

Increased serum levels of nitrotyrosine, a marker for peroxynitrite production, in systemic sclerosis

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Abstract

Objective

To determine serum levels of nitrotyrosine (NT), an end product of peroxynitrite (ONOO⁻), and its clinical association in patients with systemic sclerosis (SSc).

Methods

Serum NT levels from 25 patients with limited cutaneous SSc (lSSc) and 34 patients with diffuse cutaneous SSc (dSSc) were examined by enzyme-linked immunosorbent assay.

Results

Serum NT levels were elevated in SSc patients compared with normal controls ($n = 27$), the levels being similar between lSSc and dSSc patients ($P < 0.001$). SSc patients with elevated NT had higher serum levels of anti-agalactosyl IgG Ab, IgG and IgA than those with normal NT levels ($P < 0.05$). NT levels correlated inversely with the percentage diffusion capacity for carbon monoxide (DLco) ($P < 0.02$, $r = -0.414$, $n = 47$).

Conclusion

These results suggest that ONOO⁻ may play an important role in the clinical manifestations of SSc, especially vascular damage to the lungs, and that ONOO⁻ may be related to immunological abnormalities in SSc.

Key words

Systemic sclerosis, nitrotyrosine, diffuse capacity for carbon monoxide (DLco), immunoglobulin, anti-agalactosyl IgG antibody.

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Introduction

Nitric oxide (NO) is a gaseous radical whose role has been clarified in many diseases, ever since NO was found to be an endothelium-derived relaxing factor in 1991 (1). NO is produced from L-arginine by NO synthases (NOS), three isoforms of which have been identified: endothelial NOS, neuronal NOS, and inducible NOS. Endothelial NOS and neuronal NOS are constitutively expressed in endothelial and neuronal cells, respectively, while NOS expression is induced in certain cells by various stimuli. It has been suggested that NO may be involved in the etiology of many inflammatory diseases, including systemic sclerosis (SSc) (2).

SSc is a connective tissue disease of unknown etiology and the prognosis can be affected by the dysfunctioning of internal organs such as the lung, heart, and kidney. Kahaleh observed in his review that the destruction of microvessels leading to the clinically recognized state of chronic organ ischemia and tissue underperfusion is fundamental in the pathogenesis of SSc, and that the earliest signs of vascular dysfunction include enhanced vascular permeability and the dysregulated control of vascular tone (3). NO is thought to be involved in this aspect of SSc. Furthermore, a recent study has shown that alveolar NO concentrations are increased, and that they are inversely correlated with the diffusion capacity for carbon monoxide (DLco), in lung diseases associated with SSc. This suggests that an isolated decrease in %DLco could indicate vascular damage and that NO may play a role in the development of the lung involvement in SSc (4).

NO reacts with superoxide (O_2^-) and immediately produces peroxynitrite ($ONOO^-$), which can cause severe cytotoxicity. $ONOO^-$ causes the nitration of tyrosine and produces nitrotyrosine (NT) as an end product. Recently, antibodies (Abs) against NT have been produced and used to examine the effects of $ONOO^-$, particularly *in vivo* (5). Overexpression of inducible NOS and the reduced expression of endothelial NOS, accompanied by an accumulation of NT, have been demonstrated in the microvessels of involved cutane-

ous tissue in SSc (6). It has also been reported that serum NT levels are correlated with lupus disease activity and could offer a potentially useful laboratory measure of lupus disease activity (7).

However, there have as yet been no investigations of serum NT levels and their clinical associations in SSc. We undertook this study to measure serum NT levels in SSc patients and examine the correlation between serum NT and clinical findings in order to determine the contribution of the tissue damage induced by $ONOO^-$ to the development of SSc.

Materials and methods

Serum samples

Serum samples were obtained from 59 Japanese patients with SSc (9 males and 50 females; age 47 ± 17 years, disease duration 5.1 ± 7.4 years), all of whom fulfilled the criteria proposed by LeRoy *et al.* (8). Patients were divided into two subgroups: 25 patients (2 males and 23 females) with limited cutaneous SSc (lSSc) and 34 patients (7 males and 27 females) with diffuse cutaneous SSc (dSSc). None of the patients were being treated with steroids, D-penicillamine, prostanoids, calcium channel blockers (CCB), nitrates or immunosuppressive therapy, and none of them had a recent history of infection or abnormal liver function at the time of serum sampling. The current vascular therapy consists principally of oral Beraprost sodium, an active prostacyclin analogue. Anti-nuclear Ab was determined by indirect immunofluorescence using HEp-2 cells as substrate, and autoantibody specificities were further assessed by ELISA and immunoprecipitation. Assays were positive for anti-centromere Ab in 17 patients, anti-topoisomerase I Ab in 21 patients, anti-U1RNP Ab in 2 patients, anti-U3RNP Ab in 1 patient, anti-RNA polymerase Ab in 4 patients, anti-Th/To Ab in 1 patient, and an autoantibody with unknown specificities in 1 patient.

Twenty-seven healthy Japanese subjects (age 49 ± 14 years old, 4 males and 23 females) matched for age and sex with the SSc patients were used as normal controls.

Fresh venous blood samples were centrifuged shortly after clot formation. All samples were stored at -70°C until used. Approval for this study was obtained from the Nagasaki University Graduate School of Biomedical Sciences and all investigations were carried out in accordance with the principles of the Declaration of Helsinki.

Clinical assessment

Out of the 59 SSc patients, complete data from 47 patients were available to study the clinical and laboratory correlations. Organ system involvement was defined as described previously (9-11): lung: bibasilar fibrosis on chest radiography and high resolution computed tomography; joint: inflammatory polyarthralgias or arthritis; heart: pericarditis, congestive heart failure, or arrhythmias requiring treatment; and kidney: malignant hypertension and rapidly progressive renal failure without any other explanation. Pulmonary function, including vital capacity (VC) and DLco, were also tested. There were no patients with pulmonary hypertension without pulmonary fibrosis.

Enzyme-linked immunosorbent assay (ELISA) for serum nitrotyrosine

The concentrations of serum NT were measured by a specific ELISA kit (HK501, Hycult, biotechnology, The Netherlands). ELISA for NT was performed according to the manufacturer's procedure. Each sample was tested in duplicate.

Measurement of anti-agalactosyl IgG Ab

Serum levels of anti-agalactosyl IgG Abs were measured using a lectin enzyme immunoassay kit, Eitest CARF (Eizai Co., Ltd, Japan) with human agalactosyl IgG as antigen. The agalactosyl IgG was prepared from enzymatically served oligosaccharides of human IgG. Human IgG was subsequently treated with neuraminidase in 0.1M acetate buffer (pH5.0) for 48 hours at 37°C and β -galactosidase in 0.1M citrate-phosphate buffer (pH 7.0) for 24 hours at 37°C . Agalactosyl IgG was purified using a protein G coupled to agarose as an affinity column for chromatography.

ELISA was performed according to the manufacturer's procedure. Each sample was tested in duplicate.

Statistical analysis

Statistical analysis was performed using the Mann-Whitney U test for the comparison of NT levels, Fisher's exact probability test for the 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 less than 0.05 was considered statistically significant. All data were presented as means \pm standard deviation (SD).

Results

Serum NT levels in SSc

Serum NT levels were significantly elevated in the SSc patients compared to normal controls ($p < 0.001$; Fig. 1). Concerning the SSc subgroups, there was no significant difference in se-

rum NT levels between dSSc and ISSc patients. When we divided the dSSc patients and ISSc patients into 2 subgroups based on their disease duration (> 2 years and ≤ 2 years), no significant difference in the serum levels of NT was found (data not shown). Values higher than the mean $+ 2\text{SD}$ ($0.038 \mu\text{M}$) of the control serum samples were considered to be elevated. Elevated NT levels were observed in 23% (11/47) of all SSc patients. By disease subset, serum NT levels were increased in 28% (7/25) of dSSc patients and 18% (4/22) of ISSc patients. In contrast, none of the normal controls showed elevated NT levels. Thus, serum NT levels were elevated in SSc patients, with similar levels between dSSc and ISSc.

Clinical correlation of NT levels in SSc

SSc patients with elevated serum NT levels had significantly lower %DLco ($p < 0.01$) and higher serum levels of IgG ($p < 0.05$) and IgA ($p < 0.05$) than

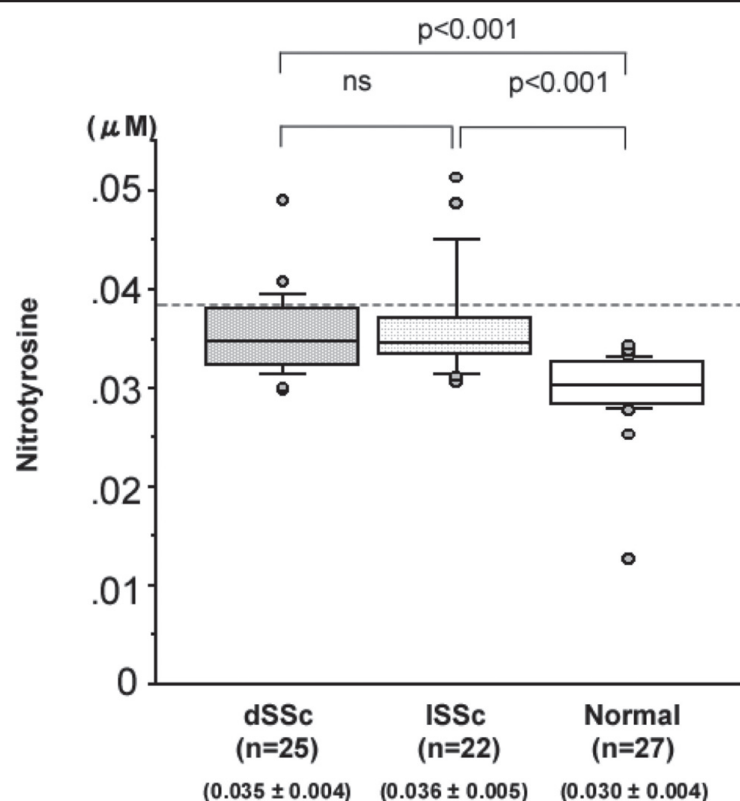


Fig. 1. Serum levels of NT in patients with dSSc or ISSc and normal controls at the first evaluation. Serum NT levels were determined by a specific ELISA. In the box and whisker plots, the 25th to 75th percentiles are represented by the horizontal lines of the box; the median is indicated by the internal horizontal line across the box, and the whiskers on each box represent the 10th to 90th percentiles. A broken line indicates the cut-off value (mean $+ 2\text{SD}$ of the control samples). The numbers of subjects examined and mean values \pm SD are indicated in parentheses.

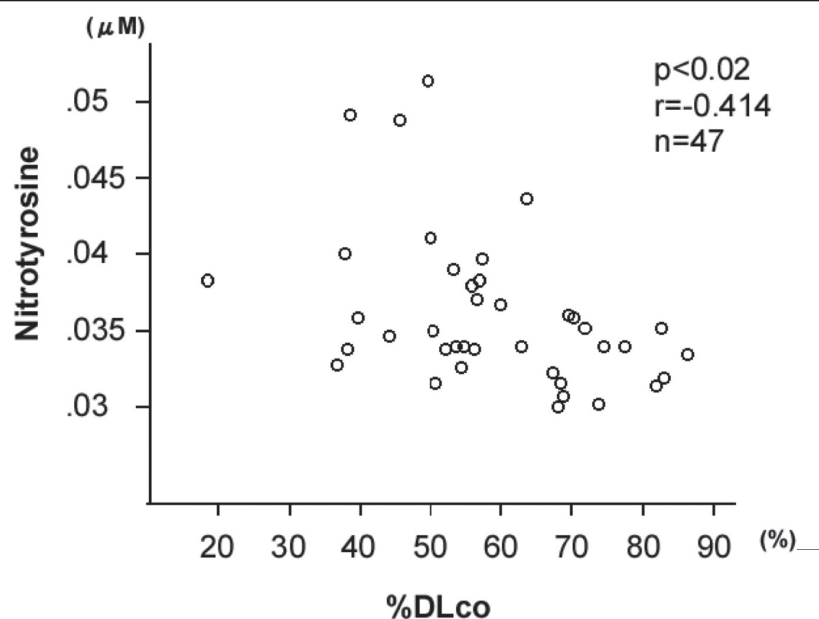
Table I. Clinical and laboratory data of patients with SSc showing elevated serum NT levels at the first evaluation.

	Elevated serum NT (n = 11)	Normal serum NT (n = 36)
Age at onset (yr) means \pm SD	37.3 \pm 18.6	45.4 \pm 17.3
Sex (male: female)	1:10	4:32
Duration (yr) means \pm SD	10.4 \pm 12.1	5.1 \pm 5.6
Clinical features:		
dSSc	64	50
ISSc	36	50
Pitting scars	55	39
Contracture of phalanges	64	39
Organ involvement:		
Lung	55	36
%VC means \pm SD	78.8 \pm 18.3	97.3 \pm 25.6
%DLco means \pm SD	47.5 \pm 12.9**	62.8 \pm 13.9
Heart	18	14
Kidney	0	3
Joint	27	19
Autoantibodies:		
Anti-topoisomerase I Ab	45	44
Anti-centromere Ab	36	36
Immunoglobulin:		
IgG means \pm SD	2034.5 \pm 657.1*	1590.7 \pm 521.6
IgA means \pm SD	421.5 \pm 213.0 *	276.0 \pm 81.7
IgM means \pm SD	242.5 \pm 106.2	181.1 \pm 88.4

Values of clinical features and organ involvement are percentages unless otherwise indicated. All the clinical and laboratory parameters and serum NT levels were obtained at the first evaluation. *P<0.05 or **P<0.01 vs SSc patients with normal NT levels.

those with normal NT levels (Table I). Furthermore, serum NT levels correlated negatively with %DLco in patients with SSc ($p < 0.02$, $r = -0.414$; Fig. 2). However, SSc patients with elevated NT levels did not show a higher prevalence of lung fibrosis. Since

pulmonary hypertension is extremely rare in Japanese SSc patients and there were no SSc patients with isolated pulmonary hypertension in this study, the correlation of serum NT levels with pulmonary hypertension could not be determined. Out of 47 SSc patients

**Fig. 2.** The negative correlation of NT levels against %DLco in patients with SSc at the first evaluation. Serum levels of NT were determined by a specific ELISA.

examined, 30 underwent the test for anti-agalactosyl IgG Ab, and 8 were found to have elevated levels while 22 were normal. Anti-agalactosyl IgG Ab levels were significantly higher in SSc patients with elevated NT levels than in those with normal NT levels ($p < 0.05$, Fig. 3), while elevated NT levels did not correlate with levels of antinuclear Ab or anti-topoisomerase I Ab determined by specific ELISA (data not shown). Thus, elevated NT levels were associated with the severity of lung involvement and elevated serum levels of Ig and anti-agalactosyl IgG Ab.

Discussion

In this study, serum NT levels were significantly increased in SSc patients compared to healthy controls. However, NT did not seem to be correlated with the extent of skin involvement, since NT levels were similar in the dSSc and ISSc patients. Many studies of serum NO levels have been conducted and some have reported high values of serum NO in SSc (2). In addition, we recently reported high values of serum 8-isoprostane, one of the markers of lipid peroxidation, in SSc patients (12). NO reacts with superoxide and immediately produces ONOO⁻, which causes the nitration of tyrosine and produces NT, but no studies have yet examined serum NT levels in SSc. Therefore, the present paper is the first to demonstrate that serum NT levels are significantly elevated in SSc. It may be noted that, while it has been reported that CCBs such as nifedipine have anti-oxidant properties in SSc (13), our patients were not being treated with CCB and therefore it is likely that our results reflected actual effects and were not influenced by the treatments.

Recently, Kingdon *et al.* reported a marked decrease in plasma protein-bound nitrotyrosine in patients with primary Raynaud's phenomenon compared to healthy controls (14). Their results are very interesting, but they did not find any difference in plasma NT levels between SSc patients and healthy controls, suggesting a possible discrepancy between their results and ours. Although some of our SSc patients had Raynaud's phenomenon,

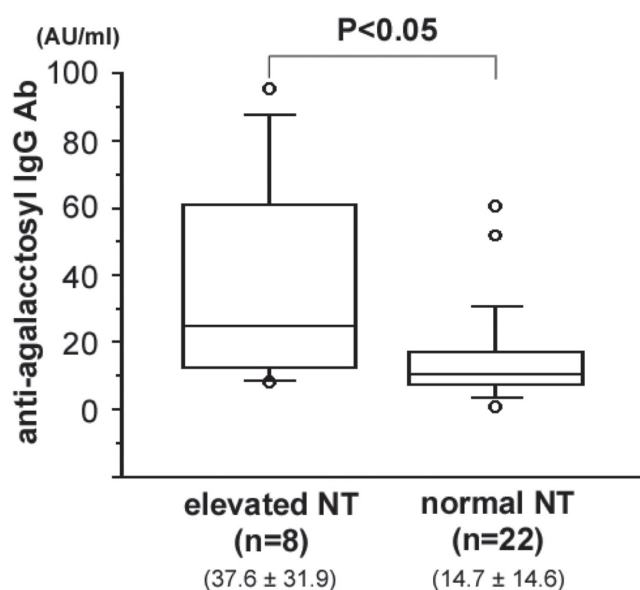


Fig. 3. Serum levels of anti-agalactosyl IgG Ab in patients with SSc showing elevated and normal serum NT levels at the first evaluation. Serum levels of anti-agalactosyl IgG Ab were determined by a specific ELISA. In the box and whisker plots, the 25th to 75th percentiles are represented by the horizontal lines of the box; the median is indicated by the internal horizontal line across the box, and the whiskers on each box represent the 10th to 90th percentiles. The numbers of subjects examined and mean values \pm SD are indicated in parentheses.

no significant correlation was seen between Raynaud's phenomenon and serum NT values in the present study (data not shown). It may be noted that in an earlier paper (12) we reported a significant positive correlation between serum levels of 8-isoprostane and NT in the same cohort of SSc patients under study here ($p = 0.0013$, $r = 0.450$, unpublished data).

Adcock *et al.* reported that superoxide could induce NO synthase mRNA through NF κ B activation (15). Since NO reacts with superoxide to produce peroxynitrite, this supports our findings of significantly increased serum NT values in SSc patients compared to healthy controls. Furthermore, they recognized that the highest plasma NT values in their study were seen in the SSc group and suggested that some patients may exhibit an increased formation of reactive nitrogen species (14). SSc is a heterogeneous disease and there is considered to be a racial difference in SSc patients. Although the reasons remain unclear, it is possible that the discrepancies in the distribution of SSc may be caused by the racial differences, patient populations or the sampling method used.

Serum NT levels were inversely cor-

related with %DLco in SSc patients, while they were not associated with lung fibrosis or %VC. Furthermore, since there were no SSc patients with isolated pulmonary hypertension in the current study, the correlation of serum NT levels with pulmonary hypertension remains unknown. Nonetheless, as an isolated decrease in %DLco indicates lung vascular damage in SSc (16), the negative correlation of serum NT levels with %DLco suggests that oxidative injury induced by ONOO⁻ contributes to lung vascular damage in SSc. Cotton *et al.* discovered the accumulation of immunodetectable NT within endothelial cells in the lesional skin from SSc, which contributed to the free radical damage implicated in the pathogenesis of SSc (16). They identified the involvement of NT in cutaneous endothelial damage and our results suggest that serum NT could be utilized as a useful serological marker for assessing vascular damage, in addition to %DLco, in the lungs of SSc patients.

Our results led us consider the possibility that ONOO⁻ might play an important role in the clinical manifestations of SSc, especially in vascular damage to the lung. Tissue damage due to free

radicals, in which NO may be crucial, is considered to be an important mechanism in the development of SSc. Increased exhaled NO has been reported in SSc-associated lung diseases and the suggested sources of NO include various types of cells, such as macrophages, epithelial cells, and infiltrating inflammatory cells in the diseased lung (18, 19). One paper reported that NO was produced by cultured fibroblasts derived from SSc sclerotic skin (20). Therefore, NO may be produced by various cells in the lung, including fibroblasts, epithelial cells, and inflammatory cells, which could cause the increase in serum NT levels in SSc patients.

In the present study, serum levels of IgG and IgA were correlated with raised serum NT levels in SSc patients. Some reports have shown that ONOO⁻ could cause the nitration of tyrosine within Ig and that ONOO⁻ treatment reduced the activity of IgG (21, 22). It was hypothesized that ONOO⁻, whose production is probably increased in SSc, could cause the nitration of amino acids within Ig, thus reducing its activity. Therefore, negative feedback mechanisms induced by lowered Ig activity might stimulate the production of Ig, leading to the hyper- γ -globulinemia that is one of immunologic abnormalities of SSc.

Anti-agalactosyl IgG Abs are autoantibodies present in the sera of SSc patients as well as rheumatoid arthritis patients (23, 24). Agalactosyl IgG is a glycoform of IgG that is found as a proportion of total IgG in all normal individuals. Rheumatoid factors show better binding to agalactosyl IgG than to galactosylated IgG. The association of elevated serum NT levels with anti-agalactosyl IgG Ab suggests that oxidative injury may play a role in the production of this autoantibody. It has been hypothesized that immune responses to autoantigens are induced by cryptic self-epitopes that are generated by the modification of self-antigens (for example, novel cleavage, altered conformation, or tertiary structures) (25). The exposure of cryptic self-epitopes activates potentially autoreactive T cells that have not previously encountered

the cryptic self, thereby breaking T cell tolerance. In this regard, reactive oxygen species have been shown to induce modification of the self-antigens, such as metal-dependent cleavage of SSc-related autoantigens (26). Therefore, the production of anti-agalactosyl IgG Ab may be related to the modification of IgG by oxidative injury.

This study has shown that serum NT is increased in patients with SSc compared to normal controls and that there is an inverse correlation with %DLco in SSc patients. These results suggest that ONOO⁻ may be involved in the processes of vascular damage to the lung in SSc. Therefore, serum NT is a potentially useful and sensitive marker whose introduction in the routine monitoring of SSc patients could be considered.

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