

Increasing levels of serum antioxidant status, Total Antioxidant Power, in systemic sclerosis

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Running title: Total antioxidant power levels in SSc

Key words: systemic sclerosis, oxidative stress, total antioxidant power, biomarker

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ABSTRACT

Purpose: Oxidative stress is one of the important factors that contribute to tissue damage in systemic sclerosis (SSc). Since the physiological response to oxidative stress is regulated by multiple antioxidant systems, it is important to measure quantitatively the total antioxidant capacity in the biological specimens. To determine the clinical significance of total antioxidant power (TAP) in SSc, we investigated the prevalence and clinical correlation of serum TAP levels in SSc patients.

Methods: Serum TAP levels were examined in 49 patients with SSc by colorimetric microplate assay. The assay measures the total abilities for reducing Cu^{++} into Cu^{+} . Clinical evaluation including medical history, physical examination, and laboratory tests were conducted for all SSc patients.

Results: Serum TAP levels were significantly elevated in SSc patients compared to normal controls ($p < 0.01$). When values higher than the mean + 2SD of the control serum samples were considered to be elevated, TAP levels were elevated in 24% of total SSc patients with 26% of diffuse cutaneous SSc patients and 23% of limited cutaneous SSc patients. Serum TAP levels were correlated positively with C-reacting protein ($r = 0.35$, $p < 0.05$). However, no other significant correlation was observed between serum TAP levels and clinical features in SSc patients.

Conclusion: These results suggested that oxidative stress is enhanced in SSc patients and serum TAP levels increase as an indicator of the global response to oxidative stress.

Introduction

Systemic sclerosis (SSc) is a connective tissue disorder characterized by fibrosis and vascular changes in the skin and other visceral organs, with autoimmune background. Although the pathogenesis of SSc remains unknown, oxidative stress has been suggested to contribute to clinical manifestations associated with SSc [1, 2]. Indeed, ischemia/reperfusion injury following Raynaud's phenomenon can generate reactive oxygen species (ROS) that may result in vascular endothelial damage [3, 4]. SSc patients exhibit notable evidence of oxidative stress, which is shown by abnormalities of nitric oxide and nitric oxide synthase and increased levels of other biomarkers, including 8-isoprostane, which indicates excess oxidative stress and heat shock protein 70 (Hsp70) [5-7].

To counteract oxidative stress to lipid, proteins, and DNA, organisms are equipped with antioxidant defense mechanism that composed of enzymatic and nonenzymatic antioxidants [8-10]. Antioxidant enzymes include the families of superoxide dismutase, catalase, glutathione peroxidase, glutathione *s*-transferase, and thioredoxin. The nonenzymatic antioxidants include glutathione, ascorbate, urate, α -tocopherol, bilirubin, and lipoic acid [11]. Because of the multiplicity of antioxidant pathways, their centrality in the prevention of oxidative stress, and the individual antioxidant capacity, it is important to evaluate the total antioxidant capacity or antioxidant power with biological specimens.

Therefore, in the present study, the levels of antioxidant power in the SSc patient sera and their clinical relevance were investigated by a specific colorimetric microplate assay kit.

Patients and Methods

Serum samples

Serum samples were obtained from all SSc patients who visited our scleroderma clinic over last 7 years. They were 49 Japanese patients with SSc (41 females, 8 males; age 50.3 ± 17.1 years) who fulfilled the criteria proposed by the American College of Rheumatology [12]. They were grouped according to the classification system proposed by LeRoy et al. [13]: 22 patients (20 females, 2 males; age 53.7 ± 12.8 years) had limited cutaneous SSc (lSSc) and 27 patients (21 females, 6 males; age 47.7 ± 19.6 years) had diffuse cutaneous SSc (dSSc). The disease duration of lSSc and dSSc patients was 10.3 ± 10.1 and 2.9 ± 3.1 years, respectively. None of SSc patients was treated with oral steroid, D-penicillamine, other immunosuppressive therapy, or antihypertensives at the evaluation. Antinuclear antibody (Ab) was determined by immunofluorescence and autoantibody specificities were further assessed by enzyme-linked immunosorbent assay and immunoprecipitation. Anticentromere Ab was positive for 17 patients, anti-topoisomerase I Ab for 22, anti-U1 RNP Ab for 2, anti-U3 RNP Ab for 1, anti-RNA polymerases I and III Ab for 5 and Th/To Ab for 1. The remaining patient was negative for autoantibody. Healthy 23 Japanese people with similar age and gender to the patients were used as normal controls. Smokers were excluded in this study. Blood samples were collected before any glucocorticoid/immunosuppressive therapy and were centrifuged shortly after clot formation. All samples were stored -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 within 3 to 5 weeks after serum collection. 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 modified Rodnan total skin thickness score (modified Rodnan TSS) as previously described [14]. 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 modified Rodnan TSS was derived summation of the scores from all the 17 areas (range 0–51). Organ system involvement was defined as previously with some modification [15]: lung = bibasilar fibrosis on chest radiography and high-resolution computed tomography; esophagus = hypomotility shown by barium radiography; joint = inflammatory polyarthralgias or arthritis; heart = pericarditis, congestive heart failure without any other explanation; muscle = proximal muscle weakness and elevated serum creatine kinase. Isolated pulmonary hypertension was defined as clinical evidence of pulmonary hypertension and increased systolic pulmonary arterial pressure (>35 mmHg) by Doppler echocardiography, in the absence of severe pulmonary interstitial fibrosis; there were no patients with isolated pulmonary hypertension in this study. Renal vascular damage was determined as pulsatility index (PI) by color flow Doppler ultrasonography of both kidneys (Aloka SSD-2000 scanner, Aloka, Tokyo, Japan), using a 3.5 MHz broadband convex probe [16]. The PI, which represents vascular impedance, was calculated as $A-B/\text{mean}$, where A is the peak systolic frequency, B is the end diastolic frequency, and the mean is the time averaged frequency. The PI was calculated as an average value obtained with 8 waveforms on the renal segmental and interlobar arteries of both kidneys. Peripheral circulatory insufficiency was evaluated by the presence of digital pitting scars or ulcers at the first physical examination.

The protocol for the study was approved by our institutional review board and

informed consent was obtained from all patients according to the declaration of Helsinki.

Colorimetric microplate assay

Total antioxidant power levels were examined by colorimetric microplate assay (Oxford biochemical research, Oxford, MI). The assay measures the total ability for reducing Cu^{++} into Cu^{+} . This reduced form of copper will selectively form a 2:1 complex with chromogenic reagent, which is stable and has an absorption maximum at ~490 nm. A known concentration of uric acid is used to create a reference curve to compare those readings obtained by the samples. Data can be expressed as μM uric acid equivalents. Each sample was tested in duplicate.

Statistical analysis

Statistical analysis was performed using Mann-Whitney U-test for significance of differences and Bonferroni's test for multiple comparisons. Spearman's rank correlation coefficient was used to examine relationship between two continuous variables. Multivariate analysis was conducted using a logistic model. A p value of less than 0.05 was considered statistically significant. All data are shown as mean \pm S.D.

Results

Serum TAP levels in SSc patients

Serum TAP levels were significantly elevated in patients with SSc compared with controls ($591.5 \pm 94.0 \mu\text{M}$, $n=49$ vs. $522.2 \pm 75.3 \mu\text{M}$, $n=23$; $p<0.01$; Figure 1). Regarding the disease subset, serum TAP levels in lSSc patients were significantly elevated ($593.3 \pm 87.1 \mu\text{M}$, $n=22$) compared to normal control ($p<0.05$). Likewise, dSSc patients exhibited significantly increased serum TAP levels ($590.0 \pm 101.0 \mu\text{M}$, $n=27$) relative to normal control ($p<0.05$). However, no significant difference in serum TAP levels was observed between lSSc and dSSc patients. When the values higher than the mean + 2SD of the control serum samples ($672.8 \mu\text{M}$) were considered to be elevated, elevated serum TAP levels were observed in 24% (12/49) of SSc patients, with 23% (5/22) of lSSc patients and 26% (7/27) of dSSc patients. Serum TAP levels did not correlate with age and disease duration. However, serum TAP levels tended to increase with age of the patients ($r=0.27$, $p=0.07$). Thus, serum TAP levels were elevated in SSc patients.

Clinical correlation of serum TAP levels

Concerning the direct correlation of serum TAP levels with laboratory parameters in SSc patients, TAP levels positively correlated with C-reacting protein (CRP; $r=0.35$, $p<0.05$). However, no other significant correlation was observed between serum TAP levels and clinical parameters. To investigate the clinical association of high TAP levels, physical and laboratory findings were compared between SSc patients with high levels of TAP and those with low levels of TAP (Table 1). SSc patients with high TAP levels exhibit lower frequency of pitting scars than those with low levels of TAP (6% vs. 34%). The prevalence of other clinical features, organ involvement, and laboratory findings in

SSc patients tended to be lower in the group with high levels of TAP. However, there was no significant correlation and association observed.

Discussion

In this study, serum TAP levels were significantly elevated in samples from SSc patients relative to normal controls by specific colorimetric microplate assay. There was no clinical correlation of serum TAP levels observed without CRP.

In SSc, ischemic/reperfusion injury followed by Raynaud's phenomenon increases the ROS production [17]. The ROS production provokes endothelial cell damages and autoimmune response to various nuclear antigens, leading to autoantibody formation and further recruitment of inflammatory cells [18]. Previously, we demonstrated that serum levels of 8-isoprane, which is one of the markers of lipid peroxidation, and Hsp70, which is a biomarker of cellular stress, were increased in SSc [6, 7]. In addition, autoantibody against peroxiredoxin I, an antioxidant enzyme, is detected in SSc patients and is associated with the SSc disease severity [19]. Furthermore, recent study demonstrated the direct role for ROS in the pathogenesis of SSc including autoantibody and collagen production in mouse model and human skin[20]. These results suggest that oxidative stress plays a role for development of SSc.

For preventing the effect of oxidative stress, several antioxidant defense mechanisms are equipped in our body. Indeed, a reduced concentration of antioxidant including ascorbic acid, α -tocopherol, and β -carotene has been reported in SSc [1, 23, 24]. Increased level of lipid peroxidation and decreased levels of antioxidant capacity in SSc patient has been reported [25]. On the other hand, the levels of superoxide dismutase, one of the main components of antioxidant defense, increase in SSc than in healthy control [26]. These results suggest that antioxidant defense system is complicated and varies in diseases. Therefore, it is important to measure total antioxidant capacity in biological samples by simple and quantitative methods.

TAP measurement in plasma from SSc patients has been reported with

conflicting result [21, 22]. In the first paper reported by Italian group, TAP level was not decreased against odds, moreover, it was even higher than in healthy control. TAP levels correlated negatively with disease duration (data not shown) [21]. On the other hand, in the second paper reported by Romanian group, TAP levels decreased in SSc patients than in control. Regarding clinical correlation, there was no influence of age, disease duration, skin score, lung involvement (DLco), or inflammation (ESR) on TAP level. [22]. In both reports, there was no difference observed about TAP levels between dSSc and lSSc patients. The number of SSc patients included these reports were smaller than our study (17, 23, and 49; respectively). Therefore, we showed clear evidence of an increase in serum TAP levels in SSc patients than in control.

Collectively, these results indicate that oxidative stress plays an important role and serum TAP levels increase, as an indicator of global response to oxidative stress, in SSc patients with respect to controls.

Acknowledgements

We thank Ms. A. Usui, M. Yozaki, and K. Shimoda for technical assistance.

This work was supported by a grant of Research on Intractable Diseases from the Ministry of Health, Labour and Welfare of Japan.

Disclosures

None.

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Figure Legends

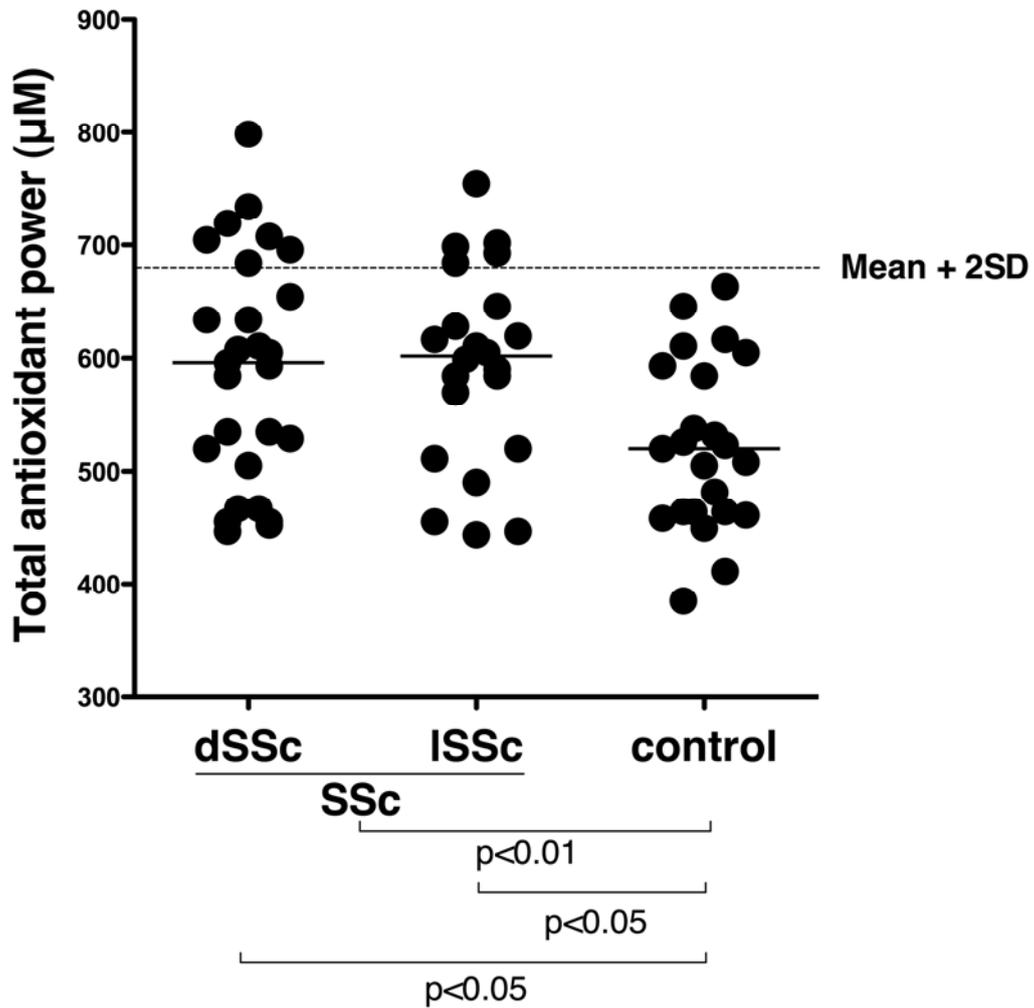


Figure 1. Serum TAP levels in serum samples from patients with dSSc, those with ISSc, and normal controls. Serum TAP levels were determined by specific colorimetric microplate assay. A broken line indicates the cut-off value (mean + 2SD of the control samples).

Table 1. Clinical and laboratory data of patients with SSc showing elevated serum TAP levels at the first evaluation. Values of clinical features and organ involvements are percentages.

	high TAP level n=12	low TAP level n=37
Age at onset, yrs, mean \pm SD	48 \pm 16	44 \pm 18
Sex, F:M	9:3	32:5
Duration, yrs, mean \pm SD	5.4 \pm 7.3	6.3 \pm 8.1
<i>Clinical features</i>		
Pitting scars	6	34
Short sublingual frenulum	14	37
Contracture of phalanges	10	35
Diffuse pigmentation	14	39
modified Rodnan TSS, mean \pm SD	13.7 \pm 12.6	13.8 \pm 9.7
<i>Organ involvement</i>		
Lung		
Pulmonary fibrosis	12	29
Pulmonary hypertension	2	10
Decreased %VC	9	19
Decreased %DLco	26	51
Esophagus	17	52
Heart	6	8
Kidney		
Increased vascular resistance	5	8
Renal crisis	0	2
Joint	8	12
Muscle	8	12
<i>Laboratory findings</i>		
Anti-topoisomerase I antibody	12	32
Anticentromere antibody	6	29
Anti-U1RNP antibody	4	6
Positive rheumatoid factor	4	11
Elevated ESR	10	14
Elevated CRP	4	10
Increased IgG	10	22

Values are percentages. The cut off value was set to 672.8 μ M.

TAP: total antioxidant power, ESR: erythrocyte sedimentation rates, CRP: C-reacting protein, TSS: total skin thickness score