Salivary heat shock proteins and their interactions with oral microenvironment

Shailja Chatterjee1, Satyawan G. Damle2, Anil K. Sharma3

1Department of Oral and Maxillofacial Pathology, M.M.C.D.S.R., M. M. University, Mullana (Haryana), 133207, India  
2Department of Pedodontics, M.M.C.D.S.R., M. M. University, Mullana (Haryana), 133207, India  
3Department of Biotechnology, M.M.E.C., M. M. University, Mullana (Haryana), 133207, India

Correspondence: Anil K. Sharma  
E-mail: anibiotech18@gmail.com  
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Salivary proteomics is an intriguing area of immunobiological interactions. Heat shock proteins are uniquely conserved molecules that maintain high percentage of homology in all species. These proteins mainly act by inducing antiapoptotic and immunoregulatory mechanisms in intra- as well as extracellular milieu. They have been proposed to play an important role in saliva by maintenance of microbiological population. Their contributory protective role in cancer biology is an interesting area of research having immense implications in therapeutics.

Keywords: Heat shock proteins; oral microflora; cancer; immunoregulatory

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Introduction

Heat shock proteins (HSPs) were discovered by Ferruccio Ritossa in 1962 when they observed that temperature shock had induced odd puffing patterns in salivary polytene chromosomes in Drosophila melanogaster. HSPs are ubiquitous, highly conserved proteins found in all prokaryotic and eukaryotic species. These proteins can be categorized into several families based on their approximate molecular weights for example, the Hsp 60 kDa, Hsp 70 kDa and Hsp 90 kDa families. These proteins while functioning as molecular chaperones participate in protein assembly, stabilization, folding and translocation of oligomeric proteins, whereas as proteases, such as ubiquitin-dependent proteasomes mediate the degradation of damaged proteins. The term ‘heat shock’ protein is a misnomer, since in addition to raised temperature, other conditions like oxidative stress, nutritional deficiencies, UV irradiation, chemicals like ethanol, viral infections, ischemia-perfusion injuries can induce the protein expression [1].

Heat shock proteins can be classified into groups according to phylogeny and structure or molecular mass: High molecular mass heat shock proteins (HSPs ≥ 100kDa), heat shock protein 90 group (81 to 99 kDa), heat shock protein 70 group (65 to 80 kDa), heat shock protein 60 group (55 to 64 kDa), heat shock protein 40 group (35 to 54 kDa) and small heat shock proteins (≤ 34 kDa) [2].
Salivary proteomes: Heat shock proteins and oral microbiota

The total amount of protein in whole saliva ranges between 0.5 to 3 mg/ml. This proteome consists of roughly 1000 distinct protein sequences of which around 300 are of human origin. Co-existence of protein sequences and their mRNA is detectable in about 70% to 93% of salivary proteins \[3,4\]. Heat shock proteins, Hsp 60, 70 and 90 are present in saliva. Hsp 70 has important extracellular actions as evident from experiments involving exogenous Hsp 70 administration to human promonocyte cells prior to tumor necrosis factor-alpha (TNF-α) exposure where a significant reduction in numbers of apoptotic and necrotic cells were observed \[2\]. Hsp 70 in saliva involves passive transport from blood serum \[3\]. Human Hsp90 share almost 40% identity with \textit{E. coli} HspG. HSP90 is present in periodontopathogens like \textit{P. gingivalis}, fungal commensals such as \textit{Candida albicans} and other commensals like \textit{Streptococcus mutans}, \textit{Streptococcus pyogenes} \[5,6,7\]. Hsp90 is a stress-induced protein involved in cellular processes such as regulation of signal transduction and steroid hormone response pathways in higher eukaryotic cells. \textit{Candida albicans} hsp90 has a molecular mass of 82 kDa and has been implicated as a virulence factor. The 47 kDa C-terminal fragment of hsp90 is a target for immune response to \textit{C. albicans} infection. Antibodies against 47 kDa C-terminal portion of hsp90 cross-react with a 92 kDa heat-inducible protein identified as native hsp90 while other clonal studies indicate that native Candida hsp90 has a mass of approximately 80 kDa at N-terminal. The major function of hsp90 is in antigen presentation to MHC Class I molecules. Human hsp90 on the other hand, is intracellular and is involved in assembly and disassembly of proteins, binding to cellular structural proteins in order to maintain them in an inactive state. Sera from systemic lupus erythematosus patients have occasionally been found to cross-react with human hsp90 to recognize \textit{C. albicans} Hsp90 and its breakdown products \[8\].

Immune responses to hsp60 have been found in other microbial infections as well. CD4+ αβ T cells specific for heat shock proteins have been found to be increased in infected animals and mediate significant protection against...
Infection with *Yersinia enterocolitica* when adoptively transferred. Similarly, in infants, levels of antibodies against hsp60 have been observed to be significantly increased after vaccination with a trivalent vaccine against tetanus, diphtheria, and pertussis. These findings suggest that priming of the immune system to hsp60 is a phenomenon, occurring at an early stage of life. Similarly to hsp60, other members of the hsp family have been described as dominant antigens in several infectious diseases. Increased antibody levels to hsp70 have been identified in sera of patients suffering from malaria, leishmaniasis, schistosomiasis, filariasis, and candidiasis. In contrast to hsp60, response to pathogen-derived hsp70 seems to be more restricted and sometimes, exclusively species specific. An important role of the humoral response against hsp90 was demonstrated in systemic candidiasis. The hsp90-specific antibodies contribute directly to protection against *Candida albicans* infection. Antibodies specific for conserved domain of hsp90 of *C. albicans* have also been identified in healthy individuals, implying that hsp of nonpathogenic commensal organisms can activate hsp90-reactive antibodies.

**Mechanisms of heat shock protein induction and expression**

Transcription of heat shock protein genes is mediated by interaction of heat shock factor (HSF) with heat shock elements in hsp gene promoter regions. Four HSPs have been identified in vertebrates, of which HSF1 and HSF2 are ubiquitously expressed. HSF1 is present in cytosol as a latent monomeric molecule. On exposure to stress, an intracellular flux of newly synthesized immature proteins activates the HSF1 molecule to a hyperphosphorylated form mediated by mitogen-activated protein kinases. Thus, HSF1 gets converted to phosphorylated trimers with a capacity to bind DNA. This phosphorylated trimeric form of HSF1 binds to DNA, and gets translocated from nucleus to cytoplasm. On the other hand, HSF2 is a temperature-sensitive protein that is inactivated at high temperatures. Thus, it is prevented from interfering with HSF1 activity in stressed cells. Various mechanisms exist for regulation of heat shock proteins. One mechanism regulates by hsp70 binding to HSF1 transactivation domain leading to a repression of heat shock gene transcription. Second mechanism involves interaction of heat shock binding protein factor 1 (HSBP 1), the active trimeric HSF1 form and Hsp70, resulting in inhibition of HSF1 to DNA binding. HSBP1 is mainly localized within the nucleus.

**Role of HSPs as intracellular signaling molecules**

Heat shock proteins are released from necrotic cells and their extracellular detection indicates non-physiological
tissue damage and therefore, induction of proinflammatory responses. Hsp60 induces expression of adhesion molecules like E-selectin, ICAM-1 (Intracellular Adhesion molecule-1) and VCAM-1 (Vascular cell adhesion molecule) on endothelial cells and IL-6 expression from endothelial cells, smooth muscle cells and macrophages. Thus, the intracellular localization of these proteins in healthy states, their release from infectious agents and the capacity of both human and bacterial Hsps to elicit innate and adaptive proinflammatory responses establishes their role as links between pathogenic conditions like necrosis and induction of innate and adaptive immunity. New evidence has proposed that Hsps are present in extracellular environment under physiological conditions as well. Both Hsp60 and Hsp70 have a normal immunoregulatory response due to their self T-cell reactivity [3].

Immunoregulatory role of Hsps

Heat shock proteins play a specific role in induction of antigen-specific immunity. Hsps bind to peptides derived from cells from which they are isolated due to their specific ‘antigenic fingerprint’ on cellular repertoire. For example, tumor-derived gp96 can induce tumor-specific immunity. Dendritic cells internalize gp96 by receptor-mediated endocytosis through α-macroglobulin receptor (CD91) or a CD91-independent mechanism or both. These chaperoned peptides are incorporated in the MHC Class I pathway for presentation to CD8+ T cells. CD91 is common to other heat shock proteins such as Hsp 70 and Hsp 90, as well.

Heat shock proteins as apoptosis inhibitors

HSPs play an important role as cellular lifeguards against apoptosis. Hsp27 belongs to a small subfamily of proteins that undergoes dephosphorylation in response to various stressors such as mitogens, inflammatory cytokines such as tumor necrosis factor-α, hydrogen peroxide. Hsp27 can prevent caspase activation by direct sequestration of cytochrome c. This protein binds to F-actin preventing cytoskeletal disruption following heat shock, cytochalasin D etc. This also indirectly inhibits apoptosis by regulation of upstream signaling pathway factors such as nerve growth factor or platelet-derived growth factors. Hsp27 increases proteosomal degradation of NF-κB inhibitor, IκBα by increasing the IκBα ubiquitination/degradation. This results in an increase in NF-κB activity, thus increasing cell survival. HSP70 protein functions as ATP-dependent molecular chaperones by assisting in folding of newly synthesized polypeptides, assembly of multiprotein complexes and transport across cell membranes. Hsp70 activation blocks the apoptotic pathway by reducing caspase activation, suppression of mitochondrial damage and nuclear fragmentation. This molecule interacts with procaspase-3 and -7 preventing their activation by apoptosome modulation. Hsp70 blocks Bax translocation thereby, preventing mitochondrial outer membrane permeabilization and inhibiting cytochrome c and AIF mitochondrial release. This protein can also bind to death receptors, DR4 and DR5 inhibiting TRAIL-induced assembly and activation of death-inducing signaling complex. Bag-1 is an apoptosis regulatory protein that acts as a chaperone of Hsp70 and simultaneously regulates activity of bcl-2 and raf-1. Hsp70 also inhibits cathepsins and lysosomal proteases involved in apoptotic mechanism [11]. Figure 1 clearly shows the diverse role of Hsp70 especially its involvement in apoptotic and signal transduction mechanism. Similarly Hsp90 gets associated with a variety of signaling proteins, including ligand-dependent transcription factors such as MyoD, tyrosine kinases like v-Src and serine/threonine kinases such as Raf-1. Hsp90 promotes the conformational maturation of these receptors and signal-transducing kinases. Hsp90 also binds to ATP and undergoes a conformational change preventing apoptosis [11].

Extracellular role of heat shock proteins

Hsps play an important extracellular role immunologically as well. However the mechanism of transport to plasma membrane, membranous anchorage and export is unclear. Cytosolic heat shock proteins do not contain any peptide that can enable membranous localization, however, they do get transported across membranes. Hence, it can be assumed that Cytosolic heat shock proteins get cotransported along with other proteins possessing transmembrane domains. Gp96, a member of Hsp90 family residing in endoplasmic reticulum is transported across plasma membrane by masking of endoplasmic reticulum-retention sequence, KDEL. Hsp70 bypasses the ER-Golgi complex route via vesicular transport and by Exosomes from peripheral blood mononuclear cells. Extracellular Hsps- 70 and -90 play an important immunoregulatory function as HSP-chaperoned peptides are cross-presented through MHC class I molecules to antigen-specific CD8+ T cells. The uptake of HSP-peptide complex by antigen-presenting cells is concentration-dependent and specific. HSP70 is involved in stimulation of dendritic cell migration to draining lymph nodes. On binding to Hsp70, MHC class II and costimulatory molecules such as CD86, CD83 and CD40 were found to be upregulated. Thus, Hsp70 as well as Hsp90 have the capacity to stimulate innate immune system. Hsp70-membrane positive tumor cells can be biochemically lysed through granzyme, B-cell mediated and perforin-mediated apoptosis [10].

Heat shock protein expression in oral diseases

Heat shock protein 27 (Hsp27) is a small molecular weight molecule. Its activity is regulated via the MAP
kinase phosphorylation pathway. It has been shown to play a significant role in tumor progression, metastasis and inhibition of apoptosis [12].

Heat shock protein 27 (Hsp27) functions to increase the cellular resistance to oxidative stress due to reactive oxygen species (ROS) and nitric oxide (NO). Tumors originating from different sites have distinct malignant phenotypes, clinical presentations and etiologic risk factors and hence, different outcomes. Therefore, hsp27 expression is variably associated with different outcomes in various malignancies. Wang et al (2009) identified hsp27 overexpression in oral squamous cell carcinoma (OSCC) [12]. A significant correlation between Ki67 index and OSCC grading was observed (P<0.05), though, a significant inverse correlation was evident between hsp27 expression and OSCC grading (P<0.01) [12].

Similar overexpression has been observed with poor prognosis in malignancies of gastric, liver and prostate [13,14,15]. However, on the other hand, overexpression has been found to be associated with good prognosis in endometrial adenocarcinoma and esophageal carcinoma [16,17].

Hsp 70 has been shown to activate T lymphocyte recognition of melanoma differentiation antigens in an antigen-specific and HLA class I-dependent fashion. Also, hsp70 is involved in protein-oncogene interactions for example between p53 and c-myc oncogenes. This cell-mediated immune response is regulated by the major histocompatibility complex (MHC) class II (HLA-DR, -DP and –DQ) molecules. MHC class II molecules are immunoregulatory glycoproteins expressed on cell surfaces of lymphocytes, macrophages and endothelial cells. MHC Class II molecule, HLA-DR is found to be expressed in melanoma cells. A concomitant overexpression of c-myc, HLA-DR and hsp70 was observed in melanoma cells indicating a direct correlation between poor prognosis and elevated expression of these cellular markers [17].

Prajitno et al (2009) evaluated the expression of hsp60 and hsp10 in a series of carcinogenetic models, for example, “dysplasia-carcinoma” sequences of uterine exocervix, large intestine and prostate [18]. They evidently found that these proteins are overexpressed during transformation of dysplasia to carcinoma in HPV-positive oral lesions [18].

Heat shock proteins are released from dying tumor cells and are thereafter, taken up by antigen-presenting cells (APCs) and can stimulate tumor-specific T cells. HSP60 is normally expressed in mitochondrial, however, during stress, an intracellular redistribution and cell surface expression occurs. Similarly, HSP65 is expressed on monocytes after IFN-γ stimulation and on T cells undergoing apoptosis. Local hsp60 and hsp70 overexpression has been observed in epidermis of Behcet’s disease (Figure 2) [19].

Thubashani et al (2011) evaluated the role of HSP70 in progression of oral submucous fibrosis (OSMF) to oral squamous cell carcinoma (OSCC) [20]. They observed a significant increase in HSP70 expression (P<0.000) as OSMF progresses towards malignancy. Intense hsp70 immunoexpression was observed in advanced as compared to mild cases of OSMF. These findings suggest that HSP70 plays a significant role in tumor progression and aggressiveness [20].

Sugerman et al (1995) studied 70kDa (HSP70) expression in OSCC, epithelial dysplasias and benign oral mucosal lesions by comparing their staining intensity [21]. Median staining intensity was significantly greater in SCC (6.22 k omega), epithelial dysplasias (9.61 k omega) and benign oral mucosal lesions (8.28 k omega) as compared to normal mucosa (5.64 k omega, P <0.5). However, staining intensity in poorly differentiated squamous cell carcinoma (7.66 k omega) was greater than that in moderately differentiated SCC (4.77 k omega), though no statistical significance was observed (P=0.06) [21].

Heat shock proteins are implicated in protein-protein interactions such as folding, translocation and prevention of inappropriate protein aggregation. Kawanishi et al (1999) performed an immunohistochemical analysis for HSPs 27 and -70 in esophageal carcinomas to study their expression for prognosis [22]. When compared with Clinicopathological features, expression of both hsp-27 and -70 correlated negatively with lymph node metastasis (P<0.05). HSP under-expression was considered to be significantly associated with poor postoperative survival (P<0.0001). Thus, it was concluded that both heat shock proteins- 27 and 70 are independent prognostic indicators for esophageal carcinoma [22]. Studies have reported that tumors expressing heat shock proteins are more resistant to adjuvant therapies than those without, examples include breast carcinoma, Osteosarcoma and prostate carcinoma [22].

Human oral Keratinocytes are sources of self-HSP60. In diseases of periodontium, hsp overexpression occurs in gingiva, inflammatory infiltrate and oral bacteria. HSP60 secretion can be influenced by lipopolysaccharides from different bacterial microorganisms. Bacterial HSPs, namely DnaK and GroEL-like protein can stimulate the expression as well as release of cytokines and adhesion molecules like E-selectin, ICAM-1 and VCAM-1 in human monocytes and endothelial cells [23].

Hsp70 of glandular origin is a combination of both constitutively expressed and stress-inducible forms. Hsp70 transport may involve passive transport via salivary glands or through lipid rafts or through Exosomes. Salivary hsp70
may entrap and agglutinate bacteria by binding both gram positive (mutans streptococci and mitis) and gram-negative (Escherichia coli) bacteria. Salivary hsp70 had the propensity to form dimers, oligomers and micelles. Salivary hsp70 binds to hydroxypatite of teeth and hence, has a role in acquired pellicle formation [23].

Heat shock proteins have been implicated in multidrug resistance, apoptosis regulation and p53 modulation. Hsp27 is constitutively expressed at lower levels in cell cytoplasm. After induction, this protein gets phosphorylated and translocated to nucleus. Hsp27 modulates reactive oxygen species via a glutathione-dependent pathway protecting intracellular proteins and also, rendering immunity against chemotherapeutic agents. Hsp60 and hsp10 coregulate protein chaperoning and folding. Elevated hsp70 levels indicate an antiapoptotic effect. Hsp70 interacts with p53 to stabilize its mutant form. However, it does not interact with wild-type p53 though, which down regulates hsp70 expression [24].

**Heat shock protein and antigen presentation**

Heat shock proteins (HSPs) released from necrotic cells have been observed to activate dendritic cells. HSP60 can induce dendritic cells maturation with increased MHC class II, CD40, CD54 and CD86 expression and allogeneic T-cell proliferation, preferably Th1. HSP60 activates mitogen-activated protein kinase p38, c-jun N-terminal kinase, intracellular signal-regulated kinase and 1kβ in dendritic cells. Both hsp65 and hsp70 upregulate CD8+ T cell derived β-chemokine expression (RANTES, MIP-1α and MIP-1β) both directly and as an adjuvant linked to peptides indirectly. This innate immunity stimulation drives adaptive responses and attract antigen presenting cells (dendritic cells, macrophages) and effector T cells. Hence, it can be concluded that HSPs stimulate both innate and adaptive immune response [19].

The HSP-APC interaction is important for maintenance of innate immunity. HSPs are the primary products of autologous origin that act as mediators of dendritic cells. Heat shock proteins (HSPs) react with HSP receptors on APCs such as macrophages and dendritic cells. The HSP-peptide complexes are engulfed in non-acidic compartments. These peptide molecules are processed and re-presented by MHC class I molecules of the antigen presenting cells (APCs). HSPs bind to the macrophages in a similar fashion to elaborate cytokines and induce expression of higher levels of co-stimulatory molecules on the dendritic cells. This process is mediated by the NF-κβ pathway [25].

Heat shock proteins satisfy all criteria of Polly Matzinger's endogenous danger signal model as follows: 1. These signals are upregulated, released or modified in injured or stressed cells. 2. These signaling molecules are confined to intracellular compartments from where they can be released following injury, thereby, getting exposed to dendritic cell receptors. 3. They can be detected by dendritic cells thus, inducing their maturation. 4. They can induce a dendritic cell maturation program effectively. 5. Their exclusion is beyond doubts of experimental artifacts due to contamination with microbial components [25].

**Conclusions**

Heat shock proteins play a cytoprotective and immunoregulatory role both in intracellular and extracellular environment. Their secretion in saliva renders a unique immunological niche. This unique presence makes oral cavity an intricate ecological habitat for resident and pathogenic microflora. Heat shock proteins are versatile molecular chaperones having diverse role not only as scaffold proteins which protect other peptides and proteins within cellular compartments but also induce dendritic cell maturation and transfer of antigenic peptides from cells undergoing apoptosis. The role of HSP70 or gp96 as cancer vaccination agents is also being established. Heat shock proteins are known to satisfy all five criteria of Polly Matzinger’s endogenous danger signal model making them ideal indicators for predicting danger signs.

**Conflict of interest**

The authors declare that there is no conflict of interest.

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