Heat shock proteins as regulators of the immune response

A Graham Pockley

Until recently, heat shock proteins (also known as heat stress proteins) have mostly been regarded as intracellular molecules that mediate a range of essential housekeeping and cytoprotective functions. However, interest in their role as intercellular signalling molecules has been fuelled by the observations that these molecules can be released and are present in the extracellular environment under physiological conditions. They can elicit cytokine production by, and adhesion molecule expression of, a range of cell types, and they can deliver maturation signals and peptides to antigen presenting cells through receptor-mediated interactions. These functions suggest that heat shock proteins could be immunoregulatory agents with potent and widely-applicable therapeutic uses. Furthermore, the induction of self heat shock protein immune reactivity can attenuate autoimmunity and delay transplant rejection, and heat shock proteins derived from tumours and pathogens can elicit specific, protective immunity. This review will focus on this rapidly evolving area of heat shock protein biology.

“Discovery is to see what everyone else has seen and to think what no one else has thought”

Albert von Szent-Györgyi Nagyapolt
1937 Nobel Laureate in Medicine

Heat shock proteins were discovered in 1962 when Ferruccio Ritossa and his co-workers noted that temperature shock had induced odd puffing patterns and an unusual profile of gene expression in the polytene chromosomes of salivary glands in Drosophila melanogaster larva. However, not until 1974 were the first products of these genes identified and the term heat shock protein coined. Heat shock proteins are highly conserved molecules that are present, and can be induced, in all eukaryotic and prokaryotic species, including plants.

Heat shock proteins are categorised into several families that are named on the basis of their approximate molecular weight (eg, the 60 kDa Hsp60 family). Heat shock proteins are localised in various intracellular compartments, and in physiological conditions some of these proteins function as intracellular molecular chaperones or proteases (panel). Chaperones take part in the assembly, stabilisation, folding, and translocation of oligomeric proteins, whereas proteases, such as the ubiquitin-dependent proteasome, mediate the degradation of damaged proteins.

Heat shock proteins are constitutively expressed (making up to 5-10% of the total protein content in healthy growth conditions), but their intracellular concentrations can be increased two to three times by insults that induce protein unfolding, misfolding, or aggregation, and a flux of newly-synthesised non-native proteins. The term heat shock proteins is something of a misnomer, since in addition to raised temperature, exposure to oxidative stress, nutritional deficiencies, ultraviolet irradiation, chemicals, ethanol, viral infection, and ischaemia-reperfusion injury can also induce the expression of these proteins—including members of the Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and small heat shock protein families.

Induction and regulation of heat shock protein expression

Regulation of transcription of heat shock protein genes is mediated by the interaction of heat shock factor (HSF) transcription factors with heat shock elements in the heat shock protein gene promoter regions. In vertebrates, four HSFs have been identified, of which HSF1 and HSF2 are ubiquitously expressed and conserved. The main heat shock factor with a role in vertebrates’ response to physiological and environmental stress is HSF1, whereas activity of HSF2 is more selective, and is mostly induced during differentiation and early development.

Usually, HSF1 is present in the cytoplasm as a latent monomeric molecule that is unable to bind to DNA. When exposed to stress, an intracellular flux of newly synthesised non-native proteins activates HSF1, which is hyperphosphorylated in a ras-dependent manner by mitogen-activated protein kinases. HSF1 is converted to phosphorylated trimers that have the capacity to bind DNA, and which translocate from the cytoplasm to the nucleus (figure 1). HSF2 has the characteristics of a temperature-sensitive protein; it is inactivated when exposed to raised temperature, and sequestered to the cytoplasm, and is thereby prevented from interference with HSF1 activity in stressed cells. The consequences of binding of HSF1 to its target, and the events that result

Search strategy and selection criteria

Literature pertinent to this review was identified by current reading in the specialty, and by searches in Medline and PubMed databases. Articles were selected on the basis of their relevance to the immunoregulatory activity of extracellular heat shock proteins, their potential clinical value, and the excellence of methods and the importance of the findings presented therein. There was no positive or negative bias to either the authors of the literature cited, or the journals in which work has been published.
Mammalian heat shock protein families and their intracellular location and function

<table>
<thead>
<tr>
<th>Major family, and members</th>
<th>Intracellular localisation</th>
<th>Intracellular function</th>
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<tr>
<td><strong>Small Hsps</strong></td>
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<tr>
<td>αB-crystallin</td>
<td>Cytoplasam</td>
<td>Cytoskeletal stabilisation</td>
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<td>Hsp27</td>
<td>Cytoplasam/nucleus</td>
<td>Actin dynamics</td>
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<td>Haem oxygenase, Hsp32</td>
<td>Cytoplasam</td>
<td>Haem catabolism, antioxidant of properties</td>
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<td><strong>Hsp40</strong></td>
<td>Cytoplasam/nucleus</td>
<td>Regulates the activity of Hsp70; binds non-native proteins</td>
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<tr>
<td>Hsp40</td>
<td>Cytoplasam</td>
<td>Processing of pro-collagen; processing and/or secretion of collagen</td>
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<tr>
<td>Hsp47</td>
<td>ER</td>
<td></td>
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<tr>
<td><strong>Hsp60 (or chaperonins)</strong></td>
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<tr>
<td>Hsp60</td>
<td>Mitochondria</td>
<td>Bind to partly folded polypeptides and assist correct folding. Assembly of multimeric complexes</td>
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<tr>
<td>TCP-1</td>
<td>Cytoplasam</td>
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<tr>
<td><strong>Hsp70</strong></td>
<td>Cytoplasam/nucleus</td>
<td>Bind to extended polypeptides. Prevent aggregation of unfolded peptides. Dissociate some oligomers.</td>
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<tr>
<td>Cognate/constitutive: Hsc70</td>
<td>ER</td>
<td>ATP binding. ATPase activity. Hsp70 downregulates HSF1 activity</td>
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<tr>
<td>Grp78/Bip</td>
<td>Cytosplasm/peroxisome</td>
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<td>mtHsp70/Grp75</td>
<td>ER</td>
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<tr>
<td><strong>Hsp90</strong></td>
<td>Cytoplasam</td>
<td>Bind to other proteins. Regulate protein activity. Prevent aggregation of refolded peptide. Correct assembly and folding of newly synthesised protein. Hsp90 assists the maintenance of the HSF1 monomeric state in non-stressful conditions.</td>
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<td>Hsp90 (α and β)</td>
<td>ER</td>
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<td>Grp94/gp96/Hsp100</td>
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<td><strong>Hsp110</strong></td>
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<td>Apg-1 (mouse)</td>
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<td>Hsp105</td>
<td>Cytoplasam</td>
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in transcription of heat shock protein genes, have been widely reviewed.

The induction of heat shock proteins has to be tightly controlled, since their persistent presence would adversely affect protein homeostasis and intracellular functions, leading to inappropriate growth control and possibly cell death. One mechanism that regulates heat shock protein expression is the binding of Hsp70 to the transactivation domain of HSF1, leading to repression of heat shock gene transcription. The interaction between Hsp70 and HSF1 has no effect on DNA binding or the stress-induced phosphorylation state of HSF1. A second mechanism regulating heat shock protein synthesis is the interaction between heat shock protein binding factor 1 (HSBP1), the active trimeric form of HSF1, and Hsp70, resulting in inhibition of the capacity of HSF1 to bind to DNA. HSBP1 is mainly localised in the nucleus, and HSBP1 mRNA is present at high concentrations in various cell lines and animal tissues that are unaffected by heat shock.

Heat shock proteins as intercellular signalling molecules

The usual view of eukaryotic heat shock proteins is that they are intracellular molecules that are released from necrotic, but not apoptotic cells, and that their release into (and presence in) the extracellular environment indicates non-physiological tissue damage and therefore induces a range of proinflammatory responses. Findings from several studies are consistent with this idea. Human Hsp60 induces the expression of the adhesion molecules E-selectin, intracellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 on vascular endothelial cells, and the secretion of interleukin-6 from vascular endothelial cells, smooth muscle cells, and macrophages. Furthermore, bacterial and mycobacterial heat shock proteins induce proinflammatory cytokine expression, and the bacterial Hsp60 (termed GroEL) induces expression of ICAM-1 and VCAM-1 on vascular endothelial cells. Chlamydial Hsp60 activates expression of E-selectin, ICAM-1, and VCAM-1 on human vascular endothelial cells, and secretion of interleukin-6 from vascular endothelial cells, smooth muscle cells, and macrophages.

The finding that proteins in the Hsp60 family could induce adhesion molecule expression and cytokine secretion from various cell types prompted the search for Hsp60 receptors. The CD14 antigen has been identified as the receptor for this protein on human peripheral blood mononuclear cells and monocytes; thus, Hsp60 uses the signalling pathway also used by lipopolysaccharide. Toll-like receptor 4, which is an important mediator of innate immunity and lipopolysaccharide signalling in mouse cells, is needed for signalling. Data from another study suggest that Toll-like receptor 2 plays a part in human Hsp60 signalling. The CD14 molecule also has a role in Hsp70-mediated activation of monocytes, the consequences of which are intracellular calcium fluxes and induction of proinflammatory cytokines (interleukin-1β, interleukin-6, tumour necrosis factor [TNF] α). Toll-like receptors 2 and 4 and the CD40 molecule are also involved. A CD14-independent, but calcium-dependent response that leads to production of TNF α has also been identified.
However, some investigators have expressed concern that the in-vitro responses induced by recombinant heat shock proteins produced in *Escherichia coli* might result from the effects of lipopolysaccharide or other proteins, present either as a contaminant of the preparation or chaperoned by the heat shock protein under investigation.\(^{38,39}\) This is especially in view of the similarity in the receptors apparently used by heat shock proteins and lipopolysaccharide. Therefore, controls are essential in such experiments.

The intracellular localisation of eukaryotic heat shock proteins in healthy circumstances, the typical release of heat shock proteins from infectious agents,\(^{40}\) and the capacity of both human and prokaryotic (bacterial) heat shock proteins to elicit innate and adaptive proinflammatory responses seem to be consistent with the proposed role for these molecules as links between pathogenic processes involving necrotic cell death, and the induction of innate and adaptive immunity. However, further evidence suggests an alternative to this point of view.

**Extracellular heat shock proteins**

Heat shock proteins can be released from some viable (non-necrotic) mammalian cell types, including cultured rat embryo cells,\(^{41}\) human islet cells,\(^{42}\) rat glial cells and a human neuroblastoma cell line,\(^{43}\) and cultured vascular smooth muscle cells exposed to oxidative stress.\(^{44}\) These findings have profound implications for the perceived role of these proteins as exclusively proinflammatory intercellular signalling molecules and danger signals. Furthermore, Hsp60 and Hsp70 are present in the peripheral circulation of healthy individuals.\(^{45-51}\) Hsp60 is also present at high concentrations in people with early atherosclerosis,\(^{45,46}\) and Hsp70 concentrations are raised in patients with peripheral and renal vascular disease.\(^{52}\)

The mechanism by which heat shock proteins are released in vitro and in vivo, and their origin in vivo, has received little attention, and is not yet fully understood. However, findings of a seminal study in the late 1980s with cultured rat embryo cells, which showed the release of heat shock proteins from viable cells, suggested that release does not seem to be mediated by the common secretory pathway, since the inhibitors colchicine and monensin do not block secretion.\(^{47}\) Nor does release seem to be due to cell damage, since Hsp70 was not readily released from cells exposed to low concentrations of nonionic detergents. Rather, the mechanism seems to be selective, since release of Hsp70 is inhibited by the presence of the lysine analogue aminoethyl cysteine, suggesting that an altered structure or function prevents correct interaction with the specific secretory mechanisms.\(^{48}\)

Since evidence has emerged that heat shock proteins are present in, or can be released into, the extracellular environment in physiological conditions, and that these molecules can elicit a range of biological activities though interactions with specific cell surface receptors, their physiological function in this context should be re-examined.

**Sequence versus functional conservation**

One of the dogmas of heat shock protein biology is that the high degree of sequence homology between equivalent heat shock protein family members derived from prokaryotes and eukaryotes (about 50%) has led to a high degree of functional conservation. However, the rigidity of this concept is questioned by results of several studies, all of which suggest that heat shock proteins of the same family, but from different species, might have markedly different functions. First, a few bacteria, one of which is *Mycobacterium tuberculosis*, contain multiple genes encoding Hsp60 (chaperonin 60), and despite having greater than 73% aminoacid similarity, mycobacterial chaperonin 60-1 is between ten and 100 times more active in induction of cytokine secretion than is chaperonin 60-2 (otherwise known as Hsp65).\(^{53}\)

Secondly, chaperonin 60-3 from *Rhizobium leguminosarum* induces the production of a range of cytokines from human monocytes, but chaperonin 60-1, which has a 74% aminoacid sequence homology with chaperonin 60-3, shows no such activity.\(^{54}\) Thirdly, Hsp60 from the oral bacterium *Actinobacillus actinomycetemcomitans* and from *E coli* are potent stimulators of bone

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**Figure 1: Induction and regulation of heat shock protein expression**

Physical or chemical stress induces production of unfolded or misfolded proteins. Heat shock factor monomers in the cytoplasm form trimers, are phosphorylated, and translocate into the nucleus. HSF homotrimers bind to heat shock protein gene promoter regions, leading to induction of gene transcription. *Hsp70* gene transcription is downregulated by interaction of Hsp70 or HSBP1 with the HSF trimers.
resorption, whereas equivalent molecules from mycobacteria are not. Finally, the sequence-sensitivity of the biological function of these molecules is eloquently revealed by the recent observation that a sole aminoacid substitution in the Hsp60 molecule from E coli (GroEL) can transform the protein into a potent insecticidal toxin.

**Autoreactivity and anti-inflammatory action**

Heat shock proteins are immunodominant molecules and a substantial amount of the immune response to pathogenic microorganisms is directed towards peptides derived from these proteins. In view of the phylogenetic similarity between microbial and mammalian forms of these molecules, and the consequent potential for crossreactivity, these findings prompted the suggestions that heat shock proteins might act as potentially harmful autoantigens, and that immune recognition of crossreactive epitopes of heat shock proteins provides a link between infection and autoimmunity. Findings that implicate immunity to heat shock proteins (especially Hsp60 and Hsp70) in the pro-inflammatory cytokine TNF response to self-Hsp60 suppress the production of the synovial fluid of patients with rheumatoid arthritis in an autoimmune, inflammatory disease. The anti-inflammatory capacity of self-Hsp60 reactivity seems to be the dominant function; the administration of whole mycobacterial Hsp65, which contains the epitope that induces T-cell activation and can induce arthritis in rats when administered alone, does not induce the disease, due to the concomitant presence of conserved (self) epitopes that can dominantly downregulate the arthritogenic capacity of the non-conserved (non-self) epitopes. Although somewhat speculative, this finding might also provide insight into the way in which heat shock proteins derived from infectious agents induce immune responses; antibody responses to non-conserved (non-self) epitopes might be promoted by the concomitant generation of Th2 cytokines, such as interleukin-4, induced by conserved (self) epitopes.

In addition to arthritis, in which the induction or presence of self-Hsp60 and Hsp70 immune reactivity seems to attenuate the disease process, immunisation of recipient mice with mouse (self)-Hsp60, or Hsp60 peptides that have the capacity to shift Hsp60 reactivity from a proinflammatory Th1 phenotype towards a regulatory Th2 phenotype, can delay mouse skin allograft rejection.

Overall, these findings suggest that, rather than being proinflammatory, self-Hsp60 and self-Hsp70 T-cell reactivity are part of a normal immunoregulatory response that has the potential to dominantly control proinflammatory responses and inflammatory disease. Investigations aimed at specifically inducing immunoregulatory T cells reactive with self heat shock protein, and monitoring their influence on pro-inflammatory disease processes such as autoimmunity, cardiovascular disease and organ transplant rejection are therefore timely and warranted.

**Peptide-specific immunity**

**Tumours**

Work in the 1980s showed that heat shock proteins might be useful for induction of antigen-specific immunity. At this time, Pramod Srivastava and colleagues noted that immunisation of mice with a 96 kDa protein fractionated from a tumour cell lysate induced resistance to the same tumour cell from which the 96 kDa protein had originally been isolated. As intracellular chaperones, heat shock proteins bind to many peptides derived from the cells from which they are isolated—the so-called antigenic fingerprint or repertoire of that cell. The protein that induced tumour protection in Srivastava’s early work has been shown to be the heat shock protein gp96 (otherwise known as grp94); immunisation of mice with Hsp70, Hsp90, and gp96 isolated from mouse tumour cells induces tumour-specific cytolytic T cells and immunity against tumours. This immunity is towards tumour-derived peptides associated with the heat shock protein, rather than the heat shock proteins themselves. The heat shock proteins calreticulin, Hsp110, and grp170 have also been shown to elicit similar effects by apparently similar mechanisms.

Protection against tumours can also be elicited by Hsp70 and gp96 in amphibians (Xenopus), and the evolutionarily conserved nature of these responses strongly suggests that such strategies could be successfully developed for clinical use. Indeed, in preliminary clinical trials, cancer-specific CD8 T-cell responses were induced in six of twelve patients immunised with gp96-peptide complexes prepared from their own tumour. The great advantage of use of tumour-cell-derived heat shock proteins as the immunogen, is that identification of relevant tumour antigens is not necessary.

An interesting and potentially important feature of gp96 is its capacity to both induce and inhibit tumour peptide
Induction of pathway for MHC class I-restricted presentation to CD8+ proteins and peptides are directed into the intracellular these cells (figure 2).105  

Induces the maturation of, and cytokine secretion from, molecules such as CD80 and CD86, and gp96 also they must be mature and express essential costimulatory

In part, been elucidated. Antigen-presenting cells, such as dendritic cells, spontaneously internalise gp96 by

Specific immunity, dependent on the dose of gp96 that is administered. Low doses purified from methylcholanthrene-induced (Meth A) fibrosarcoma given to mice (2×1 μg intradermally) induces protective immunity to the tumour, whereas high doses (2×10 μg intradermally) do not.106 High doses of tumour-derived gp96 elicit an active, antigen-specific downregulation of tumour-specific immunity that can be adaptively transferred by CD4+ T cells purified from animals treated with high doses of tumour-derived gp96.106 However, the precise identity and phenotype of these cells has yet to be determined. These findings suggest that immunisation with gp96, either purified from appropriate tissues or chaperoning appropriate peptides, might be an effective strategy for downregulation of several antigen-specific inflammatory conditions. The mechanism by which high doses of gp96 can induce specific immunoregulatory activity is unknown, but is being actively investigated in the author’s laboratory.

By contrast, the mechanism by which tumour-derived gp96 can induce tumour-specific immunity has, at least in part, been elucidated. Antigen-presenting cells, such as dendritic cells, spontaneously internalise gp96 by receptor-mediated endocytosis100,102 through the α2-macroglobulin receptor (CD91 molecule)103 or a CD91-independent mechanism,104 or both. Chaperoned proteins and peptides are directed into the intracellular pathway for MHC class I-restricted presentation to CD8+ T cells.100,102 For dendritic cells to fully activate T cells, they must be mature and express essential costimulatory molecules such as CD80 and CD86, and gp96 also induces the maturation of, and cytokine secretion from, these cells (figure 2).105

The CD91 molecule is also common to other heat shock proteins that have the capacity to induce tumour-specific immunity (such as calreticulin, Hsp70, Hsp90).106 α2-macroglobulin, which is a serum protein and was originally described as the ligand for CD91, is also able to direct exogenous antigens into the endogenous pathway of antigen presentation through the same receptor; such a mechanism could allow continual sampling of the antigenic profile of the organism, and hence appropriate regulation of immune responses.107 Hsp60 does not bind to CD91; nor does tumour-derived Hsp60 induce tumour-specific immunity. The identification on macrophages of an Hsp60 binding site that is unique to this receptor suggests that Hsp60 has a role in immunoregulation that is distinct from that of the other heat shock proteins.108

Pathogens
The capacity of heat shock proteins to elicit specific immunity to infectious agents is also being investigated.109 Treatment of mice with a fusion protein (HspE7) incorporating an early viral protein (E7) of the human papillomavirus 16 has been shown to induce specific cytotoxic T-cell activity, and to confer protection against a mouse tumour cell expressing E7.110,111 Heat shock proteins isolated from SV40-transformed and influenza-infected cells, or a mixture of gp96 or Hsp70 reconstituted with specific cytotoxic T lymphocyte epitopes from SV40 and influenza virus, have been shown to elicit peptide-specific cytolytic T cells and protective antiviral immunity in mice.112-115 Similar results have been obtained with lymphocytic choriomeningitis virus.116 Immunity can also be induced to proteins covalently linked to appropriate heat shock proteins; immunisation of mice with the HIV-1 p24 protein covalently linked to mycobacterial Hsp70 elicits antibody, cytokine, and lymphocyte proliferative responses.117 Furthermore,
administration of recombinant fusion protein, consisting of mycobacterial Hsp65 and portions of the nucleoprotein antigen of influenza virus, elicits MHC class I restricted, nucleoprotein-specific cytotoxic T cells.\textsuperscript{11,12}

Clearly, in view of this evidence, heat shock proteins chaperone intracellular peptides and can deliver these peptides, concomitant with a proinflammatory stimulus, to antigen presenting cells for subsequent activation of peptide-specific T cells. This response provides an adaptive advantage, in that the release of heat shock proteins from cells undergoing necrosis promotes the activation of innate and adaptive immunity, and the crosspresentation of cytoplasmic protein-derived peptides. This method allows the organism to respond to cell death, and is especially advantageous in viral infections.\textsuperscript{13,14}

**Summary**

Much remains to be learnt about heat shock protein biology. In the past, reactivity to heat shock proteins has been associated with several kinds of disease, yet evidence now suggests that immune reactivity to self-derived molecules is anti-inflammatory, and attenuates proinflammatory conditions such as arthritis and organ transplant rejection. The physiological and immunological role of extracellular heat shock proteins and their intercellular signalling capacity should also be reconsidered, since these proteins can be released from viable cells, and are present in the peripheral circulation of healthy individuals.

Heat shock proteins are extremely potent molecules, the importance of which to physiological and immunological processes is indicated by the high degree to which their structure and function are phylogenetically conserved. Enhanced understanding of the various immunoregulatory mechanisms in which heat shock proteins are involved could help us to harness the power of these molecules for the control of inflammatory processes and the treatment of human disease.

**Conflict of interest statement**

None declared.

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