Increased Serum Levels of N$^{\varepsilon}$-(hexanoyl)lysine, A New Marker of Oxidative Stress, in Systemic Sclerosis

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ABSTRACT. ***Objective.*** To determine serum levels of N$^{\varepsilon}$-(hexanoyl)lysine (HEL), a new marker of oxidative stress, and its clinical association in patients with systemic sclerosis (SSc).

Methods. Serum HEL levels from 26 patients with limited cutaneous SSc (lSSc), 34 with diffuse cutaneous SSc (dSSc), 20 with systemic lupus erythematosus (SLE), 20 with dermatomyositis (DM), and 40 healthy individuals were examined by enzyme linked immunosorbent assay.

Results. Serum HEL levels were elevated in patients with SSc compared with healthy controls (n = 40) with similar levels between patients with lSSc and dSSc ($p < 0.0001$). SSc patients with elevated HEL levels had increased serum levels of anti-agalactosyl IgG antibody, rheumatoid factor (RF), and IgM than those with normal HEL levels ($p < 0.05$). HEL levels correlated positively with anti-agalactosyl IgG antibody ($p = 0.013$, $r = 0.408$) and RF titer ($p = 0.0028$, $r = 0.426$).

Conclusion. Our results suggest that oxidative stress may play an important role in immunological abnormalities of SSc, especially in the production of autoantibodies including anti-agalactosyl IgG antibody and RF. (First Release Sept 1 2008; J Rheumatol 2008;35:2214–9; doi:10.3899/jrheum.080191)

Key Indexing Terms:
N$^{\varepsilon}$-(HEXANOYL)LYSINE
ANTI-AGALACTOSYL IgG ANTIBODY
SYSTEMIC SCLEROSIS
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Systemic sclerosis (SSc) is a multisystem disorder of connective tissue characterized by sclerotic changes in the skin and internal organs. Further, many immunologic abnormalities, including the presence of autoantibodies and hyper-γ-globulinemia, have been detected in patients with SSc, suggesting that SSc has an autoimmune background. SSc-specific antibodies including anti-topoisomerase I, anticentromere, and anti-RNA polymerase antibody have been identified[1-3].

Although the pathogenesis of this disease remains unclear, oxidative stress has been suggested to contribute to clinical manifestations associated with SSc, such as vascular damage, fibrosis, and immunological abnormalities[4-7]. We have reported high values of serum 8-isoprostane, one of the markers of lipid peroxidation, in patients with SSc[8]. To further assess the role of oxidative stress in the development of SSc, a new marker that directly reflects oxidative damages *in vivo* would be expected.

Recently, one marker of oxidative stress, N$^{\varepsilon}$-(hexanoyl) lysine (HEL), has been identified, a novel adduct formed by the reaction of linoleic acid hydroperoxide and lysine[9]. The monoclonal antibody to HEL has been available[10] and examinations of HEL in some diseases have been reported using this antibody[11-13]. However, to our knowledge there have been no studies examining the involvement of HEL in patients with SSc.

We conducted this study to measure serum HEL levels in patients with SSc and examine the correlation between serum HEL and clinical findings in order to investigate the involvement of oxidative stress in patients with SSc.

MATERIALS AND METHODS

Serum samples. Serum samples were obtained from 60 Japanese patients with SSc (49.2 ± 16.8 yrs old, 52 women, 8 men). All patients fulfilled the criteria for SSc proposed by LeRoy, *et al*[14]. The disease duration of patients with SSc was 5.6 ± 7.2 years. Patients were classified into the following 2 subgroups: 26 with limited SSc (lSSc; 25 women, 1 man) and 34 with diffuse SSc (dSSc; 27 women, 7 men). None of the patients with SSc were treated with steroids, D-penicillamine, prostanoids, calcium channel blockers, or other immunosuppressive therapy, and none had a recent history of infection and abnormal liver function at the time of serum sampling. Antinuclear antibody was determined by indirect immunofluorescence using HEp-2 cells as the substrate and autoantibody specificities were further assessed by ELISA and immunoprecipitation. Anticentromere antibody was positive for 21 patients, anti-topoisomerase I antibody for 26, anti-U1RNP antibody for 2, anti-U3RNP antibody for 1, anti-RNA polymerase antibody for 6, anticentromere and anti-topoisomerase antibody for 1, and autoantibody with unknown specificities for 2 and negative for 1.

Forty healthy Japanese persons (45.2 ± 10.1 yrs old, 35 women, 5 men) matched for age and sex with SSc patients served as controls. Twenty patients with systemic lupus erythematosus (SLE; 38.7 ± 12.5 yrs old, 19 women, 1 man) and with dermatomyositis (DM; 48.3 ± 14.6 yrs old, 17

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women, 3 men) were included as disease controls and they fulfilled the criteria for SLE proposed by the American College of Rheumatology[15] and that for DM proposed by Bohan and Peter[16]. Smokers were excluded from our study. Fresh venous blood samples were centrifuged shortly after clot formation. All samples were stored at –70°C prior to use. All investigations were performed after approval by the Nagasaki University Graduate School of Biomedical Sciences and according to the Declaration of Helsinki principles.

Clinical assessment. Complete medical histories, examinations, and laboratory tests were conducted for all patients at their first visit. Out of 60 patients with SSc studied for serum HEL levels, clinical and laboratory correlation was examined for 50 patients because of the limited data available. Organ system involvement was defined as described[17-19]: 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, or 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. Pulmonary function, including vital capacity (VC) and diffusion capacity for carbon monoxide (DLCO), was also tested. There were no patients with pulmonary hypertension without pulmonary fibrosis.

Enzyme linked immunosorbent assay (ELISA) for serum HEL. The concentrations of serum HEL were measured by a competitive ELISA kit (Japan Institute for Aging, Shizuoka, Japan). ELISA for HEL was performed according to the manufacturer's procedure. Namely, each serum was diluted 4 times with phosphate buffered saline. Then, diluted serum was incubated with α-chymotrypsin at 37°C overnight and spun at 5000 g for 1 hour using centricon-10 (Millipore filter) in order to remove protein. Fifty milliliters of centrifuged filtrate was applied as a sample. Each sample was tested in duplicate.

Measurement of anti-agalactosyl IgG antibody. Anti-agalactosyl IgG antibody in serum was measured with Lectin Enzyme Immunoassay kit, Eitest CARF (Eizai Co. Ltd., Tokyo, Japan) using 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 0.1 M acetate buffer (pH 5.0) for 48 h at 37°C and ß-galactosidase in 0.1 M citrate-phosphate buffer (pH 7.0) for 24 h 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.

Measurement of rheumatoid factor (RF). RF in serum was measured by a nephelometric method and values > 20 IU/ml were considered positive.

Statistical analysis. Statistical analysis was performed using Mann-Whitney U-test for comparison of HEL levels, 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 2 continuous variables. A p value < 0.05 was considered statistically significant. All data were presented as means ± standard deviation (SD).

RESULTS

Serum HEL levels in SSc. Serum HEL levels were significantly elevated in total patients with SSc compared to healthy controls ($p < 0.0001$; Figure 1). SLE and DM were shown as disease controls in Figure 1, and serum HEL levels were also significantly elevated in both SLE and DM patients compared to healthy controls ($p < 0.0001$; Figure 1). Concerning the SSc subgroups, there was no significant

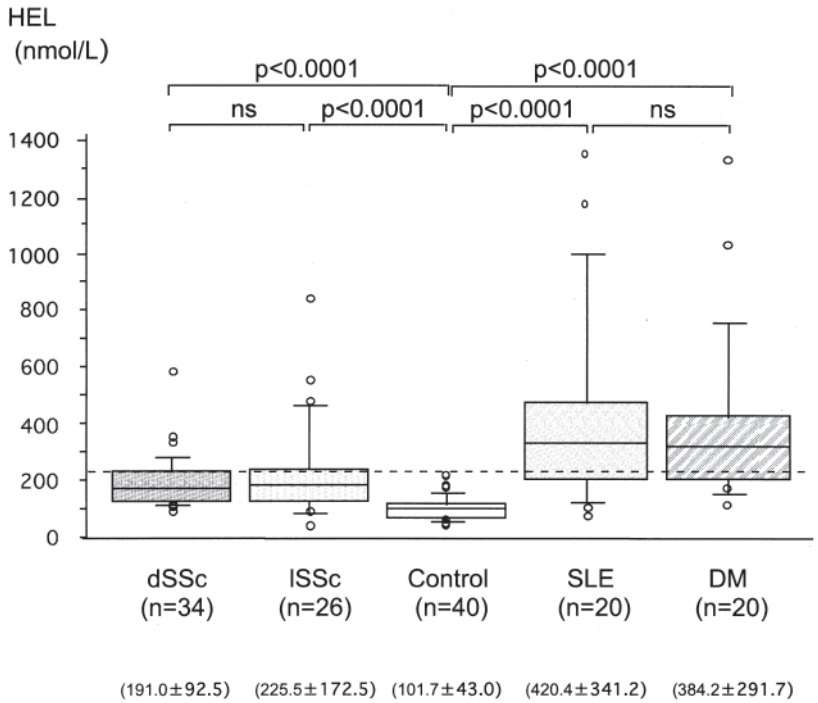

Figure 1. Serum levels of HEL in patients with dSSc or lSSc, SLE, and DM, as compared to healthy controls at the first evaluation. SLE and DM are shown as disease controls. Serum HEL levels were determined by a specific ELISA. The 25th to 75th percentiles are represented by horizontal lines, median by the internal horizontal line, and 10th to 90th percentiles by the whiskers. N = subjects examined and mean values ± SD are in parentheses. Broken line indicates cutoff value (mean + 3 SD of control samples).

difference in serum HEL levels between dSSc and lSSc patients. Values higher than the mean + 3 SD (230.7 nmol/l) of the control serum samples were considered to be elevated in our study. Elevated HEL levels were observed in 25% (15/60) of total patients with SSc. Regarding the subsets of SSc, serum HEL levels were increased in 24% (8/34) of patients with dSSc and 27% (7/26) of patients with lSSc. By contrast, no healthy control showed elevated HEL levels. Thus, serum HEL levels were elevated in SSc patients with similar levels in dSSc and lSSc.

Clinical correlation of HEL levels in SSc. SSc patients with elevated serum HEL levels had significantly elevated serum levels of anti-agalactosyl IgG antibody ($p < 0.01$), IgM ($p < 0.05$), and RF ($p < 0.05$) than those with normal HEL levels (Table 1). Further, serum HEL levels correlated positively with anti-agalactosyl IgG antibody ($p = 0.013$, $r = 0.408$; Figure 2A) and with RF titer ($p = 0.0028$, $r = 0.426$; Figure 3A) in patients with SSc. However, SSc patients with elevated HEL levels did not show higher prevalence of lung fibrosis and lower %DLCO. Since pulmonary hypertension is extremely rare in Japanese patients with SSc and there were no SSc patients with isolated pulmonary hypertension in our study, correlation of serum HEL levels with pulmonary hypertension could not be determined. Out of 60 patients with SSc examined, 38 patients had the test for anti-agalactosyl IgG antibody. Among these, 11 patients were considered to be elevated and 27 were normal for levels of serum HEL. Anti-agalactosyl IgG antibody levels were significantly higher in SSc patients with elevated HEL levels than those with normal HEL levels ($p = 0.0011$; Figure 2B). Meanwhile, out of 60 patients with SSc, 50 had the test for RF. Among these, 13 patients were considered to be elevated and 37 were normal for levels of serum HEL. RF levels were significantly higher in SSc patients with elevated HEL than those with normal HEL levels ($p = 0.0174$; Figure 3B). Elevated HEL levels did not correlate with levels of anti-centromere antibody and anti-topoisomerase I antibody determined by specific ELISA (data not shown). Thus, elevated HEL levels were associated with elevated serum levels of IgM, RF, and anti-agalactosyl IgG antibody.

Table 1. Clinical and laboratory data of patients with SSc showing elevated serum HEL levels at the first evaluation. Values of clinical features and organ involvement are percentages unless otherwise indicated. All the clinical and laboratory measures and serum HEL levels were obtained at the first evaluation.

	Elevated Serum HEL, n = 15	Normal Serum HEL, n = 45
Age at onset, yrs, mean ± SD	43.4 ± 15.0	44.1 ± 17.7
Sex (male:female)	0:15	8:37
Duration, yrs, mean ± SD	8.2 ± 11.1	4.7 ± 5.2
Clinical features		
dSSc	53	58
lSSc	47	42
Pitting scars	53	42
Contracture of phalanges	53	42
Organ involvement		
Lung	60	38
% VC, mean ± SD	89.1 ± 30.4	96.6 ± 22.4
% DLCO, mean ± SD	58.9 ± 18.3	59.5 ± 17.4
Esophagus	50	56
Heart	13	16
Kidney	0	4
Joint	27	22
Muscle	7	24
RF	33**	7
RF titer, IU/ml, mean ± SD	26.6 ± 44.1*	3.3 ± 10.6
Anti-agalactosyl IgG antibody (AU/ml), mean ± SD	36.3 ± 28.8**	12.3 ± 9.9
Autoantibodies		
Anti-topoisomerase I (U/ml)	47	44
Anti-centromere (index)	40	33
Immunoglobulin		
IgG, mg/dl, mean ± SD	1666.1 ± 491.6	1696.6 ± 606.5
IgA, mg/dl, mean ± SD	309.8 ± 124.2	324.0 ± 158.1
IgM, mg/dl, mean ± SD	241.4 ± 100.8*	189.1 ± 130.7

* $p < 0.05$ or ** $p < 0.01$ vs SSc patients with normal HEL levels. HEL: N^{ε}-(Hexanoyl)lysine; dSSc: diffuse cutaneous systemic sclerosis; lSSc: limited cutaneous SSc; SD: standard deviation; VC: vital capacity; DLCO: diffusing capacity carbon monoxide; RF: rheumatoid factor.

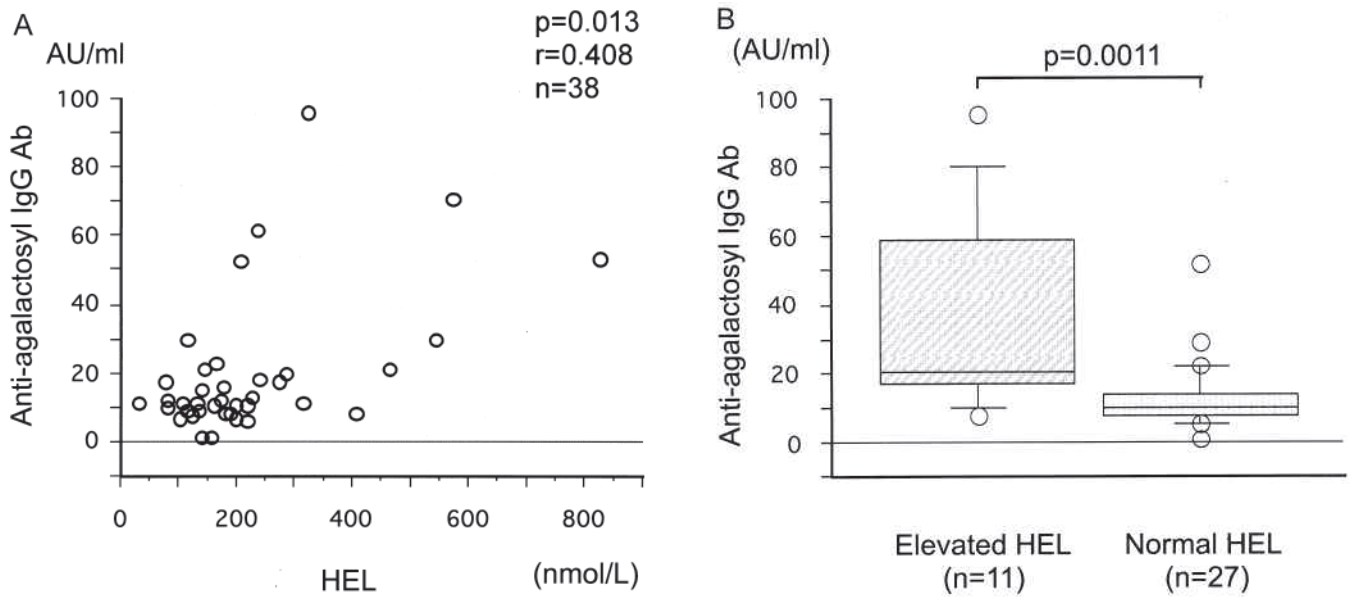

Figure 2. A. Positive correlation of serum HEL levels against anti-agalactosyl IgG antibody in patients with SSc at first evaluation. Serum levels of HEL were determined by a specific ELISA. B. Serum levels of anti-agalactosyl IgG antibody determined by a specific ELISA in patients with SSc showing elevated and normal serum HEL levels at the first evaluation. The 25th to 75th percentiles are represented by horizontal lines, median by the internal horizontal line, and 10th to 90th percentiles by whiskers. N = subjects examined and mean values ± SD are in parentheses.

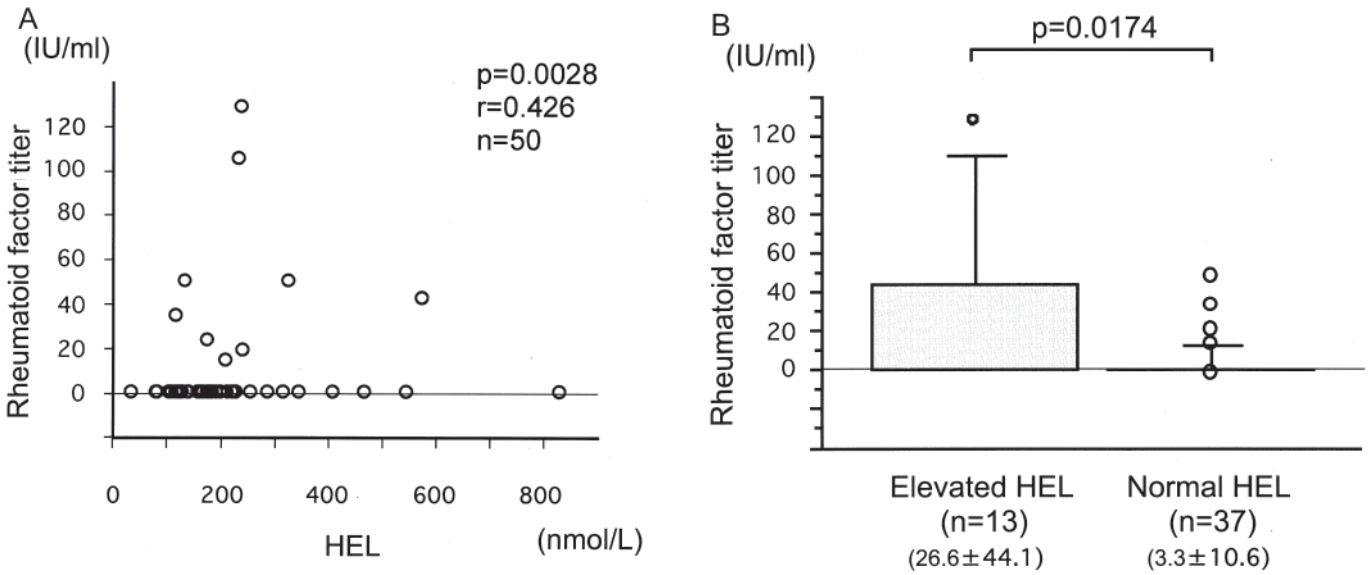

Figure 3. *A.* Positive correlation of serum HEL levels against RF in patients with SSc at first evaluation. Serum levels of HEL were determined by a specific ELISA and serum levels of RF titer by a nephelometric method. B. Serum levels of RF in patients with SSc showing elevated and normal serum HEL levels at first evaluation. The 25th to 75th percentiles are represented by horizontal lines, median by the internal horizontal line, and 10th to 90th percentiles by whiskers. N = subjects examined and mean values ± SD are in parentheses.

DISCUSSION

HEL was identified as one of the lipid hydroperoxide-modified lysine residues and considered to be a new marker of oxidative stress[9]. Using the antibody to HEL, examinations of HEL have been reported in some diseases[10,12]. We have already reported high values of serum 8-isoprostane, one of the markers of lipid peroxidation, in patients with SSc[8]. Although SSc is considered to be among the oxidative stresses, to our knowledge there have been no reports concerning HEL as an oxidative stress marker in patients with SSc. Our study is the first to reveal that serum HEL levels were significantly increased in SSc patients compared to healthy controls. Further, we confirmed the positive correlation between serum 8-isoprostane levels and serum HEL levels (p = 0.0031, r = 0.407; Figure 4). Therefore, it was strongly confirmed that oxidative stress levels were

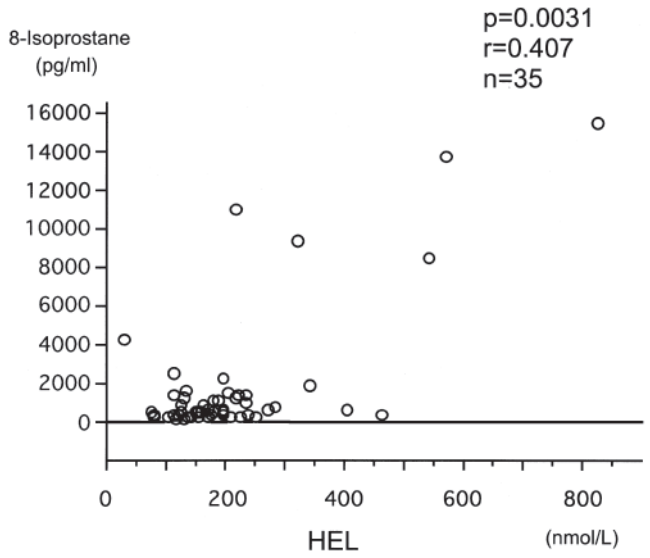

Figure 4. Positive correlation of serum HEL levels against serum 8-isoprostane in patients with SSc at the first evaluation. Serum levels of HEL and 8-isoprostane were determined by specific ELISA kits. For details of 8-isoprostane in SSc, refer to Ogawa, *et al*[8].

enhanced in patients with SSc. However, HEL levels did not correlate with the extent of skin sclerosis since HEL levels were similar between patients with dSSc and lSSc.

We demonstrated that serum HEL levels were significantly elevated not only in patients with SSc but also in those with SLE and DM, compared to healthy controls. We also confirmed the positive correlations in patients with SSc between serum HEL levels and anti-agalactosyl IgG antibodies as well as RF, both frequently detected in systemic autoimmune diseases including rheumatoid arthritis (RA). Ryo, *et al* reported that HEL levels were significantly elevated in salivary glands of patients with Sjögren's syndrome (SS) compared to normal controls and non-SS patients, and then referred to the involvement of oxidative stress in the pathogenesis of SS[12]. Moreover, Kageyama, *et al* reported that urinary HEL levels were significantly reduced by the administration of etanercept, a tumor necrosis factor-α (TNF-α) inhibitor, along with the improvement of other clinical and laboratory measures including C-reactive protein, swollen joint number, and tender joint number in patients with RA[11]. TNF-α was reported to cause the generation of reactive oxygen species, which played an essential role in the proliferative synovitis and joint destruction of RA[11]. Kageyama, *et al* thought that the improvement of oxidative stress presented by HEL and other oxidative markers might lead to the improvement of RA. Thus, the measurement of HEL levels not only in serum but also in urine and other tissues was thought to be important for consideration of the involvement of oxidative stress in autoimmune diseases.

On the other hand, SSc patients with elevated serum HEL levels had significantly elevated serum levels of IgM ($p < 0.05$) compared to those with normal HEL levels. Serum HEL levels correlated positively with RF titer ($p = 0.0028$, $r = 0.426$) in them, and RF was of the IgM class in our study. There are some reports showing that oxidative stress could cause damage of IgG and lead to the production of IgM[20]. Thus, it was considered that oxidative stress induced in SSc could cause the damage of Ig and that the activity of Ig became less effective. Therefore, negative feedback mechanisms induced by ineffective Ig activity might stimulate production of Ig, leading to hyper-γ-globulinemia that is one of the immunologic abnormalities in SSc.

Newkirk, *et al* reported that advanced glycation endproduct (AGE)-damaged IgG occurred as a result of oxidative stress and that IgM anti-AGE-damaged IgG autoantibodies were detected in patients with early synovitis. These antibodies were highly significantly associated with RF[21]. RF is sometimes detected as a less specific autoantibody in serum samples from patients with SSc and serum HEL levels correlated with RF titer in patients with SSc in our study. Therefore, it was considered that a similar mechanism could occur in the production of autoantibodies in patients with SSc. Anti-agalactosyl IgG antibody is among the autoantibodies detected in sera from patients with SSc as well as those with RA[22,23]. Agalactosyl IgG is a glycoform of IgG that is found as a proportion of total IgG in all normal individuals. The Fc portion of IgG is the target of RF that is very frequently detected in patients with RA. RF is shown to bind better to agalactosyl IgG than to galactosylated IgG. The present association of elevated serum HEL levels with anti-agalactosyl IgG antibody suggests that oxidative injury may play a role in production of this autoantibody. It has been hypothesized that immune responses to autoantigens are induced by cryptic self-epitopes that are generated by modification of the self-antigens (for example, novel cleavage, altered conformation, or tertiary structure)[24]. 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[25]. Therefore, the production of anti-agalactosyl IgG antibody may be related to the modification of IgG by oxidative injury.

Serum HEL levels were increased in patients with SSc as compared to healthy controls and showed a positive correlation with RF and anti-agalactosyl IgG antibody in patients with SSc in our study. These results led us to the possibility that oxidative stress may be involved in immunologic abnormalities in SSc. The measure of serum HEL can be considered a useful marker for evaluation of oxidative stress, and more detailed and sensitive examination will become possible by adding the measure of serum HEL to routine examinations.

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