Evaluation of circulatory and salivary levels of heat shock protein 60 in periodontal health and disease

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ABSTRACT

Background: Self-antigens such as heat shock protein 60 (HSP 60) have recently been implicated in the periodontal disease pathogenesis. There is scant evidence regarding HSP 60 levels in circulation and saliva following periodontal disease and its possible relation to systemic inflammation.

Aim of the Study: The aim was to evaluate the circulatory and salivary levels of HSP 60 in periodontal health and disease and to correlate it with high sensitivity C-reactive protein (hs-CRP).

Materials and Methods: Forty-five peripheral blood samples were collected from two groups of patients (periodontally healthy - Group A [22 patients] and periodontal disease - Group B [23 patients]). Serum, cell lysates, and saliva samples were used to detect HSP 60 levels in both groups by enzyme linked immunosorbent assay technique. Measurement of hs-CRP was performed using an immunoturbidimetric assay. Statistical analysis was done using the student *t*-test and Pearson's correlation.

Results: Circulatory HSP 60 was significantly increased in periodontal disease compared to health (*P* - 0.038). There was a significant correlation between the totals circulating HSP 60 and hs-CRP (*P* - 0.052), but there was no significant correlation between the salivary HSP 60 and hs-CRP levels in periodontal disease.

Conclusion: Circulating HSP 60 levels may play a role in the systemic inflammatory state produced by periodontal disease. Salivary HSP 60 may not be used as a surrogate to determine systemic inflammation.

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Periodontitis is a chronic inflammatory disorder that results in loss of tooth supporting apparatus. *Porphyromonas gingivalis*, a Gram-negative anaerobic bacteria has been strongly implicated in the pathogenesis of chronic periodontitis, either singly or in combination with other red complex bacteria such as *Tannerella forsythia, Treponema denticola*.[1] The major antigenic determinants of these pathogens are lipopolysaccharides (LPS), capsule, fimbriae, outer membranous protein, and other lipid antigens.[2] Although the inflammatory response in periodontal disease is evoked primarily in response to these exogenous antigens, recent evidence suggests that the immune system may be activated against host antigens as well.[3]

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Heat shock proteins (HSP) are perhaps, the most extensively investigated self-antigens involved in periodontal disease.[4] These proteins, produced by cells in response to environmental stimuli such as high temperature, mechanical stress, and infection,[4] participate in vital physiological processes in the cell such as folding, assembly, and translocation of polypeptides across membranes. They are hence referred to as molecular chaperones (Cpn). HSP 60 or Cpn 60 is a mitochondrial chaperonin that assists in folding linear amino acid chains into their respective three-dimensional structure. Human HSP 60 and their bacterial counterparts share more than 50% amino acid sequence homology. This structural homology may lead to inadvertent mediation of immune responses.[5]

Ueki *et al.*,[6] have described a role for HSP 60 in periodontal disease. These authors have demonstrated an upregulation of HSP 60 expression in gingival tissues affected by

periodontal disease. HSP 60 was shown to induce secretion of proinflammatory cytokines in professional antigen presenting cells,[7,8] activate T-cells in primary stimulation and thereby perpetuate the overall inflammatory process.[7]

Heat shock protein 60 has been identified in serum even though they are typically regarded as intracellular components.[9] The source of this freely circulating HSP 60 is not fully understood and has hence, been the object of much speculation.[10] Regardless of the source; circulating HSP 60 levels may assume clinical significance because it has been implicated in the etiopathogenesis of cardiovascular disease (CVD). HSP 60 has been reported to upregulate E-selectin, intercellular adhesion molecule-1, vascular cell adhesion molecule-1 expression and interleukin-6 (IL-6) production in endothelial cells and thereby contribute to atheromatous plaque formation.[11]

Periodontal disease has been documented to evoke a low-grade systemic inflammatory response as evidenced by leukocytosis and elevated high sensitivity C-reactive protein (hs-CRP) levels. This systemic inflammatory response is accompanied by a spillover of several inflammatory mediators such as IL-6, IL-4, IL-10, and tumor necrosis factor-α (TNF-α). This spillover is thought to modulate the course and severity of other chronic disorder such as CVD. However, there is insufficient evidence in the literature regarding circulatory HSP 60 levels following periodontal disease and its possible influence on the systemic inflammatory response.

Saliva has been used as a diagnostic tool to investigate periodontal disease activity as it is readily available and contains locally produced microbial and host response mediators. Several inflammatory markers and host-derived products in saliva such as IL-1, TNF-α, matrix metalloproteinases, human neutrophil elastase, etc., have been evaluated for use as markers for periodontal disease activity. However, there is scant literature regarding the use of salivary HSP 60 as a biomarker.

In recent years, saliva has been postulated to be capable of reflecting not only periodontal disease, but also systemic diseases such as, CVD, Rheumatoid arthritis, Sjogrens syndrome. There is insufficient evidence in the literature regarding the reliability of using saliva as a surrogate for evaluating systemic inflammatory status and its mediators.

The purpose of this study was to estimate the circulatory and salivary levels of HSP 60 in periodontal disease and to assess whether it correlates with hs-CRP.

MATERIALS AND METHODS

Study population

This study was reviewed and approved by Institutional Review Board, Ragas Dental College and Hospital, Chennai, India. Forty-five patients (21 males, 24 females, and mean age 35 ± 15 years) who attended the outpatient Department of Periodontology, Ragas Dental College and Hospitals, Chennai during the period January 2009 to June 2009 were enrolled in the study. Patients were assigned to the healthy gingiva group (Group A - 22 patients) or periodontal disease group (Group B - 23 patients) based on the following criteria: Group A patients exhibiting no signs of periodontal disease, determined by the absence of clinical attachment loss (CAL), absence of bleeding on probing (BOP) and probing depth (PD) of ≤3 mm. Group B patients exhibited PD of ≥5 mm and CAL of ≥3 mm and radiographic evidence of bone loss in at least 6 teeth.[12]

Patients excluded from the study were those with: (a) History of periodontal therapy or antibiotic therapy in the past 6 months, (b) history of systemic diseases that may affect the periodontal status, (c) pregnancy and Lactation, (d) smokers. (e) Evidence of any other active oral infections, e.g. pulpal pathology. Informed consent was obtained from all patients.

Clinical evaluation

Clinical evaluation was done using a mouth mirror and William's periodontal probe. The PD, CAL, and BOP were evaluated.

Sample preparation for enzyme linked immunosorbent assay and immunoturbidimetric assay

A volume of 3 ml of peripheral blood was drawn from patients using venepuncture from the antecubital fossa. Peripheral blood was drawn prior to onset of Phase I periodontal therapy in periodontitis patients. All patients underwent a complete physical examination and hematological investigation in Ragas General Hospital to rule out systemic diseases and conditions.

Serum preparation

From the 3 ml of blood drawn by venepuncture in the antecubital fossa, 2 ml of blood was allowed to clot at room temperature for 30 min and centrifuged at 3000 rpm for 10 min. The obtained serum was then divided into 2 aliquots and then transferred to a labeled poly propylene tube and stored at -70°C and used for further analysis.

Cell lysate preparation

Of 3 ml; 1 ml of blood was added to 2 ml of phosphate buffer saline. This mixture was carefully layered in a centrifuge tube containing 3 ml of histopaque-1077 (Sigma - Aldrich Co) centrifugation at 3000 rpm for 30 min. The buffy coat layer containing lymphocytes and mononuclear cells was separated and washed twice with 10 ml phosphate buffered saline at 2000 rpm for 10 min. One milliliter 1 × extraction reagent was added per 1 ml of blood. The extract was incubated for 30 min

on ice. The extract was centrifuged in polypropylene microcentrifuge tube at 21,000 rpm for min in a refrigerated microfuge (Remi) at 4°C. The supernatants were obtained and stored at –70°C and used for further analysis.

Saliva collection

Salivary collection was done according to the technique by Navazesh 1993.[13] The saliva was collected in a sterile disposable plastic container and the samples were stored at –70°C and used for further analysis.

Measurement of high sensitivity C-reactive protein

Measurement of hs-CRP was performed using an immunoturbidimetric assay performed on a Randox Daytoner analyzer (Randex Laboratories, Crumlin Co., Autrium, UK). The assay range was 0.1-20 mg/l and detection limit was 0.03 mg/l.

Enzyme-linked immunoassay for heat shock protein 60

Appropriate enzyme-linked immunosorbent assay kit (Assay Designs and Stressgen) was purchased. Serum, cell lysate and saliva samples were analyzed using Elisa system according to the manufacturer's recommended procedure and 96-well plate precoated with appropriate antibodies was used. First, serum/cell lysate/saliva samples and standards were added and incubated for 3 h. Then, the conjugate antibody was added and incubated 1 h at room temperature. The plates were washed again, and substrate was added to develop color change and incubated for 30 min at room temperature in the dark. Finally, the optical densities were read at 450 nm, and the samples were compared to the standards. The results for HSP 60 were expressed at pg/µl.

Statistical analysis

Student's *t*-test was used for statistical analysis of the circulatory and salivary HSP 60 values in health and disease, which was expressed in terms of mean and standard deviation. Correlation coefficient of circulatory and salivary HSP 60 with hs-CRP in periodontal health and disease was analyzed using Pearson's correlation. $P < 0.05$ was considered to be statistically significant at the 5% level.

RESULTS

There was a significant increase in the hs-CRP level in periodontal disease (2.25 ng/dl) when compared with health (1.16 ng/dl) at $P < 0.05$ (0.024) [Table 1].

There was a significant increase in the circulatory HSP 60 level (total serum and cell lysate) in periodontal disease (94.67 ng/dl) when compared with health (59.05 ng/dl) at $P < 0.05$ (0.038) [Table 1].

When the circulating HSP 60 levels were examined individually as cell lysate and serum levels, the results obtained were as follows:

There was a significant increase in the cell lysate level of HSP 60 in periodontal disease (80.89 ng/dl) when compared to health (53.54 ng/dl) at $P < 0.05$ (0.044) [Table 1].

There was no significant difference in the serum (20.19 ng/dl) and salivary HSP 60 levels (30.15 ng/dl) in periodontal disease when compared to serum (15.68 ng/dl) and salivary HSP 60 levels (26.04 ng/dl) in health at $P > 0.05$ [Table 1].

There was a statistically significant correlation between total circulatory HSP 60 and hs-CRP (57%) at $P < 0.05$ (0.052) in periodontal disease. There was no statistically significant correlation between total circulatory HSP 60 and hs-CRP (13%) at $P < 0.05$ (0.754) in periodontal health. There was also no significant correlation between salivary HSP 60 and hs-CRP (29%) at $P < 0.05$ (0.864) in periodontal disease [Table 2].

There was no significant correlation between salivary HSP 60 and circulating HSP 60 (either total or individual) in both health and disease [Tables 2 and 3].

DISCUSSION

Although periodontopathic bacteria are the primary etiological agents in periodontal disease, the ultimate determinant of disease progression is an aberrant host immune response. The immune response is typically activated through exposure to bacterial antigens with specific Pathogen associated molecular patterns (PAMPs) such as LPS, fimbriae, and outer membranous protein, etc.[2] In addition to these PAMPs, self-antigens have also been reported to be involved in host inflammatory/immune response.[3,14]

Among the most studied self-antigen in periodontal disease is the HSP, especially HSP 60. HSP 60 has been reported to be significantly elevated in gingival tissues affected by periodontal disease; however, there is as yet, no conclusive evidence linking it to disease activity. HSP's

Table 1: hs-CRP, HSP 60 in total circulation, cell lysate and serum in periodontal health and disease

Variables	hs-CRP (mg/l)	Total circulatory HSP 60 (ng/dl)	Cell lysate (ng/dl)	Serum (ng/dl)	Saliva (ng/dl)
Health (mean±SD)	1.16±0.033	59.05±35.20	53.54±30.52	15.68±10.02	26.04±14.36
Disease (mean±SD)	2.25±0.29	94.67±36.74	80.89±49.46	20.19±16.57	30.15±24.34
P value	0.024*	0.038*	0.044*	0.488	0.628

**P*<0.05=Statistically significant, *P*>0.05=Not significant. hs-CRP=High sensitivity C-reactive protein, HSP 60=Heat shock protein 60, SD=Standard deviation

Table 2: Pearson's correlation for total circulatory levels and hs-CRP and HSP 60 levels in serum and cell lysates of periodontal disease

Samples	Samples	Correlation coefficient %	*P* value
Total circulatory levels	hs-CRP	57	0.052
Saliva	hs-CRP	29	0.864
Serum	Cell lysate	15	0.967
Cell lysate	Saliva	11	0.280
Saliva	Serum	5	0.347

**P*<0.05=Statistically significant, *P*>0.05=Not significant, hs-CRP=High sensitivity C-reactive protein, HSP 60=Heat shock protein 60

Table 3: Pearson's correlation for total circulatory levels and hs-CRP and HSP 60 levels in serum and cell lysates of periodontal health

Samples	Samples	Correlation coefficient %	*P* value
Total circulatory levels	hs-CRP	13	0.754
Saliva	hs-CRP	17	0.657
Serum	Cell lysate	70	0.995
Cell lysate	Saliva	10	0.419
Saliva	Serum	3	0.558

**P*<0.05=Statistically significant, *P*>0.05=Not significant, hs-CRP=High sensitivity C-reactive protein, HSP 60=Heat shock protein 60

are typically regarded as intracellular proteins but free serum HSP 60 has been reportedly identified and linked to systemic inflammation and CVD.[10,15-17] The source of this free circulating HSP 60 remains a controversy. This study was undertaken to investigate if periodontal disease could contribute to this free circulating HSP 60 levels in a manner similar to that of circulating proinflammatory cytokines and thereby affect systemic inflammation.

There was a significant increase in hs-CRP levels following periodontal disease in our study. This study adds to the existing body of literature that has linked periodontal disease to the systemic inflammatory marker hs-CRP.[18,19] There was a significant increase in total circulating HSP 60 in periodontal disease when compared with health. These results are in conformity with previous studies of Yamazaki *et al.*,[20] Ueki *et al.*[6] In order to further clarify its role in the pathogenesis, cell lysate, and serum HSP 60 levels were examined individually. There was a significant increase in the cell lysate, but not serum HSP 60 levels in periodontal disease when compared with health. These results could be interpreted to mean that periodontal disease may not contribute significantly to the free circulating HSP 60 levels. However, these results also indicate that there is immune cell activation in response to HSP 60. It is not clear whether this activation occurred in relation to human HSP 60 or HSP 60 of *P. gingivalis* or indeed if this is a predominantly mononuclear or lymphocytic response. Further studies are needed to clarify this point. The expression of human HSP 60 has been confirmed on endothelial cells, fibroblast/smooth muscle cells in atherosclerotic lesions in humans. HSP 60 has agonist activity and is able to activate immune cells and vascular endothelial cell.[21]

Previous studies have attempted to designate a putative role for HSP 60 in the etiopathogenesis of CVD.[7,15,16] In addition to activating Natural Killer Cells and γ δ T cells, HSP 60 can result in increase of proinflammatory cytokines like TNF-α.[10,15-17,22] The role of inflammation in the disease process is now well-accepted and is postulated to be a common link between periodontal disease and CVD.

High sensitivity C-reactive protein have been shown to be independently associated with CVD and may play a direct active role in the atherosclerotic process.[23] There was a strong positive correlation between total HSP 60 and hs-CRP levels, but when examined independently neither cell lysate nor serum HSP 60 showed any such correlation. These results suggest that HSP 60 may be associated with systemic inflammatory state, the underlying mechanisms however could not be determined. This may in part be due to the small size of our study. This finding could have important implications as this may be one of the pathways through which periodontal disease modulate the course of CVD.

There has been a resurgent interest in salivary diagnostics in the last few years. Recent evidence demonstrated its ability to predict not only local inflammatory conditions such as chronic periodontal disease, but also systemic conditions such as CVD, breast cancer, etc., Our results suggest that salivary HSP 60 levels exhibited no significant difference between periodontal health and disease. There is scant previous literature with which we can compare these levels. Previous studies on HSP are related to HSP 70 levels and have focused largely on salivary gland pathology. Saliva has been reported to be a second hand marker of periodontal disease as most of its constituents have gained entry from gingival crevicular fluid. The lack of association between salivary HSP 60 levels and periodontal disease is probably due to its etiopathogenesis.

Salivary HSP 60 did not correlate with the circulating HSP 60 levels or hs-CRP. The cell lysate HSP 60 levels indicate that the predominant systemic response to HSP 60 is cellular in nature. As saliva is generally thought to be reflective of the plasma or serum constituents; there was no significant correlation between salivary and circulating HSP 60. Consequently, it did not correlate with hs-CRP either. This data must not be interpreted to mean that saliva cannot be used as a diagnostic tool to evaluate systemic inflammation; only that salivary HSP 60 is a poor marker of both periodontal disease and its systemic inflammatory response.

Salivary HSP 60 has limited value as a biomarker of periodontal disease. Further, it correlates poorly with circulating HSP 60 levels and hs-CRP and is, therefore, not an ideal surrogate marker of systemic inflammation. Further studies are needed to confirm this hypothesis.

REFERENCES

1. Haffajee AD, Socransky SS. Microbiology of periodontal diseases: Introduction. Periodontol 2000 2005;38:9-12.
2. Handfield M, Levesque RC. Strategies for isolation of *in vivo* expressed genes from bacteria. FEMS Microbiol Rev 1999;23:69-91.
3. Yamazaki K, Ohsawa Y, Yoshie H. Elevated proportion of natural killer T cells in periodontitis lesions: A common feature of chronic inflammatory diseases. Am J Pathol 2001;158:1391-8.
4. Morimoto RI. Regulation of the heat shock transcriptional response: Cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes Dev 1998;12:3788-96.
5. Ellis JR, Van Eden W, Young D. Stress Proteins as Molecular Chaperones. Stress Proteins in Medicine. Elsevier 1996. p. 1-26.
6. Ueki K, Tabeta K, Yoshie H, Yamazaki K. Self-heat shock protein 60 induces tumour necrosis factor-alpha in monocyte-derived macrophage: Possible role in chronic inflammatory periodontal disease. Clin Exp Immunol 2002;127:72-7.
7. Yamazaki K, Ohsawa Y, Itoh H, Ueki K, Tabeta K, Oda T, *et al.* T-cell clonality to *Porphyromonas gingivalis* and human heat shock protein 60s in patients with atherosclerosis and periodontitis. Oral Microbiol Immunol 2004;19:160-7.
8. Honda T, Domon H, Okui T, Kajita K, Amanuma R, Yamazaki K. Balance of inflammatory response in stable gingivitis and progressive periodontitis lesions. Clin Exp Immunol 2006;144:35-40.
9. Pockley AG, Bulmer J, Hanks BM, Wright BH. Identification of human heat shock protein 60 (Hsp60) and anti-Hsp60 antibodies in the peripheral circulation of normal individuals. Cell Stress Chaperones 1999;4:29-35.
10. Kleindienst R, Xu Q, Willeit J, Waldenberger FR, Weimann S, Wick G. Immunology of atherosclerosis. Demonstration of heat shock protein 60 expression and T lymphocytes bearing alpha/beta or gamma/delta receptor in human atherosclerotic lesions. Am J Pathol 1993;142:1927-37.
11. Kol A, Lichtman AH, Finberg RW, Libby P, Kurt-Jones EA. Cutting edge: Heat shock protein (HSP) 60 activates the innate immune response: CD14 is an essential receptor for HSP60 activation of mononuclear cells. J Immunol 2000;164:13-7.
12. Armitage GC. Development of a classification system for periodontal diseases and conditions. Ann Periodontol 1999;4:1-6.
13. Navazesh M. Methods for collecting saliva. Ann N Y Acad Sci 1993;694:72-7.
14. Wassenaar A, Reinhardus C, Thepen T, Abraham-Inpijn L, Kievits F. Cloning, characterization, and antigen specificity of T-lymphocyte subsets extracted from gingival tissue of chronic adult periodontitis patients. Infect Immun 1995;63:2147-53.
15. Chung SW, Kang HS, Park HR, Kim SJ, Choi JI. Immune responses to heat shock protein in *Porphyromonas gingivalis*-infected periodontitis and atherosclerosis patients. J Periodontal Res 2003;38:388-93.
16. Karnoutsos K, Papastergiou P, Stefanidis S, Vakaloudi A. Periodontitis as a risk factor for cardiovascular disease: The role of anti-phosphorylcholine and anti-cardiolipin antibodies. Hippokratia 2008;12:144-9.
17. Pockley AG, Wu R, Lemne C, Kiessling R, de Faire U, Frostegård J. Circulating heat shock protein 60 is associated with early cardiovascular disease. Hypertension 2000;36:303-7.
18. Renvert S, Lindahl C, Roos-Jansåker AM, Lessem J. Short-term effects of an anti-inflammatory treatment on clinical parameters and serum levels of C-reactive protein and proinflammatory cytokines in subjects with periodontitis. J Periodontol 2009;80:892-900.
19. Nakajima T, Ueki-Maruyama K, Oda T, Ohsawa Y, Ito H, Seymour GJ, *et al.* Regulatory T-cells infiltrate periodontal disease tissues. J Dent Res 2005;84:639-43.
20. Yamazaki K, Ohsawa Y, Tabeta K, Ito H, Ueki K, Oda T, *et al.* Accumulation of human heat shock protein 60-reactive T cells in the gingival tissues of periodontitis patients. Infect Immun 2002;70:2492-501.
21. Maguire M, Coates AR, Henderson B. Chaperonin 60 unfolds its secrets of cellular communication. Cell Stress Chaperones 2002;7:317-29.
22. Slade GD, Offenbacher S, Beck JD, Heiss G, Pankow JS. Acute-phase inflammatory response to periodontal disease in the US population. J Dent Res 2000;79:49-57.
23. Pepys MB, Hirschfield GM. C-reactive protein: A critical update. J Clin Invest 2003;111:1805-12.

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