Clinical and Experimental Rheumatology 2008; 26: 659-662.

Serum levels of heat shock protein 70, a biomarker of cellular stress, are elevated in patients with systemic sclerosis: association with fibrosis and vascular damage

F. Ogawa, K. Shimizu, T. Hara, E. Muroi, M. Hasegawa, K. Takehara*, S. Sato

*Department of Dermatology, Nagasaki University Graduate School of Biomedical Sciences and *Department of Dermatology, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan.*

Fumihide Ogawa, MD, PhD;
Kazuhiro Shimizu, MD, PhD;
Toshihide Hara, MD;
Eiji Muroi, MD;
Minoru Hasegawa, MD, PhD;
Kazuhiko Takehara, MD, PhD;
Shinichi Sato, MD, PhD.

Please address correspondence and reprints requests to:
Shinichi Sato, MD, PhD, Department of Dermatology, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan.
E-mail: s-sato@nagasaki-u.ac.jp

Received on June 7, 2007; accepted in revised form on January 22, 2008.

Key words: Systemic sclerosis, heat shock protein 70, oxidative stress, fibrosis, vascular damage.

Competing interests: none declared.

ABSTRACT

Objective. *To determine the clinical significance of heat shock protein (Hsp) 70, a sensitive biomarker for monitoring cellular stress, in systemic sclerosis (SSc), we investigated the prevalence and clinical correlation of serum Hsp70 levels in SSc patients.*

Methods. *Serum Hsp70 levels were examined in 48 patients with SSc by enzyme-linked immunosorbent assay.*

Result. *Serum Hsp70 levels were significantly elevated in SSc patients compared to normal controls (n=30), and were similar between patients with diffuse cutaneous SSc (n=26) and those with limited cutaneous SSc (n=22). Serum Hsp70 levels were elevated in 27% of total SSc patients with 30% of diffuse cutaneous SSc patients and 23% of limited cutaneous SSc patients. Hsp70 levels were significantly increased in SSc patients with pulmonary fibrosis or contracture of phalanges compared with those without pulmonary fibrosis or contracture of phalanges. Serum Hsp70 levels correlated positively with modified Rodnan total skin thickness score, renal vascular resistance, serum levels of monocyte chemotactic protein-1, C-reacting protein, and serum levels of 8-isoprostane.*

Conclusion. *Serum Hsp70 levels were increased in SSc patients and were associated with pulmonary fibrosis, skin sclerosis, renal vascular damage, oxidative stress, and inflammation. These results suggest that Hsp70 is a useful serological marker for evaluating cellular stresses and the disease severity in SSc.*

Introduction

Systemic sclerosis (SSc) is a connective tissue disorder characterized by fibrosis, vascular changes in the skin and other visceral organs, with autoimmune background. SSc patients exhibit notable evidence of oxidative stress, shown by abnormalities of nitric oxide (NO), nitric oxide synthase, and increased levels of other biomarkers including 8-isoprostane that indicate excess oxidative stress (1,2). Oxidative stress has been suggested to contribute to clinical manifestations associated with SSc, such as vascular damage, fibrosis, and autoantibody production (3, 4)

Heat shock proteins (Hsps) are a family of highly conserved proteins found in all organisms cells and function as molecular chaperons facilitating protein folding, assembly, and intracellular transport (5). Their synthesis is increased greatly in response to a variety of stressful stimuli, such as hyperthermia, hypertension, hypoxia, ischemia-reperfusion injury, inflammation, and autoimmunity (5). Previous studies have shown that Hsp70 plays an important role in protecting against acute lung injury and oxidative stress, such as NO (6, 7). Thus, Hsp70 has been considered a sensitive biomarker for monitoring not only oxidative stress, but also other cellular stresses, including inflammation and tissue injury.

To evaluate stressful stimuli as mentioned above and its significance in SSc, we assessed serum Hsp70 levels and their clinical correlation in SSc patients. Hsp70 levels were also compared with one of the oxidative stress marker, 8-isoprostane, and with inflammation initiator, monocyte chemotactic protein-1 (MCP-1), in SSc.

Patients and Methods

Serum samples

Serum samples were obtained from all SSc patients who visited our scleroderma clinic over the last 7 years. They were 48 Japanese patients with SSc (41 females, 7 males; age 49.1±17.1 years) who fulfilled the criteria proposed by the American College of Rheumatology. They were grouped according to the classification system: 22 patients (20 females, 2 males; age 53.7±12.8 years) had limited cutaneous SSc (lSSc) and 26 patients (21 females, 5 males; age 45.3±19.4 years) had diffuse cutaneous SSc (dSSc). The disease duration of lSSc and dSSc patients was 10.3±10.1 and 3.1±3.1 years, respectively. None of the SSc patients was treated with oral steroid, D-penicillamine, or other immunosuppressive therapy at the evaluation. Anticentromere Ab was positive for 17 patients, antitopoisomerase I Ab for 21, anti- U1 RNP Ab for 2, anti-U3 RNP Ab for 1, anti-RNA polymerases I and III Ab for 4, and Th/To Ab for 1.

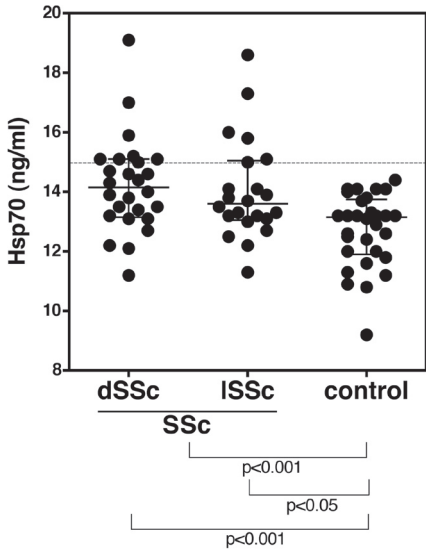

Fig. 1. Inducible Hsp70 levels in serum samples from patients with dSSc, those with lSSc, and normal controls. Inducible Hsp70 levels were determined by a specific ELISA. Horizontal lines show the median values and interquartile range. A broken line indicates the cut-off value (mean +2 SD of the control samples).

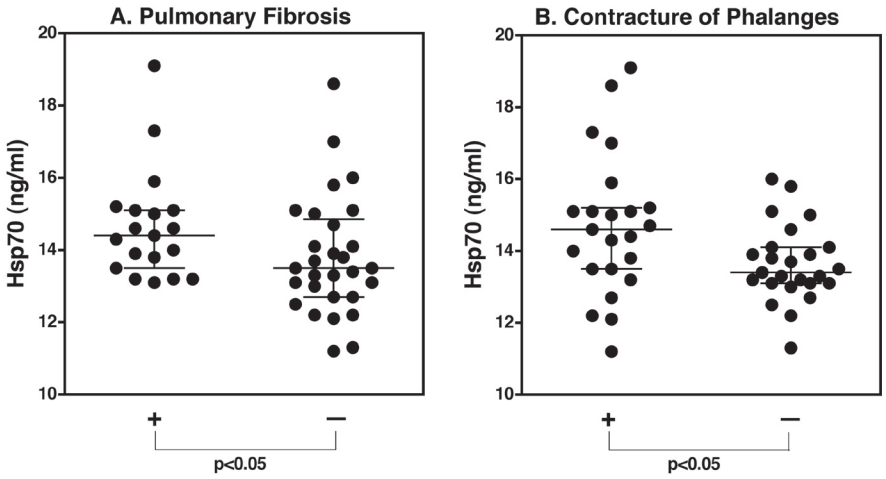

Fig. 2. Hsp70 levels in SSc patients in the presence and absence of pulmonary fibrosis (**A**) and contracture of phalanges (**B**). Inducible Hsp70 levels were determined by specific ELISA. Horizontal lines show the median values and interquatile range.

Thirty healthy Japanese people with similar age and gender (27 females, 3 males; age 44.6±11.3 years) to the patients were used as normal controls. Smokers were excluded from this study. Blood samples were centrifuged shortly after clot formation. All samples were stored at -80ºC prior to use.

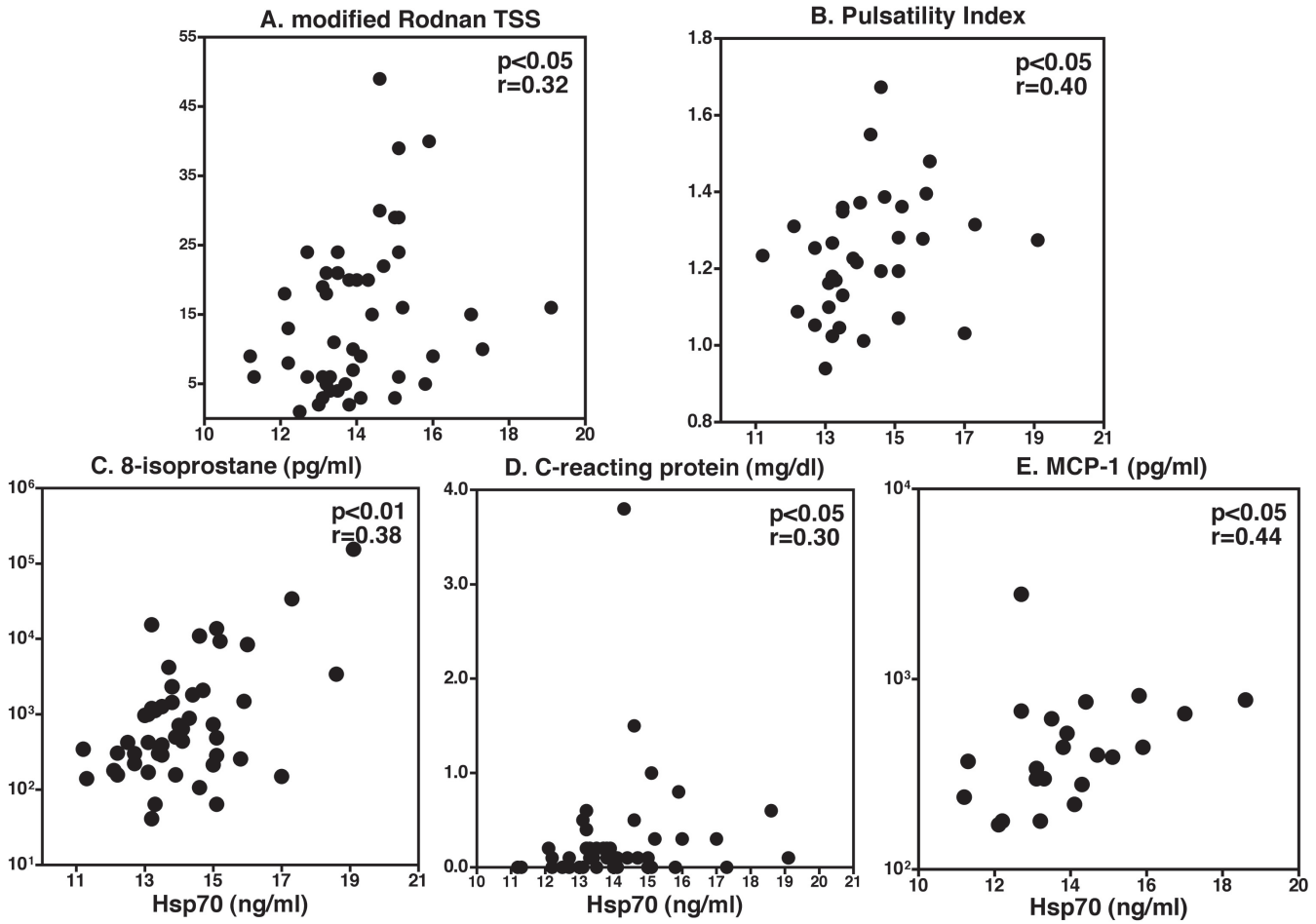

Fig. 3. The correlation of serum Hsp70 levels against modified Rodnan TSS (A), pulsatility index (PI; B), and serum levels of 8-isoprostane (**C**), MCP-1 (**D**), and C-reactive protein (**E**) in SSc patients. Serum levels of Hsp70, 8-isoprostane, and MCP-1 were determined by specific ELISA. The PI is a parameter for renal vascular resistance determined by color-flow Doppler ultrasonography of the renal interlobar arteries of both kidneys.

Table I. Clinical and laboratory data of patients with SSc showing elevated serum Hsp70 levels at the first evaluation. Values of clinical features and organ involvement are percentages.

	Elevated Hsp70 n=13	Normal Hsp70 n=35
Age at onset, yrs, mean ± SD	46 ± 14	43 ± 19
Sex, F:M	11:2	32:5
Duration, yrs, mean ± SD	4.4 ± 6.8	6.9 ± 8.0
Clinical features:		
Diffuse cutaneous SSc	62	54
Limited cutaneous SSc	38	46
Pitting scars	54	41
Short sublingual frenulum	69	47
Contracture of phalanges	69	41
Diffuse pigmentation	54	54
Organ involvement:		
Lung		
Pulmonary fibrosis	54	38
Decreased %VC	54*	23
Decreased %DLco	77	77
Esophagus	39	58
Heart	15	19
Kidney		
Increased vascular resistance	18	14
Renal crisis	8	3
Joint	31	22
Muscle	15	24
Autoantibodies:		
Anti-topoisomerase I antibody	46	46
Anticentromere antibody	15	41
Anti-U1RNP antibody	8	3

All the clinical and laboratory parameters and serum Hsp70 levels were obtained at the first evaluation. *$p<0.05$ *vs.* SSc patients with normal Hsp70 levels.

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). Renal vascular damage was determined as a pulsatility index (PI) by color flow Doppler ultrasonography of both kidneys (8). The protocol for the study was approved by local ethical committee of Kanazawa University School of medicine and Kanazawa University Hospital, and informed consents were obtained from all the patients according to the declaration of Helsinki.

Enzyme-linked immunosorbent assay (ELISA)

Serum Hsp70 (Stressgen, Victoria, Canada), 8-isoprostane (Cayman, MI, USA), and MCP-1 (Pharamingen, San Diego, CA, USA) levels were examined by a specific ELISA kit according to the manufacturer's protocol. Each sample was tested in duplicate.

Statistical analysis

Statistical analysis was performed using Mann-Whitney U-test for comparison of Hsp70 levels, Bonferroni's test for multiple comparisons, and Spearman's rank correlation coefficient for the relationship between two continuous variables. A p-value of less than 0.05 was considered statistically significant.

Results

Serum Hsp70 levels in SSc

Serum Hsp70 levels in SSc patients (mean±SD, 14.1±1.6 ng/ml) were significantly elevated compared with healthy controls (12.7±1.2, $p<0.001$; Fig. 1). Patients with dSSc (14.2±1.6) and lSSc (14.0±1.7) had significantly higher inducible Hsp70 levels than healthy controls ($p<0.001$, $p<0.05$, respectively). However, Hsp70 levels were similar between dSSc and lSSc patients. Values higher than mean + 2 SD of healthy control serum samples were considered elevated in this study. Increased Hsp70 levels were detected in 27% (13/49) of total SSc patients, with 30% (8/27) of dSSc patients and 23% (5/22) of lSSc patients. By contrast, none of the healthy controls showed elevated Hsp70 levels.

Clinical correlation of serum Hsp70 levels

Inducible Hsp70 levels in SSc patients with pulmonary fibrosis (n=19, 14.7±1.5) were significantly higher than in those without it (n=29, 13.8±1.6, $p<0.05$). Similarly, Hsp70 levels in SSc patients with contracture of phalanges (n=23, 14.7±2.0) were significantly increased than in those without it (n=25, 13.6±1.1, $p<0.05$; Fig. 2). Serum Hsp70 levels correlated positively with modified Rodnan TSS ($r=0.32$, $p<0.05$) and renal vascular resistance ($r=0.40$, $p<0.05$). However, Hsp70 levels were similar between SSc patients with digital pitting scar/ulcers and those without each complication. Serum Hsp70 levels correlated positively with serum level of 8-isoprostane ($r=0.38$, $p<0.01$), C-reacting protein ($r=0.30$, $p<0.05$), and MCP-1 ($r=0.44$, $p<0.05$; Fig. 3). The clinical characteristics of patients with elevated Hsp70 levels were described in Table I. Thus, elevated inducible Hsp70 levels correlated with the severity of pulmonary fibrosis, skin sclerosis, renal vascular damage, oxidative stress, and inflammation.

Discussion

The present study is the first to reveal that serum Hsp70 levels were significantly elevated in SSc patients, suggesting that SSc patients are subject to various cellular stresses. Up-regulated Hsp70 transcription levels are observed in fibroblasts derived from SSc patients (9) and its expression is increased by TGF-β stimulation (10).

Therefore, increased Hsp70 levels may be related to the enhanced TGF-β signaling in SSc. Furthermore, serum Hsp70 levels increased in SSc patients with pulmonary fibrosis or contracture of phalanges, and correlated positively with modified Rodnan TSS, renal vascular resistance, and inflammation markers such as C-reacting protein and MCP-1. These results suggest that serum Hsp70 level is a useful serologic marker of fibrotic process and vascular damage in SSc patients. MCP-1 is expressed in inflammatory mononuclear cells, endothelial cells, keratinocytes, and fibroblasts in the skin from earlier onset of SSc, leading to enhanced leukocyte migration into the affected tissues (11). Cellular stresses induced by MCP-1 might be related to up-regulation of Hsp70 expression in SSc, which may result in the positive correlation of serum Hsp70 levels with serum MCP-1 levels and the extent of skin fibrosis in this study.

We also demonstrated that serum Hsp70 levels positively correlated with serum levels of 8-isoprostane, a stable biomarker that closely reflects oxidative stress, in SSc patients. This suggests that enhanced levels of oxidative stress contribute to up-regulation of Hsp70 expression. Furthermore, the positive correlation of serum Hsp70 levels with renal vascular damage in this study also suggests that Hsp70 induction reflects oxidative stress, since ischemia and reperfusion injury following Raynaud's phenomenon can generate reactive oxygen species that may result in vascular endothelial damage (12). Therefore, serum Hsp70 level may be a serological marker for oxidative stress and renal vascular damage in SSc. Previous studies have shown the cyto-protective capacity of increased Hsp70 against toxic stimuli and ischemic-reperfusion injury (7, 13, 14). Since endothelial cells from SSc patients exhibit augmented apoptosis (15), serum Hsp70 levels may be elevated to protect apoptosis and ischemic-reperfusion injury in SSc.

In conclusion, although the functional significance of serum inducible Hsp70 remains unknown in this study, our results suggested that inducible Hsp70 is related to the excessive oxidative stress associated with SSc.

References

1. OGAWA F, SHIMIZU K, MUROI E *et al*.: Serum levels of 8-isoprostane, a marker of oxidative stress, are elevated in patients with systemic sclerosis. *Rheumatology* (Oxford) 2006; 45: 815-8.
2. ANDERSEN GN, CAIDAHL K, KAZZAM E *et al*.: Correlation between increased nitric oxide production and markers of endothelial activation in systemic sclerosis: findings with the soluble adhesion molecules E-selectin, intercellular adhesion molecule 1, and vascular cell adhesion molecule 1. *Arthritis Rheum* 2000; 43: 1085-93.
3. SAMBO P, BARONI SS, LUCHETTI M *et al*.: Oxidative stress in scleroderma: maintenance of scleroderma fibroblast phenotype by the constitutive up-regulation of reactive oxygen species generation through the NADPH oxidase complex pathway. *Arthritis Rheum* 2001; 44: 2653-64.
4. LUCZYNSKA M, SZKUDLAREK U, DZIANKOWSKA-BARTKOWIAK B *et al*.: Elevated whole blood chemiluminescence in patients with systemic sclerosis. *Clin Exp Rheumatol* 2005; 23: 173-9.
5. KIANG JG, TSOKOS GC: Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. *Pharmacol Ther* 1998; 80: 183-201.
6. GANTER MT, WARE LB, HOWARD M *et al*.: Extracellular heat shock protein 72 is a marker of the stress protein response in acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2006; 291: L354-61.
7. KIM HP, WANG X, ZHANG J *et al*.: Heat shock protein-70 mediates the cytoprotective effect of carbon monoxide: involvement of p38 beta MAPK and heat shock factor-1. *J Immunol* 2005; 175: 2622-9.
8. NISHIJIMA C, SATO S, HASEGAWA M *et al*.: Renal vascular damage in Japanese patients with systemic sclerosis. *Rheumatology* (Oxford) 2001; 40: 406-9.
9. DEGUCHI Y, SHIBATA N, KISHIMOTO S: Elevated transcription of heat shock protein gene in scleroderma fibroblasts. *Clin Exp Immunol* 1990; 81: 97-100.
10. CAO Y, OHWATARI N, MATSUMOTO T, KOSAKA M, OHTSURU A, YAMASHITA S: TGF-beta1 mediates 70-kDa heat shock protein induction due to ultraviolet irradiation in human skin fibroblasts. *Pflugers Arch* 1999; 438: 239-44.
11. HASEGAWA M, SATO S, TAKEHARA K: Augmented production of chemokines (MCP-1, MIP-1α, and MIP-1β) in patients with systemic sclerosis: MCP-1 and MIP-1α may be involved in the development of pulmonary fibrosis. *Clin Exp Immunol* 1999; 117: 159-65.
12. SIMONINI G, PIGNONE A, GENERINI S *et al*.: Emerging potentials for an antioxidant therapy as a new approach to the treatment of systemic sclerosis. *Toxicology* 2000; 155: 1-15.
13. YANG CW, LI C, JUNG JY *et al*.: Preconditioning with erythropoietin protects against subsequent ischemia-reperfusion injury in rat kidney. *Faseb J* 2003; 17: 1754-5.
14. GIFFARD RG, XU L, ZHAO H *et al*.: Chaperones, protein aggregation, and brain protection from hypoxic/ischemic injury. *J Exp Biol* 2004; 207: 3213-20.
15. JUN JB, KUECHLE M, HARLAN JM, ELKON KB: Fibroblast and endothelial apoptosis in systemic sclerosis. *Curr Opin Rheumatol* 2003; 15: 756-60.