Original article

Anti-heat shock protein 70 levels in gingival crevicular fluid of Japanese patients with chronic periodontitis

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Abstract: Periodontitis is an inflammatory disease involving complex tripartite cross-interactions among bacterial, host and environment factors. Heat shock proteins (Hsps) are a protein family produced in response to stress conditions. Hsps protect cells under adverse circumstances such as infection, inflammation and disease. One of the causes of periodontal disease is thought to be an imbalance in the expression of Hsps and anti-Hsp antibodies. Hsps are classified according to their molecular weight, and the Hsp70 family has been intensively investigated. Among the Hsp70 family members, stress-inducible Hsp72 and constitutively expressed Hsp73/Hsc70 are the best known [4,5].

Introduction

Periodontitis is an inflammatory disorder affecting the periodontium, and in severe cases it can provoke progressive alveolar bone loss, with subsequent loosening and loss of teeth. Periodontitis is a condition involving complex interactions among bacterial, host and environment factors and characterized by compromised immune responses, leading to gingival and periodontal ligament destruction, as well as alveolar bone resorption [1,2]. Wound healing is a complicated biological process involving sequential molecular and cellular events that are regulated spatially and temporally. Heat shock proteins (Hsps) are a protein family produced in response to stress conditions, and serve as a sensitive biological marker of thermal stress. Hsps are present in all organisms, being expressed in response to several environmental stressors to protect cells from damage, and promoting a variety of biological processes by acting as molecular chaperones under non-stressed conditions [3]. Hsps are classified according to their molecular weight, and the Hsp70 family has been intensively investigated.

Among the Hsp70 family members, stress-inducible Hsp72 and constitutively expressed Hsp73/Hsc70 are the best known [4,5]. Anti-Hsp70 antibodies are found in smokers and patients with Graves’ disease [6]. Patients with uveitis have circulating anti-Hsp70 antibodies whose levels may depend on disease severity [7]. The serum levels of various Hsps antibodies, including anti-Hsp70, are reportedly higher in patients with dilated cardiomyopathy than in healthy controls [8]. The relationship between the level of anti-Hsp70 antibody and several types of vascular disease suggests that Hsp70 might contribute to the pathogenesis and progression of atherosclerosis [9]. It has been suggested that the plasma levels of anti-Hsp70 or anti-Hsp71 antibodies might be associated with hypertension and harsh working conditions, and are increased in patients with severe heat exhaustion [10,11]. It has been reported that patients with graft-versus-host disease after allogeneic stem cell transplantation possess circulating anti-Hsp70 and anti-Hsp90 antibodies [12]. Hsp70 is thought to be a potential autoantigen in multiple sclerosis [13], and high levels of expression of Hsp27, Hsp70 and Hsp73 in myelin may act as an additive immune target involved in progression of the disease [14,15]. Anti-Hsp70 antibody could also be a causal factor of schizophrenia, especially in patients who have not been medicated for it [16,17]. Blood levels of anti-Hsp70 antibody and formation of Hsp70-antibody complexes in the placenta may induce preterm birth [18]. Moreover, the levels of Hsp70 and its antibody can be used as diagnostic and prognostic indicators in patients with gynecological malignancies [19,20]. Inflamed periodontal tissue shows marked down-regulation of Hsp70 family members [21], and it has been reported that levels of anti-Hsp70 antibody in periodontitis patients did not change significantly during periodontal treatment for 6 months [22]. This relationship between Hsp70 family members and periodontal inflammation suggests that Hsp70 and its antibody might contribute to the pathogenesis and progression of periodontitis.

The purpose of the present study was to clarify the levels of anti-Hsp70 antibody in gingival crevicular fluid (GCF) from both diseased and healthy control (HC) gingival sulci in patients with chronic periodontitis (CP), and to consider the possible significance of Hsp70 antibody levels in relation to periodontal disease severity.

Materials and Methods

Study population

Nine patients with chronic periodontitis (mean age, 62.8 ± 9.1 yr) were recruited. All received initial periodontal therapy at Nihon University Hospital School of Dentistry at Matsudo, Japan. The ethics committee at Nihon University School of Dentistry at Matsudo approved the study (EC17-017, EC18-17-017-1). All patients provided written informed consent to participate after the investigative approach had been fully explained to them. Periodontitis was assessed clinically in terms of probing pocket depth (PPD), clinical attachment level (CAL), bleeding on probing (BOP), plaque index (PII) and gingival index (GI) [23,24]. PPD and CAL were measured using a PCP11 probe (Hu-Friedy, Chicago, IL, USA). Patients were defined as having CP if they had at least two sites with a PPD of ≥5 mm and an attachment loss of ≥5 mm [25]. All patients were physically well and had no history of periodontal therapy or antibiotic treatment for at least 3 months prior to participation in the study.

Sample size

For sample size calculation, EZR on R commander Ver. 1.27 was used [26]. To confirm the required sample size for this study, 80% power with a two-sided comparison (α = 0.05), the predicted difference in the mean value between two groups (550 ng/mL) and the standard deviation of two groups (400 ng/mL) were set and calculated. The required sample size for each group was 9. Therefore, the number of samples taken was appropriate.

Clinical protocol

Clinical examinations were performed 4 times (first visit [1st], 2nd examination [2nd] after supragingival scaling, 3rd examination [3rd] at 3 months after scaling and root planing [SRP], and 4th examination [4th] at 6 months after SRP under infiltration anesthesia) by two trained periodontists (HT...
The significance of differences between the 1st, 2nd, 3rd, and 4th examinations was analyzed by chi-squared test.

Clinical parameters were presented as mean ± standard error (SE) in Table 1.

Statistical analysis
Clinical parameters were presented as mean ± standard error (SE) in Table 1. The significance of differences between the 1st, 2nd, 3rd, and 4th examinations for clinical parameters, GCF volume and anti-Hsp70 antibody levels among the groups were determined by Steel-Dwass test, and presented as the median and interquartile range. Difference in BOP at the 1st, 2nd, 3rd, and 4th examinations were analyzed by chi-squared test. The level of significance was adjusted at 5%. Statistical analyses were performed by using Excel add-in software, Statcel4 (provided with “Statcel-the useful addin forms on Excel” 4th ed., OMS Ltd., Higashikurume, Japan).

Results
The age, sex, PPD, CAL, PII, GI, and BOP distributions for the 9 patients are listed in Table 1. Average PPD and CAL at the HC sites (PPD ≤3 mm) were 2.9 ± 0.1 mm and 3.2 ± 0.2 mm, respectively. In contrast, average PPD and CAL at the CP sites (PPD ≥5 mm) were 6.1 ± 0.3 mm and 7.9 ± 0.9 mm, respectively. PI, GI, and BOP scores at the CP sites (2.2 ± 0.4, 1.8 ± 0.1 and 0.9 mm, respectively) were higher than those at the HC sites (0.3 ± 0.2, 0 and 0%). Changes in the concentrations of anti-HSP70 antibody in GCF from HC sites was significantly higher than that from CP sites. Furthermore, the concentrations of anti-Hsp70 antibody at HC and CP sites did not change significantly during the periodontal therapy (Table 3). In contrast, at CP sites, PPD was reduced from 6.1 ± 0.3 mm at the 1st examination to 4 (3-6) mm at the 4th examination (P < 0.05). Changes in BOP at the 1st, 2nd, 3rd, and 4th examinations were analyzed by chi-squared test.

Table 1 Patient characteristics

| Age | Healthy control sites | Chronic periodontitis sites |
|-----|-----------------------|-----------------------------|
| Males | 62.8 ± 9.1 | 2 (22%) |
| Females | 7 (78%) | 4 (44%) |

PPD, probing pocket depth; CAL, clinical attachment level; PII, plaque index; GI, gingival index; BOP, bleeding on probing; mean ± SE (n = 9).

Table 2 Changes in the concentrations of anti-Hsp70 antibody in GCF collected from HC and CP sites during the therapy

| Concentration (ng/mL) | 1st | 2nd | 3rd | 4th |
|-----------------------|-----|-----|-----|-----|
| HC sites | 1001.4 (520.2-4388) | 1466 (276.8-2012.2) | 1513 (139.5-2913) | 1595.9 (305.2-2845.2) |
| CP sites | 718.2 (33.3 -1068.5) | 719.2 (173.9-1143.9) | 690.6 (404.2-2740.1) | 1030.9 (356.2-1560.5) |

GCF, gingival crevicular fluid; HC, healthy control; CP, chronic periodontitis; 1st, first visit; 2nd, 2nd examination after supragingival scaling; 3rd, 3 months after scaling and root planing (SRP); 4th, 6 months after SRP; *P < 0.05; median and interquartile range (n = 9).

Table 3 Changes in clinical parameters at HC sites

| PPD | 1st | 2nd | 3rd | 4th |
|-----|-----|-----|-----|-----|
| CAL | 3 (2-3) | 3 (2-4) | 3 (2-4) | 3 (2-3) |
| PI | 3 (0-3) | 2 (0-3) | 2 (0-3) | 2 (0-3) |
| GI | 0 (0-1) | 0 (0-1) | 0 (0-1) | 0 (0-1) |
| BOP | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |

HC, healthy control; PPD, probing pocket depth; CAL, clinical attachment level; PI, plaque index; GI, gingival index; BOP, bleeding on probing; Median and interquartile range (n = 9). Changes in BOP at the 1st, 2nd, 3rd, and 4th examinations were analyzed by chi-squared test.

Table 4 Changes in clinical parameters at CP sites

| PPD | 1st | 2nd | 3rd | 4th |
|-----|-----|-----|-----|-----|
| CAL | 6 (5-8) | 5 (4-7) | 5 (4-7) | 4 (3-6)* |
| PI | 7 (6-14) | 7 (4-11) | 6 (4-13) | 7 (4-10) |
| GI | 2 (1-2) | 2 (0-2) | 1 (0-2) | 1 (0-2) |
| BOP | 7 (78%) | 5 (56%) | 3 (33%)* | 3 (33%)* |

CP, chronic periodontitis; PPD, probing pocket depth; CAL, clinical attachment level; PI, plaque index; GI, gingival index; BOP, bleeding on probing; Median and interquartile range (n = 9) compared with 1st. *P < 0.05. Differences in BOP at the 1st, 2nd, 3rd, and 4th examinations were analyzed by chi-squared test.

Enzyme-linked immunosorbent assay (ELISA)
Concentrations of anti-Hsp70 antibodies in GCF samples were measured by ELISA using the Anti-Hsp70 IgG/A/M (human) ELISA kit (Enzo Life Sciences, Plymouth Meeting, PA, USA) in accordance with the manufacturer’s instructions. Briefly, GCF samples collected in the Periopaper strips were dissolved in 750 μL of Sample Diluent 2 provided in the kit. The diluted GCF samples (100 μL) were then incubated for 2 h in recombinant human Hsp70 protein pre-coated microplate wells. After a wash to eliminate unbound substances, hydrogen peroxidase-conjugated anti-human IgG, IgA and IgM goat sera were added to the wells. After washing away any unbound conjugate, tetramethylbenzidine substrate solution was added to the wells for 15 min. Color development was stopped within 30 min, and the optical density of each well was determined using a microplate reader at 450 nm. All measurements were performed in duplicate and the concentrations of anti-Hsp70 antibodies were expressed as ng/mL.
to 56% at the 2nd, and 33% at the 3rd and 4th (P < 0.05). Changes in the volume of GCF from HC and CP sites during the course of periodontal therapy are shown in Fig. 1. Although GCF volume at these sites did not change significantly, there were significant differences in between HC and CP sites at the 1st and 3rd visits (Fig. 1).

Discussion

In the present study, it has been shown for the first time that the levels of anti-Hsp70 antibody in GCF from HC sites were significantly higher than those at CP sites at the 4th visit during periodontal therapy in patients with periodontal disease. The concentrations of Hsps are up-regulated rapidly when cells are exposed to environmental stressors such as elevated temperature. They are involved in crucial physiological processes, and are involved in response to various stimuli such as osmotic stress, and in protein repair in damaged cells [28]. The mechanisms and roles of the immune system in the pathogenesis of periodontal disease are not yet thoroughly understood, especially the possible role of Hsps in the etiology of periodontitis. Inflammation of periodontal pockets is accompanied by a rise in temperature of up to 2°C [29]. It is well known that several types of proinflammatory cytokine are produced in inflamed periodontal tissues [30], and might act as stress factors to increase the level of endogenous Hsps. However, Hsc70, Hsp70-2 and heat shock protein family A (Hsp70) member 4 are markedly down-regulated in inflamed periodontal tissues. These intracellular Hsp70 family proteins facilitate the folding of newly synthesized proteins and prevent protein aggregation [21].

In vitro [31] and in vivo [32]. If endotoxemia and fever persist in patients with sepsis, expression of Hsp70 might be often observed [33,34]. The expression patterns of Hsp70 and Hsp25 after CO2 laser irradiation of gingival tissues suggest that Hsp70 might participate in periodontal wound healing. Therefore, Hsp70 may play a regulatory role in the aging process as a molecular chaperone, since it mitigates the effects of proteotoxic stress [37,38]. In Caenorhabditis elegans, knock-in of extra copies of Hsp70, a homolog of met-2 (mortalin)/mhs70/Gpr75, has been shown to extend life-span [39], whereas knockdown of mitochondrial Hsp70 induces progeria-like phenotypes [40].

Fig. 1 Box plot showing changes and variations in GCF volume at HC and CP sites during the periodontal therapy (median and interquartile range; n = 9). *P < 0.05

auto-antibodies against Hsps. Anti-Hsp60 or anti-Hsp70 antibodies enhance the production of IL-8 and tumor necrosis factor-alpha (TNFα) induced by Hsp60 or Hsp70 in human peripheral blood monocytes and mononuclear cells [41]. Serum levels of both Hsp70 and its antibody are increased in Behcet’s disease and both can be predictive of acute coronary syndrome [42,43]. Anti-Hsp70 antibody levels are associated with nascent metabolic syndrome and are significantly higher in healthy subjects than in patients with type 1 diabetes. Therefore, the serum level of anti-Hsp70 antibody might be novel marker of protection against chronic diabetic complications [44,45].

In this study, anti-Hsp70 antibody levels in GCF were significantly higher at HC sites than at CP sites. Moreover, anti-Hsp70 antibody levels increased after initial periodontal therapy. Therefore, it has been suggested that the level of anti-Hsp70 antibody could become an appropriate indicator of the periodontitis healing process.

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Conflict of interest

The authors have no conflict of interest to declare.

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