pulmonary fibrosis, it did not correlate with the extent of skin sclerosis. The reasons for this discrepancy remained unknown.

Several longitudinal studies have shown that in dSSc patients, skin scores rapidly increase over the first several years, and then gradually decrease [29, 30]. The finding that anti-Prx I Ab correlated with longer disease duration in SSc suggests that anti-Prx I Ab is not likely to reflect ongoing skin fibrosis but reflects the accumulation of oxidative stress in some internal organs. Collectively, these results suggest that anti-Prx I Ab is not a suitable marker for predicting the activity or stage of SSc but useful in determining the accumulation of oxidative stress and the severity of visceral involvement.

Remarkably, in the current study, IgG isolated from serum samples of SSc patients, which contained anti-Prx I Abs, was able to inhibit Prx I enzymatic activity. In contrast, the Prx I activity was not inhibited by IgG isolated from serum samples of healthy individuals and those of SSc patients that had autoantibodies against topoisomerase I, centromere, or U1RNP, but not IgG anti-Prx I Ab. These results suggest that IgG anti-Prx I Ab inhibits Prx enzymatic activity. However, as IgG anti-Prx I Ab was not purified from total IgG, the current study did not formally prove that IgG anti-Prx I Ab itself inhibits Prx I activity. Nevertheless, this is the first study suggesting a possible functional relevance of anti-Prx I Ab in vivo. Moreover, anti-Prx I Ab levels closely correlated with serum levels of 8-isoprostane, a marker of oxidative stress (Fig. 2d). These results suggest the possibility that anti-Prx I Ab could enhance the oxidative stress by inhibiting Prx I enzymatic activity in SSc. However, it remains still controversial whether autoantibodies found in SSc have a pathogenic role or they are merely by-products of underlying disease process [31], because autoantigens in SSc are generally intracellular molecules, and there is little evidence that autoantibody can penetrate into viable cells. Prx I also exists in the cytosol and nucleus [16, 32], and is not secreted outside the cells under physiological condition. Therefore, it remains unknown whether the presence of anti-Prx I Ab is related to the development of SSc.

There are two possibilities that might explain the pathogenetic role of anti-Prx I Ab in SSc. One possibility is that Prx I could be secreted or leaked from cells in SSc. Oxidative stress and cellular release of Prx I are shown in patients with lung cancer and in cultured astrocyte cells [33, 34]. Prx I overexpression is also induced by cellular proliferation, differentiation, oxidative stress and pro-inflammatory cytokines [18]. Furthermore, it has been suggested that excessive Prx I leads to self-aggregation or aggregation with other intracellular proteins, resulting in cell death [18], because Prx I tends to form large insoluble aggregates [12, 35]. Taken together, it is possible that Prx I is also overexpressed/released or leaked from apoptotic cells in SSc patients. Another possibility is cross-reactivity of anti-Prx I Ab with membrane or secreted isoforms of Prxs II and IV [15]; the structure and sequence of the peroxidatic active site is highly conserved among Prxs I–IV [14, 15] and all Prxs I–IV can form both homodimers and heterodimers [18]. In fact, it has been found that Prx I and Prx IV form a heterodimer [36]. If released or leaked, Prx I could play an important role as an extracellular antioxidant enzyme like extracellular superoxide dismutase and glutathione peroxidases [37]. Alternatively, if anti-Prx I Ab cross-reacts with other extracellular Prxs, anti-Prx I Ab could contribute to enhance the oxidative stress by inhibiting enzymatic activity of extracellular Prxs. In the current study, we could not detect serum Prx I in SSc patients by immunoblotting analysis (data not shown) and did not assess whether Prx I was detected in sera from SSc patients and whether anti-Prx I Ab cross-reacted with Prxs I, II and IV. Therefore, further research will be required to address these issues.

In SSc, ischaemic-reperfusion injury due to Raynaud’s phenomenon increases the production of reactive oxygen species [38]. Because endothelial cells lack catalase, an antioxidant enzyme [39], and are susceptible to reactive oxygen species, which result in endothelial cell damage, including intimal proliferation and fibrous thickening of the media. Furthermore, the exposure of nuclear contents of damaged endothelial cells may provoke an autoimmune response to various nuclear antigens, leading to autoantibody formation and further recruitment of inflammatory cells [40]. That Prx I is also abundantly expressed in endothelial cells and overexpressed in proliferating cells, which suggests that long-term injury of endothelial cells by oxidative stress increases the chance of Prx I overexpression and fragmentation, leading to anti-Prx I autoantibody production. Consistent with this, the presence of anti-Prx I Ab correlated with longer disease duration and inflammation in SSc. On the other hand, there was strong correlation between anti-Prx I Ab levels and the severity of lung fibrosis. Several studies have shown Prx I overexpression in lung diseases, such as sarcoidosis and cancer [33, 41]. Interestingly, in patients with non-small cell lung cancer, both anti-Prx I Ab and Prx I are detected in their serum samples [33]. Collectively, anti-Prx I Ab may be secondarily produced by the exposure of the cryptic epitope produced by the long-term exposure of oxidative stress, which in turn could enhance oxidative stress in SSc.
In conclusion, our study suggests that IgG anti-Prx I autoantibody is related to enhanced oxidative stress and is a useful serological marker for the disease severity of SSc. In addition, this autoantibody may contribute to the development of SSc by inhibiting Prx I enzymatic activity.

**Rheumatology key messages**

- IgG anti-Prx I autoantibody is associated with the disease severity of SSc.
- Anti-Prx I antibody may enhance the oxidative stress by inhibiting Prx I enzymatic activity.

The authors have declared no conflicts of interest.

**References**