Clinical significance of serum HMGB-1 and sRAGE levels in systemic sclerosis:

association with disease severity

Ayumi Yoshizaki[1], Kazuhiro Komura[1], Yohei Iwata[1], Fumihide Ogawa[1], Toshihide Hara[1], Eiji Muroi[1], Motoi Takenaka[1], Kazuhiro Shimizu[1], Minoru Hasegawa[2], Manabu Fujimoto[2], and Shinichi Sato[1]

[1]Department of Dermatology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan, and [2]Department of Dermatology, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan

Address for correspondence and reprints requests: Dr. Shinichi Sato, Department of Dermatology, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki, 852-8501, Japan.

Phone: 81-95-819-7333

Fax: 81-95-849-7335

E-mail: s-sato@nagasaki-u.ac.jp

Running title: Serum levels of HMGB-1 and sRAGE in SSc

Abstract

The high mobility group box 1 protein (HMGB-1)/adcanced glycation end products (RAGE) system is recently shown to play an important part in immune/inflammatory disorders. However, the association of this system in systemic sclerosis (SSc) remains unknown. To determine clinical association of serum levels of HMGB-1 and soluble RAGE (sRAGE) in patients with SSc, sera from 70 patients with SSc and 25 healthy controls were examined by enzyme-linked immunosorbent assay. Sera from Tight-skin mice and bleomycin-induced scleroderma mice, animal models for SSc, were also examined. Skin HMGB-1 and RAGE expression was assessed by immunohistochemistry. Serum HMGB-1 and sRAGE levels in SSc were higher than those in controls. Similarly, HMGB-1 and sRAGE levels in animal SSc models were higher than those in control mice. SSc patients with elevated HMGB-1 and sRAGE levels had more frequent involvement of several organs and immunological abnormalities compared to those with normal levels. Furthermore, HMGB-1 and sRAGE levels correlated positively with modified Rodnan total skin thickness score and negatively with pulmonary function test. HMGB-1 and sRAGE expression in the sclerotic skin was more intense than normal skin. These results suggest that elevated serum HMGB-1 and sRAGE levels are associated with the disease severity and

immunological abnormalities in SSc.

Key words; HMGB-1, RAGE, autoimmune disease, systemic sclerosis, toll-like

receptor

Introduction

Systemic sclerosis (SSc) is a multisystem disorder of connective tissue characterized by excessive accumulation of extracellular matrix in the skin and various internal organs [1]. SSc is accompanied by various immunological abnormalities, including autoantibody production, hyper-γ-globulinemia, and elevated levels of erythrocyte sedimentation rates (ESR), C-reactive protein (CRP), and several cytokines, such as tumor-necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 [2, 3]. Although these abnormalities were associated with several organ involvement and systemic vascular damage, the mechanism and pathogenesis of SSc remain unknown [4]. Recent studies have shown that damaged, necrotic, and apoptotic cells release high mobility group box 1 protein (HMGB-1) [5, 6]. HMGB-1 is a non-histone nuclear protein with dual function. Inside the cells, HMGB-1 is a nuclear constituent loosely bound to chromatin and plays a role in transcriptional regulation, and outside the cells, HMGB-1 serves as an inflammatory cytokine [7, 8]. HMGB-1 released from damaged, necrotic, and apoptotic cells binds to receptor for advanced glycation end products (RAGE), toll-like receptor (TLR)2, TLR4, and also TLR9. HMGB-1 system induces the nuclear factor-κB (NFκB) phosphorylation and productions of several cytokines and chemokines, such as TNF-α, IL-1β, IL-6, macrophage inflammatory protein-1α, and

transforming growth factor-β by endothelial cells, fibroblasts, and various immune cells, such as macrophages, monocytes, T cells, and B cells [5, 6, 9-12]. RAGE, the first receptor that was identified for HMGB-1, is encoded in the MHC class III region, together with the genes encoding the receptors for several complement components, as well as the genes encoding TNF, lymphotoxin, and heat-shock protein 70 [9]. RAGE can be expressed as both a transmembrane molecule, which directly interacts with extracellular-signal-regulated kinases 1 and 2 and drives activation of the mitogen-activated protein kinase p38 and NFκB, and as a soluble molecule [9, 13]. Soluble RAGE (sRAGE) is produced by alternative splicing of RAGE mRNA [14-16]. In addition, it has also been shown that pericytes, endothelial cells, and macrophages produce and release sRAGE extracellularly, suggesting the presence of a negative feedback mechanism in RAGE signaling [9, 15, 17].

HMGB-1 and RAGE are known to be causally involved in a variety of pathophysiological processes, such as rheumatoid arthritis, acute lung injury, disseminated intravascular coagulation, Alzheimer disease, tumorigenesis, abnormalities associated with diabetes, and impaired wound healing [8, 17-22]. Although HMGB-1 is regarded as a trigger of these immune/inflammatory disorders mediated via RAGE and TLR and also affects immunological abnormalities [5, 6, 9, 12, 23], correlation of serum

HMGB-1 and sRAGE levels with immunological parameters and each organ involvement has not been investigated. Therefore, we investigated clinical correlation of serum HMGB-1 and sRAGE levels with immunological parameters, the extent of skin fibrosis and vascular damage, and the presence of various organ involvements in 70 Japanese patients with SSc. Furthermore, we showed HMGB-1 and sRAGE levels in tight-skin (TSK) mice and bleomycin-induced scleroderma models, which are animal models for SSc [24, 25].

Methods

Serum samples from patients

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 [26]. Patients were grouped according to the classification system proposed by LeRoy et al. [27]: 31 patients (28 women and 3 men) had limited cutaneous SSc (lSSc) and 39 patients (33 women and 6 men) had diffuse cutaneous SSc (dSSc). The age of patients (mean ± SD) was 49 ± 15 years. Patients with dSSc were aged 48 ± 17, while those with lSSc were 53 ± 12 years old. The disease duration of patients with lSSc and dSSc was 9.4 ± 9.7 and 2.6 ± 2.5 years, respectively. None of SSc patients was treated with oral corticosteroid, D-penicillamine, or other immunosuppressive therapy at the evaluation. Antinuclear antibody (Ab) was determined by indirect immunofluorescence using HEp-2 cells as the substrate, and specificities of autoantibody were further assessed by enzyme-linked immunosorbent assay (ELISA) and immunoprecipitation. Anti-topoisomerase I Ab for 29 (24 dSSc and 5 lSSc), anti-centromere Ab was positive for 26 patients (3 dSSc and 23 lSSc), anti-U1RNP Ab for 2 (all lSSc), anti-U3RNP Ab for 1 (dSSc), anti-RNA polymerases I and III Ab for 7 (all dSSc), and Th/To Ab for 1 (lSSc). The remaining 4 patients were

negative for autoantibodies. Twenty-five age- and sex-matched healthy Japanese individuals were used as normal controls. Fresh venous blood samples were centrifuged shortly after clot formation. All samples were stored at -70°C prior to use.

Serum samples from mice

Heterozygous TSK (TSK/+) mice, bleomycin and phosphate-buffer saline (PBS)-treated C57BL/6 mice, and non-treated C57BL/6 mice were used in this study. TSK mice and C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). TSK mice were on C57BL/6 background. Bleomycin (Nippon Kayaku Co. Ltd., Tokyo, Japan) was dissolved in PBS at a concentration, and 300 μg of bleomycin was injected subcutaneously into the shaved back of the mice daily for 4 weeks, as described previously [24]. All mice were housed in a specific pathogen-free barrier facility and screened regularly for pathogens. Serum samples were obtained from 12 TSK mice, 8 bleomycin-induced scleroderma mice, 6 PBS-treated C57BL/6 mice, and 6 non-treated C57BL/6 mice at the age of 10 weeks by a cardiac puncture and stored at -80°C prior to use. All studies and procedures were approved by Committee on Animal Experimentation of Nagasaki University Graduate School of Medical Science.

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 [28]: 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 [29]: 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, or 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 color-flow Doppler ultrasonography of the renal interlobar arteries of both kidneys [30]. The protocol was approved by Nagasaki and Kanazawa University Hospitals and informed consent was obtained from all patients.

Immunohistochemical staining for HMGB-1 and RAGE

Immunohistochemistry for HMGB-1 and RAGE was performed as previously described [31, 32]. Formalin-fixed and paraffin-embedded tissues were obtained from 10 SSc patients (5 dSSc and 5 lSSc) and 5 normal controls. Deparaffinized sections were incubated at 4ºC with a primary monoclonal Ab to human HMGB-1 (3 µg/ml; Abnova Corp., Taipei, Taiwan) or RAGE (2.5 µg/ml; Chemicon Inc., Temecula, CA) after blocking tissue peroxydase activity and non-specific binding. The slides were sequentially incubated with a horseradish peroxidase (HRP)-conjugated goat anti-mouse and mouse anti-goat immunoglobulin G Ab (0.4 µg/ml; Santa Cruz Biotechnology Inc., Santa Cruz, CA), respectively, and the reaction products were visualized using diaminobenzidine (DAKO Inc., carpinteria, CA) with methyl green as a counterstain. Primary Abs preabsorbed with excess recombinant peptides (R&D systems) were used

as negative controls.

ELISA for serum HMGB-1 and sRAGE levels

ELISA for serum HMGB-1 and serum sRAGE levels was performed as previously described [20, 21, 33] using a specific ELISA kit (Shino-Test Co., Sagamihara, Kanagawa, Japan. for HMGB-1 and R&D systems, Minneapolis, MN for sRAGE), according to the manufacturer's protocol. Each sample was tested in duplicate. The detection limit of this assay was 2.5 ng/ml for HMGB-1 and 78 pg/ml for sRAGE.

ELISA for serum TNF-α, IL-1β, and IL-6 levels

Serum levels of TNF-α (normal; 0-16 pg/ml), IL-1β (normal; 0-5 pg/ml) , and IL-6 (normal; 5-15 pg/ml) were also assessed by specific ELISA kits (R&D systems) as previously described [34].

Statistical analysis

Statistical analysis was performed using Mann-Whitney U-test for determining the level of significance of differences between sample means, 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.

Results

Serum HMGB-1 and sRAGE levels by ELISA

HMGB-1 and sRAGE levels in serum samples from SSc patients and normal controls were assessed by ELISA (Fig. 1a, b). Serum HMGB-1 and sRAGE levels in lSSc and dSSc patients were significantly higher than those found in normal controls (HMGB-1; $p < 0.05$ and $p < 0.01$, sRAGE; $p < 0.05$ and $p < 0.01$, respectively). Serum HMGB-1 and sRAGE levels in dSSc patients were significantly elevated relative to lSSc patients ($p < 0.01$ and $p < 0.05$, respectively). Furthermore, HMGB-1 levels correlated positively with sRAGE levels ($r = 0.472$, $p < 0.001$; Fig. 1c). In this assay system, the cut off value was set at 8.1 ng/ml for HMGB-1 and 364.9 pg/ml for sRAGE, which was calculated as the mean + 2 SD for normal controls (Fig. 1a, b). In total patients with SSc, serum HMGB-1 and sRAGE levels were elevated in 47% (33/70) and 34% (24/70), respectively. Serum HMGB-1 and sRAGE levels were increased in 29% (9/31) and 16% (5/31) of lSSc patients, respectively, and the frequency of serum elevated levels was significantly higher in dSSc patients (62%, 24/39, $p < 0.01$ for HMGB-1 and 49%, 19/39, $p < 0.01$ for sRAGE) relative to lSSc patients. Thus, serum HMGB-1 and sRAGE levels were elevated in SSc patients, especially dSSc patients.

HMGB-1 and RAGE expression in the fibrotic skin

HMGB-1 expression was only faintly detected in the nucleus of normal control fibroblasts (Fig. 2a). In contrast, SSc patients had higher nuclear and cytoplasmic expression of HMGB-1 in fibroblasts compared with normal controls (Fig. 2b). In addition, RAGE expression was only faintly detected in the cytoplasm and cell membrane of fibroblasts from normal controls (Fig. 2c). Remarkably, SSc patients had higher cytoplasmic and cell membrane expression of RAGE in fibroblasts compared with normal controls (Fig. 2d). HMGB-1 and RAGE expression in sclerotic skin of lSSc patients was similar to that of dSSc patients (data not shown). Thus, HMGB-1 and RAGE expression was up-regulated in the fibrotic skin from SSc patients.

Clinical correlation

Then, we assessed clinical correlation of serum HMGB-1 and sRAGE levels in SSc patients (Table 1). SSc patients with serum elevated HMGB-1 and sRAGE levels had significantly higher modified Rodnan TSS points ($p < 0.05$ and $p < 0.01$), higher frequency of dSSc ($p < 0.01$ and $p < 0.01$), decreased %VC ($p < 0.05$ and $p < 0.05$), and decreased %DLco ($p < 0.01$ and $p < 0.05$), and more frequent involvement of nailfold bleeding ($p < 0.05$ and $p < 0.05$), pitting scar/ulcer ($p < 0.05$ and $p < 0.05$), pulmonary

fibrosis (p<0.01 and p<0.01), heart (p<0.05 and p<0.05), kidneys (p<0.05 and p<0.05), and joints (p<0.05 and p<0.05) than those with normal levels. Consistent with these findings, serum HMGB-1 and sRAGE levels were significantly elevated in SSc patients with nailfold bleeding (p<0.05 and p<0.01), pitting scar/ulcer (p<0.01 and p<0.05), arthritis/arthralgias (p<0.01 and p<0.05), or pulmonary fibrosis (p<0.01 and p<0.01) than those without each clinical parameter (Fig. 3). Serum HMGB-1 and sRAGE levels also correlated inversely with %VC (r=-0.264, p<0.05 and r=-0.293, p<0.01) or %DLco (r=-0.414, p<0.001 and r=-0.293, p<0.001; Fig. 4). Furthermore, serum HMGB-1 and sRAGE levels correlated positively with modified Rodnan TSS (r=0.481, p<0.001 and r=0.480, p<0.001) and renal vascular resistance (r=0.276, p<0.05 and r=0.380, p<0.05), which was determined as the pulsatility index value in the renal interlobular arteries by color-flow Doppler scans [30]. However, serum HMGB-1 and sRAGE levels did not correlate with any other clinical parameters, including the disease duration. In addition, HMGB-1 and sRAGE levels were increased in both early dSSc patients with disease duration of 2 years or less (11.5 ± 9.4 ng/ml, p<0.01 and 329.6 ± 131.4 pg/ml, p<0.01) and late dSSc patients (20.0 ± 13.9 ng/ml, p<0.001 and 372.7 ± 141.7 pg/ml, p<0.001) relative to normal controls; however, there was no significant difference in HMGB-1 and sRAGE levels between early dSSc and late dSSc patients.

Regarding correlation of serum HMGB-1 and sRAGE levels with immunological parameters, SSc patients with elevated HMGB-1 and sRAGE levels had significantly higher frequency of elevated levels of serum IgG ($p < 0.05$ and $p < 0.05$), CRP ($p < 0.01$ and $p < 0.05$), and ESR ($p < 0.01$ and $p < 0.05$) and more frequent presence of anti-topoisomerase I Ab ($p < 0.05$ and $p < 0.05$) compared with those with normal levels (Table 1). Moreover, serum HMGB-1 and sRAGE levels correlated positively with levels of serum IgG ($r = 0.304$, $p < 0.01$ and $r = 0.243$, $p < 0.05$; Fig. 4). Regarding the correlation of serum levels of HMGB-1 and sRAGE with those of TNF-α, IL-1β, and IL-6, there was no significant difference in serum TNF-α, IL-1β, and IL-6 levels between SSc patients with elevated serum HMGB-1 levels and those with normal levels (Table 1). Similarly, these cytokine levels did not significantly differ between SSc patients with elevated serum sRAGE levels and those with normal levels. Furthermore, serum HMGB-1 and sRAGE levels did not correlate with serum levels of TNF-α ($r = 0.106$, $p = 0.096$ and $r = 0.117$, $p = 0.089$, respectively), IL-1β ($r = 0.097$, $p = 0.152$ and $r = 0.108$, $p = 0.091$), and IL-6 ($r = 0.086$, $p = 0.184$ and $r = 0.128$, $p = 0.082$). Thus, elevation of serum HMGB-1 and sRAGE levels was associated with the severity of skin and pulmonary fibrosis, many other organ involvements, and various immunological abnormalities in SSc.

Serum HMGB-1 and sRAGE levels in SSc mouse models by ELISA

TSK mice spontaneously develop cutaneous fibrosis and autoantibody production resembling human SSc [25]. Recently, Yamamoto et al. have established a new mouse model of SSc using bleomycin treatment: the subcutaneous injection of bleomycin induces fibrosis in the dermis and lung, autoantibody production, and dermal inflammatory infiltration, which more closely mimics the features of human SSc than TSK mice [24, 35]. HMGB-1 and sRAGE levels in serum samples from 12 TSK mice, 8 bleomycin-induced scleroderma mice, 6 PBS-treated C57BL/6 mice, and 6 non-treated C57BL/6 mice were assessed by ELISA (Fig. 5). TSK mice exhibited significantly higher HMGB-1 and sRAGE levels than those in non-treated C57BL/6 mice as normal controls of TSK mice (421% and 243% of non-treated C57BL/6 mice, respectively; $p<0.001$). Similarly, bleomycin-induced scleroderma mice had significantly higher HMGB-1 and sRAGE levels than those in PBS-treated C57BL/6 mice as normal controls of bleomycin-induced scleroderma mice (633% and 347% of PBS-treated C57BL/6 mice, respectively; $p<0.001$). In addition, bleomycin-induced scleroderma mice showed significantly higher HMGB-1 and sRAGE levels than those in TSK mice (158% and 143% of TSK mice, respectively; $p<0.05$). PBS-treated C57BL/6 mice

exhibited similar levels of HMGB-1 and sRAGE to those in non-treated C57BL/6 mice.

Thus, serum HMGB-1 and sRAGE levels were significantly higher in TSK and

bleomycin-induced scleroderma mice than those in normal controls.

Discussion

The present study is the first to reveal that HMGB-1 and sRAGE levels were elevated in serum samples from SSc patients relative to normal controls. We also showed that there was positive correlation between HMGB-1 and sRAGE in SSc patients and that HMGB-1 and sRAGE levels correlated positively with modified Rodnan TSS. Furthermore, elevation of HMGB-1 and sRAGE levels was accompanied by the presence of pulmonary fibrosis and decreased %VC and %DLco, indicating that HMGB-1 and sRAGE levels correlated with the severity of lung fibrosis. Remarkably, increased HMGB-1 and sRAGE levels were also associated with involvement of many other organs, such as heart, kidneys, and joints. Finally, HMGB-1 and sRAGE levels correlated with renal vascular resistance and the presence of nailfold bleeding and pitting scar/ulcer, suggesting that they reflect vascular damage in SSc. Serum HMGB-1 and sRAGE levels increased in both early-stage and late-stage SSc patients; furthermore, there was no significant difference in HMGB-1 and sRAGE levels between early-stage and late-stage SSc patients. Collectively, these results suggest that HMGB-1 and sRAGE level is a useful serological marker for evaluating the disease severity rather than disease activity.

In this study, serum HMGB-1 and sRAGE levels correlated with various

immunological parameters, including the presence of anti-topoisomerase I Ab and serum levels of IgG, CRP, and ESR. Recent studies have demonstrated that HMGB-1 may contribute to progression of the inflammatory response in several immune/inflammatory disorders [8, 17-22]. HMGB-1 is actively secreted by damaged, necrotic, and apoptotic cells after translocation from the nucleus to secretory lysosomes [5, 6]. In this study, immunohistochemical staining revealed that HMGB-1 over-expression was detected in SSc fibrotic skin compared with normal skin. Extracellular HMGB-1 exerts proinflammatory effects, at least in part, through interaction with its receptor RAGE [36]. Previous studies revealed that RAGE expression was increased in immune/inflammatory disorders. Our results also showed that stronger expression of RAGE was detected in SSc fibrotic skin than in normal skin. Moreover, engagement of RAGE by its ligands results in a sustained NFκB and various inflammatory gene activation in all cell types studied thus far, such as endotheliums, fibroblasts, monocytes, macrophages, and lymphocytes [5, 6, 9-12], and RAGE expression itself is controlled by NFκB [23, 37]. Furthermore, the finding of Yamagishi et al. suggest that circulating sRAGE levels may reflect tissue RAGE expression and may be elevated in parallel with RAGE ligands, such as HMGB-1, as a counter-system against RAGE ligand-elicited tissue damage [23, 38]. Actually, in our present study,

there was significant correlation between HMGB-1 and sRAGE. HMGB-1 is also an

endogenous ligand for TLR2, 4, and 9 that are also expressed by various immune cells,

such as macrophages, B cells, and T cells [12, 39]. Stimulation through TLR2, 4, and 9

induces B cell differentiation to immunoglobulin-secreting plasma cells and enhances

the production of cytokines, including TNF-α, IL-1β, and IL-6 [12, 40], which may

explain serum IgG, CRP, and ESR elevation and higher prevalence of

anti-topoisomerase I Ab in SSc patients with elevated HMGB-1 and sRAGE levels.

Thus, HMGB-1 and sRAGE may play a role in immunological abnormalities associated

with SSc.

HMGB-1 can act as a proinflammatory molecule and endogenous ligand for

TLRs and thereby can induce several inflammatory reactions, including cytokine

production [12, 39, 40]. Moreover, co-expression of HMGB-1, TNF-α, IL-1β, and IL-6

has been reported in several autoimmune diseases, such as systemic lupus

erythematosus and Sjögren's syndrome [41, 42]. However, the association of serum

levels of HMGB-1 and those of several cytokines, including TNF-α, IL-1β, and IL-6 has

been controversial. In chronic kidney disease, serum HMGB-1 levels positively

correlated with TNF-α, IL-1β, and IL-6 [43]. Consistent with this, HMGB-1 induced

time-dependent elevation of TNF-α, IL-1β, and IL-6 in mice [44]. In contrast, serum

HMGB-1 levels did not correlate with serum levels of TNF-α, IL-1β, and IL-6 in severe sepsis patients [45]. In addition, HMGB-1 expression was not consistently influenced by TNF-α blocking therapy in rheumatoid synovitis patients [46]. Although the reasons for this discrepancy are unknown, this may be due to difference in disease mechanism, disease cytokine profiles, and experimental conditions between studies. Our results suggest that serum HMGB-1 and sRAGE levels may serve as a possible TNF-α, IL-1β, and IL-6-independent serological marker for disease severity of SSc.

Serum HMGB-1 and sRAGE elevation was also detected in TSK and bleomycin-induced scleroderma mice, animal models for SSc. TSK mice have a spontaneous mutation within the gene encoding fibrillin 1, an extracellular matrix glycoprotein crucial for microfibril assembly, which results in increased synthesis and excessive accumulation of collagen and other extracellular matrix proteins in the skin [47]. TSK mice exhibit not only skin fibrosis but also autoantibody production [48]. In bleomycin-induced scleroderma mice, bleomycin induces fibrosis in the dermis and lung, autoantibody production, and dermal inflammatory infiltration, which closely mimics the features of human SSc [24, 35]. In these SSc model mice, elevated HMGB-1 and sRAGE may reflect immunological abnormalities and may contribute to fibrosing process. In this study, bleomycin-induced scleroderma mice had elevated serum levels

of HMGB-1 and sRAGE compared with TSK mice. This phenomenon may be related to

enhanced reactive oxygen species production induced by bleomycin that could initiate

more intense tissue damage, leading to greater generation of HMGB-1 that induces

activation of macrophage, T cell, and B cell. Then sRAGE levels were elevated as a

counter-system molecule [49]. Collectively, these results suggest that HMGB-1 and

sRAGE levels are related to skin fibrosing process.

Recently, increasing serum HMGB-1 levels have been reported in several

immune/inflammatory disorders [8, 17-22]. However, it has been controversial whether

sRAGE increases or decreases in immune/inflammatory conditions. In rheumatoid

arthritis, atherosclerosis and retinopathy with diabetes mellitus, and Alzheimer disease,

sRAGE levels inversely correlate with disease severity [22, 50, 51]. On the other hand,

one study showed that sRAGE levels positively correlated with monocyte chemotactic

protein-1 and TNF-α, suggesting that sRAGE level may become a biomarker of

vascular inflammation in type 2 diabetec patients [52]. In addition, Bopp et al. reported

that nonsurvivors had higher sRAGE levels than survivors with septic patients,

suggesting that sRAGE is related to the inflammatory severity [23]. Although the

reasons for this discrepancy are unknown, this may be due to difference in disease

pathophysiology, disease duration, patient population, and the assay system used to

detect serum sRAGE. Our results suggest that serum sRAGE levels reflect the disease severity in SSc, like HMGB-1. However, to clarify clinical significance of HMGB-1 and sRAGE levels in SSc, prospective studies with larger numbers of SSc patients will be needed.

Enhanced production of reactive oxygen species, probably due to vascular ischemia and reperfusion injury following Raynaud's phenomenon [53, 54], may increase HMGB-1, leading to production of sRAGE as a counter-system against HMGB-1/RAGE-induced inflammatory responses [23, 38]. Consistent with this, in this study, HMGB-1 and sRAGE levels were associated with vascular damage, such as renal vascular damage, nailfold bleeding, and pitting scar/ulcer. In conclusion, HMGB-1 and sRAGE may be a key molecule and serological marker that links 3 main SSc features of immunological abnormalities, fibrosis, and vascular damage.

Acknowledgement

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Table

Table 1. Clinical and loboratory features of SSc patients with elevated serum HMGB-1

and sRAGE levels

	Elevated HMGB-1 (n=33)	Normal HMGB-1 (n=37)	Elevated sRAGE (n=24)	Normal sRAGE (n=46)
Sex, number of males/females	5/28	4/33	5/19	4/42
Age at onset, mean ± S.D. yrs	49 ± 15	42 ± 18	47 ± 15	44 ± 17
Disease duration, mean ± S.D. yrs	5 ± 7	6 ± 8	5 ± 6	6 ± 8
Disease pattern, number with dSSc/lSSc	24/9**	15/22	19/5**	20/26
Clinical features				
Modified Rodnan TSS, mean ± S.D. points	17 ± 12*	11 ± 7	20 ± 12**	10 ± 7
Nailfold bleeding	85*	65	88*	67
Pitting scar/ulcer	55*	28	58*	31
Diffuse pigmentation	67*	41	67	46
Contracture of phalanges	55	38	61	38
Organ involvement				
Lungs				
Pulmonary fibrosis	58**	27	67**	28
Decreased %VC	45*	21	55*	21
Decreased %DLco	90**	74	86*	79
Pulmonary hypertension	28	22	33	21
Esophagus	55	50	43	57
Heart	24*	8	29*	9
Kidneys	26*	3	26*	7
Joints	33*	11	33*	13
Muscles	21	16	21	17
Laboratory findings				
Positive for anti-topoisomerase I Ab	55*	32	58*	33
Positive for anti-centromere Ab	30	46	25	48
Serum IgG, mean ± S.D. mg/dl	1884 ± 671*	1482 ± 231	1888 ± 627*	1576 ± 401
Serum IgA, mean ± S.D. mg/dl	348 ± 184	300 ± 103	368 ± 194	299 ± 111
Serum IgM, mean ± S.D. mg/dl	188 ± 143	193 ± 75	201 ± 147	190 ± 101
CRP, mean ± S.D. mg/dl	0.40 ± 0.53**	0.23 ± 0.66	0.40 ± 0.41*	0.32 ± 0.69

ESR, mean ± S.D. mm/h	24.1 ± 16.5**	12.7 ± 10.3	22.0 ± 14.1*	13.3 ± 8.9
Serum TNF-α, mean ± S.D. pg/ml	13.7 ± 4.8	11.3 ± 5.6	13.4 ± 6.4	13.1 ± 5.2
Serum IL-1β, mean ± S.D. pg/ml	37.8 ± 15.2	36.4 ± 18.5	38.2 ± 17.4	39.4 ± 14.8
Serum IL-6, mean ± S.D. pg/ml	168.4 ± 119.7	169.6 ± 117.1	159.2 ± 121.4	164.8 ± 105.9

Unless noted otherwise, values are percentages.

*p<0.05, **p<0.01 vs. SSc patients with normal HMGB-1 and sRAGE levels.

Figure legends

Figure 1. HMGB-1 (a) and sRAGE (b) levels in serum samples from patients with lSSc, those with dSSc, and healthy controls (CTL). The short bar indicates the mean value in each group; the broken line indicates the mean + 2 SD levels of healthy controls. (c) The correlation of HMGB-1 and sRAGE in SSc patients. Serum HMGB-1 and sRAGE levels were determined by ELISA.

Figure 2. Representative HMGB-1 and RAGE expression in the normal skin and sclerotic skin from a SSc patient (a; HMGB-1 in normal skin, b; HMGB-1 in sclerotic skin, c; RAGE in normal skin, and d; RAGE in sclerotic skin). HMGB-1 and RAGE expression was shown by immunohistochemical staining (diaminobenzidine staining, original magnification; x100).

Figure 3. Serum HMGB-1 and sRAGE levels in the presence and absence of nailfold bleeding, pitting scar/ulcer, arthritis/arthralgia, and pulmonary fibrosis in SSc patients. Serum HMGB-1 and sRAGE levels were determined by ELISA.

Figure 4. The correlation of serum HMGB-1 and sRAGE levels with modified Rodnan

TSS, %VC, %DLco, the pulsatility index (PI) value, and serum levels of IgG in patients

with SSc. The PI value is a parameter for renal vascular resistance determined by

color-flow Doppler ultrasonography of the renal interlobar arteries of both kidneys.

Serum HMGB-1 and sRAGE levels were determined by ELISA.

Figure 5. Serum HMGB-1 and sRAGE levels in TSK, non-treated C57BL/6 (C57BL/6),

bleomycin-induced scleroderma (BLM), and PBS-treated C57BL/6 (PBS) mice. Each

histogram shows the mean (± SD) results obtained for each group.