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Heat shock proteins in health and disease

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Abstract
Heat shock or stress proteins have been regarded as intracellular molecules that have a range of housekeeping and cytoprotective functions, only being released into the extracellular environment in pathological situations such as necrotic cell death. However, evidence is now accumulating to indicate that, under certain circumstances, these proteins can be released from cells in the absence of cellular necrosis, and that extracellular heat shock proteins have a range of immunoregulatory activities. The capacity of heat shock proteins to induce pro-inflammatory responses, together with the phylogenetic similarity between prokaryotic and eukaryotic heat shock proteins, has led to the proposition that these proteins provide a link between infection and autoimmune disease. Indeed, both elevated levels of antibodies to heat shock proteins and an enhanced immune reactivity to heat shock proteins have been noted in a variety of pathogenic disease states. However, further evaluation of heat shock protein reactivity in autoimmune disease and after transplantation has shown that, rather than promoting disease, reactivity to self-heat shock proteins can downregulate the disease process. It might be that self-reactivity to heat shock proteins is a physiological response that regulates the development and progression of pro-inflammatory immunity to these ubiquitously expressed molecules. The evolving evidence that heat shock proteins are present in the extracellular environment, that reactivity to heat shock proteins does not necessarily reflect adverse, pro-inflammatory responses and that the promotion of reactivity to self-heat shock proteins can downregulate pathogenic processes all suggest a potential role for heat shock proteins as therapeutic agents, rather than as therapeutic targets.

Keywords: Heat, proteins, disease, reactivity, therapeutic agents, therapeutic targets

Introduction
It was in 1962 that Ritossa and co-workers first discovered that subjecting *Drosophila melanogaster* larvae to temperature shock induced specific gene activation (Ritossa, 1962); however, it was not until 1974 that the first products of these genes were identified and the term 'heat shock protein' was adopted (Tissieres *et al*., 1974) [47]. Subsequent work has demonstrated that heat shock proteins are present, and can be induced; in all species and that they are among the most phylogenetically conserved proteins. Heat shock proteins are categorised into several families that are named on the basis of their approximate molecular mass (e.g. the 70 kDa Hsp70; Table 1). Under physiological conditions, some of these proteins function as molecular chaperones or proteases that have a number of intracellular functions. Chaperones are involved in the assembly and folding of oligomeric proteins, whereas proteases such as the ubiquitin-dependent proteasome mediate the degradation of damaged proteins (Gething and Sambrook, 1992) [17].

The term heat shock proteins is somewhat of a misnomer, as they are not induced solely by heat shock. Indeed, in addition to being constitutively expressed (making up 5–10% of the total protein content under normal growth conditions), these proteins can be markedly induced (up to 15% of the total cellular protein content) by a range of cellular insults including increased temperature, oxidative stress, nutritional deficiencies, ultraviolet irradiation, exposure to chemicals (e.g. ethanol), viral infection, and ischemia–reperfusion injury (Welch, 1993). Stressors that cause protein unfolding, misfolding or aggregation trigger a stress response that leads to the induction of gene transcription for proteins with the capacity to stabilise and re-fold proteins, thereby re-establishing the balance between protein synthesis, assembly and degradation. Regulation of heat shock protein gene transcription is mediated by the interaction of the heat shock factor (HSF) transcription factors (of which the principal one in vertebrates is HSF1) with heat shock elements (HSEs) in the heat shock protein gene promoter regions (Morimoto, 1994) [28].

Table 1. Major mammalian heat shock proteins and their function (tab001gps)

Major family and members	Cellular localisation	Cellular function
Small		
αB-crystallin	Cytoplasm	Cytoskeletal stabilisation
Hsp27	Cytoplasm/nucleus	Actin dynamics
Heme oxygenase, Hsp32	Cytoplasm	Haeme catabolism, antioxidant properties
Hsp60 or chaperonins		
Hsp60	Mitochondria	Both: bind to partially folded polypeptides and assist correct folding; assemble multimeric complexes
TCP-1	Cytoplasm	
Hsp70		
Hsp70 (inducible)	Cytoplasm/nucleus	All: bind to extended polypeptides; prevent aggregation of unfolded peptides; dissociate some oligomers; bind ATP and show ATPase activity Hsp70 is involved in regulation of HSF1 activity and the repression of heat shock protein gene transcription
Hsc70 (cognate)	Cytoplasm/peroxisome	
Grp78/BiP	ER	
mtHsp70/Grp75	Mitochondria	
Hsp90		
Hsp90 (α and β)	Cytoplasm	All: bind to other proteins; regulate protein activity; prevent aggregation of re-folded peptide; correct assembly and folding of newly synthesised protein Hsp90 appears to be involved in maintaining the HSF1 monomeric state in non-stressful conditions; represents 1–2% of total protein
Grp94/gp96/Hsp100	ER	
Hsp110		
Hsp110 (human)	Nucleolus/cytoplasm	Thermal tolerance
Apg-1 (mouse)	Cytoplasm	Protein refolding
Hsp105	Cytoplasm	

Abbreviations: ER, endoplasmic reticulum; TCP-1, tailless complex polypeptide; Grp, glucose-regulated protein; Hsp, heat shock protein; BiP, immunoglobulin heavy chain binding protein; mtHsp70, mitochondrial Hsp70; HSF1, heat shock factor 1; Apg-1, protein kinase essential for autophagy.

In the unstressed state, HSF1 is present in the cytoplasm as a latent monomeric molecule that is unable to bind to DNA. Under stressful conditions, HSF1 is hyperphosphorylated in a *ras*-dependent manner by members of the mitogen-activated protein kinase (MAPK) subfamilies (e.g. ERK1, JNK/SAPK, p38 protein kinase; (Kim, 1997). HSF1 is converted to phosphorylated trimers with the capacity to bind DNA, and translocates from the cytoplasm to the nucleus (Fig. 1).

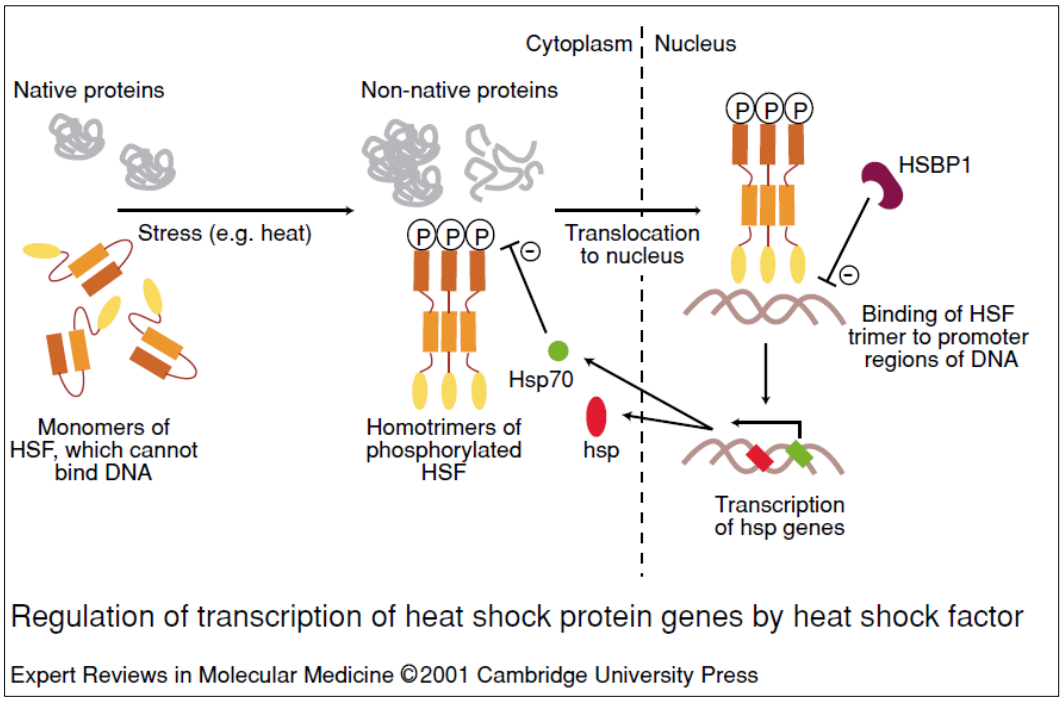

Fig 1: Regulation of transcription of heat shock protein genes by heat shock factor. Heat shock factor (HSF) is present in the cytoplasm as a latent monomeric molecule that is unable to bind to DNA. Under stressful conditions, the flux of non-native proteins (which are non-functional, prone to aggregation, protease-sensitive, and bind to chaperones) leads to phosphorylation (P) and trimerisation of HSF. The trimers translocate to the nucleus, bind the promoter regions of heat shock protein (hsp) genes and mediate hsp gene transcription. The activity of HSF trimers is downregulated by hsps (e.g. Hsp70) and the heat shock binding protein 1 (HSBP1) that is found in the nucleus.

The signal that activates HSF1 is thought to be a flux of newly synthesised non-native proteins (Morimoto, 1994) [28]. The consequences of HSF binding to its target, and the events that results in the ensuing transcription of heat shock genes, have been reviewed previously (Wu, 1995) [54]. The generation of heat shock proteins must be only transient, even if exposure to stress is over a prolonged period, as a continued presence of heat shock proteins would adversely influence protein homeostasis and a variety of intracellular functions. One mechanism by which the activity of HSF1 is regulated is via the binding of Hsp70 to its transactivation domain, thereby leading to repression of heat shock gene transcription (Shi *et al*., 1998) [42]. The interaction between Hsp70 and HSF1 has no effect on DNA binding or the stress induced phosphorylation state of HSF1 (Shi *et al*., 1998) [42]. A second mechanism that regulates heat shock protein synthesis is the interaction between heat shock protein binding factor 1 (HSBP1) and the active trimeric form of HSF1 and Hsp70, thereby inhibiting the capacity of HSF1 to bind to DNA (Satyal, 1998) [40]. HSBP1 is predominantly localised in the nucleus and levels of HSBP1 mRNA have been shown to be present at high levels in a variety of cell lines and animal tissues and to be unaffected by heat shock (Satyal, 1998) [40]. This article reviews the current literature on heat shock proteins and their influence on pathogenic processes such as autoimmunity, organ allograft rejection and vascular disease. It highlights the evolving evidence that rather than being exclusively intracellular, heat shock proteins are present in, and can be released into, the extracellular compartment under physiological conditions and elicit a range of functions. Given the functional versatility of heat shock proteins and their capacity to mediate both induction and regulation of immunity, further studies aimed at understanding the mechanisms underlying these functions might reveal strategies by which heat shock proteins can be used as therapeutic agents either for the generation of protective immunity or for the down regulation of deleterious inflammatory conditions.

Heat shock proteins as molecular chaperones:- Molecular chaperones are defined as 'proteins that assist the correct non-covalent assembly of other protein-containing structures in vivo but are not permanent components of these structures when they are performing their normal biological functions' (Ellis, 1996). An alternative definition is that 'a molecular chaperone is a protein that binds to and stabilises an otherwise unstable conformer of another protein, and by controlled binding and release of the substrate protein facilitates its correct fate in vivo, be it folding, oligomeric assembly, transport to another subcellular compartment, or controlled switching between active/inactive conformations' (Hendrick and Hartl, 1993) [19]. The precise nature of peptide binding and the factors involved appear to be chaperone dependent. Whereas ATP binding is important for the release of peptides from Hsp70, BiP (immunoglobulin heavy chain binding protein) and Hsp90 (Grenert *et al*., 1999) [18], its role in peptide binding to and unloading from gp96, the endoplasmic reticulum paralogue of Hsp90, is unclear (Wearsch and Nicchitta, 1997) [50]. Not all stress proteins function as molecular chaperones; however, those that do fulfil an essential intracellular role and, in the extracellular compartment, have the capacity to mediate the induction of peptide-specific immunity, as is described later.

Potential pathogenic role for heat shock proteins:- Heat shock proteins, particularly those of the Hsp60 and Hsp70 families, are immunodominant molecules, and a significant element of the immune response to pathogenic microorganisms is directed towards peptides derived from heat shock proteins (Kaufmann, 1990) [22]. This phenomenon, together with the ubiquitous nature of human heat shock proteins and the high degree of sequence homology between mammalian and bacterial heat shock protein cognates (~50–60% identical residues in the case of the Hsp60 family) has led to debate as to whether the immune system recognises heat shock proteins as dominant microbial antigens or potentially harmful self-antigens (Kaufmann, 1990) [22]. It has also been suggested that heat shock proteins might provide a link between infection and autoimmunity, either through recognition of conserved epitopes or via cross-reactivity/molecular mimicry (Lamb, 1989) [25]. Evidence for a link between heat shock protein reactivity and disease pathogenesis, particularly autoimmune disease, vascular disease and organ allograft rejection, has arisen from several studies.

Heat shock proteins and heat shock protein reactivity in autoimmune disease:

Hsp60: Several investigations have implicated Hsp60 and immune reactivity to members of the Hsp60 family in autoimmune diseases, the best studied of which are arthritis and diabetes.

Hsp60 is expressed in the synovial tissue of patients with rheumatoid arthritis (RA) and juvenile chronic arthritis (Boog, C.J. 1992) [6], and T cells derived from the synovial fluid are activated by mycobacterial Hsp65 (Pope *et al*., 1992) [33]. T-cell reactivity to self-Hsp60 has been reported in patients with RA; immortalised B cells from the synovial tissue of RA patients show specificity for bacterial Hsp60; and elevated levels of circulating antibodies to Hsp60 are present in children with juvenile chronic arthritis (de Graeff-Meeder, 1993) [9]. T-cell-mediated responses to mycobacterial Hsp65 have also been implicated in experimental models of arthritis, and disease can be initiated in rats by the transfer of T-cell clones specific for mycobacterial Hsp65 (van Eden, 1988) [48]. In addition, antibodies to Hsp65 are elevated in mice with pristane-induced arthritis (Thompson, 1990). Evidence of a role for Hsp60 in type 1 diabetes [insulin-dependent diabetes mellitus (IDDM)] is somewhat equivocal. Supporting such a role is evidence that naive T cells from non-obese diabetic (NOD) mice can be activated by both self-Hsp60 and mycobacterial Hsp 60, that anti-Hsp60 T cells can mediate insulitis and hyperglycaemia in the NOD mouse, and that peripheral blood T cells from patients with IDDM demonstrate a heightened proliferative response to human Hsp60 and Hsp60 peptides (Abulafia-Lapid, 1999) [1]. However, in NOD mice, immunity to auto antigens other than heat shock proteins, such as glutamic acid decarboxylase 65 (GAD), appears much earlier than responsiveness to mycobacterial Hsp65, thereby arguing against an essential role for heat shock proteins in disease induction in this model. In addition, no evidence for serological immunity to islet cell heat shock proteins has been reported in IDDM. Evidence of a role for Hsp60 in the pathogenesis of multiple sclerosis (MS) is less apparent. Hsp60 expression has been identified in chronic MS plaques (Raine, 1996) [33], and a humoral response to Hsp60 has been detected in the cerebrospinal fluid of patients with MS; however, the latter is not specific for MS

and is also present in a number of chronic degenerative conditions. Nor is peripheral blood lymphocyte reactivity to Hsp60 altered in MS patients (Salvetti, 1992) [39].

Hsp70: In contrast to the findings for Hsp60, Hsp70 has been implicated as a potential auto antigen in MS. In IDDM, the preferential expression of Hsp70 by cells, but not cells, in the islets of Langerhans might be important for the understanding of autoimmune destruction of cells in this disease (Strandell, 1995) [44]. Auto antibodies to the constitutive form of Hsp70 (Hsc70) have been identified in a proportion of patients with primary biliary cirrhosis (45.7%) and patients with autoimmune hepatitis patients (52.9%), but not in patients with chronic hepatitis B or C infection. Reactivity to Hsp70 has also been implicated in the induction of disease in toxin induced interstitial nephritis.

Heat shock proteins and heat shock protein reactivity in transplantation: In addition to autoimmune disease, heat shock proteins and reactivity to heat shock proteins have been associated with allograft rejection. Heat shock proteins are induced during graft preservation, ischemia–reperfusion and surgery (Knowlton *et al*., 1991) [24], and by the inflammatory process of the rejection response, including the localised production of cytokines by infiltrating leukocytes. In rats, Hsp70 gene and protein expression are increased in rejecting cardiac allografts, and graft-infiltrating lymphocytes proliferate in response to recombinant mycobacterial Hsp65 and Hsp71 (Mehta, 1997) [26]. Heat shock protein expression is also induced in the intestinal epithelium and lamina propria after rat small-bowel transplantation and appears, in part at least, to be resistant to immunosuppression with tacrolimus (Ogita, 2000) [30]. In humans, heat shock protein expression is increased in rejecting lungs; T cells from rejected renal grafts respond to Hsp72; and mycobacterial Hsp65-induced growth of graft-infiltrating lymphocytes from endomyocardial biopsies correlates with cardiac graft rejection (Moliterno, 1995) [27]. These findings have led to the proposition that heat shock protein expression in allograft tissue induces heat shock protein reactivity, thereby promoting the development of acute and chronic graft rejection (Duquesnoy, 1999) [10]. However, heat shock proteins are cytoprotective molecules and their induction in the peri- and immediate post-transplantation periods is likely to be a protective response targeted towards the maintenance of cell and tissue integrity. This is supported by reports that heat shock proteins attenuate preservation and ischemia–reperfusion injury (Okubo, 2001) [31], and that they protect endothelial cells from neutrophil-mediated necrosis and a variety of cell types from oxidative injury. In addition, lower levels of Hsp70 in pre-liver-transplant biopsies and organ perforates are associated with early graft loss (Flohe, 1998) [13]. The precise influence of heat shock proteins on allograft survival is currently unclear. A direct involvement of Hsp60 in the rejection process has been suggested by the observations that skin from transgenic mice over expressing Hsp60 transplanted into allogeneic recipients is rejected more rapidly than skin transplanted from wild-type donors (Birk, 1999) [4]. By contrast, skin transplanted into Hsp60-transgenic mice, in which spontaneous autoimmunity to Hsp60 is reduced, is rejected more slowly than skin grafted into wild-type recipients. However, further studies are required to define more clearly a direct role for heat shock proteins in the induction and progression of allograft rejection.

Heat shock proteins and heat shock protein reactivity in vascular disease: It is now apparent that there is an inflammatory component to vascular disease that involves the accumulation of monocytes and activated T cells in atherosclerotic lesions and the localised presence of pro-inflammatory cytokines (Frostegard, 1999) [5]. Evidence also suggests that the immunological component of the development of atherosclerosis might, at least in part, involve the expression of, and reactivity to, heat shock proteins. The evidence for this proposition has arisen from three findings. First, the intensity of heat shock protein expression positively correlates with the severity of atherosclerosis; second, there is a localised enrichment of T cells in the lesion, and this is of particular interest given the capacity of T cells to directly recognise and respond to autologous heat shock proteins; and third, immunisation with recombinant mycobacterial Hsp65 can induce atherosclerotic lesions in normocholesterolaemic rabbits. A role for Hsp60 in the induction of the inflammatory response that characterises atherosclerosis has also been suggested. Lipid laden cells formed from the uptake of oxidised low-density lipoprotein (LDL), via a 'scavenger' receptor that does not recognise native LDL, are a principal component of the atherosclerotic plaque. Exposure of the monocytic cell lines U937 and HL60 to oxidised LDL induces marked expression of Hsp60 (Frostegard, 1996) [14]. These findings suggest that the inflammatory response associated with atherosclerosis might in part be promoted by the activation of T cells reactive with the Hsp60 that is expressed on monocytes within the lesion or released locally. Localised expression of heat shock proteins might also be influenced by hemodynamic factors, as raised blood pressure has direct effects on the vasculature and vessels subjected to greater mechanical and shear stress express heat shock proteins and are more prone to the development of atherosclerosis (Schett, 1995) [41]. Humoral responses to heat shock proteins have also been implicated in vascular disease. Elevated levels of circulating antibody to the mycobacterial 65 kDa heat shock protein have been reported in carotid atherosclerosis, coronary heart disease and borderline hypertension. Levels of antibodies to human Hsp60 are also raised in peripheral vascular disease (Wright, 2000) [53]. The in vivo physiological significance of antibodies to heat shock proteins in the pathogenesis of vascular disease has yet to be clearly established. However, they have been shown to mediate endothelial cell cytotoxicity, and the observation that anti-Hsp65/60 antibodies in individuals with atherosclerosis recognise three distinct, self- Hsp65/60 sequences might implicate them in the initiation of atherosclerosis via an autoimmune type mechanism. Levels of anti-Hsp65 antibodies might have diagnostic value as titres have recently been shown to predict the 5-year mortality of patients with carotid atherosclerosis (Xu, 1999) [55].

Heat shock proteins in normal aging: Increasing age is associated with a reduced capacity to maintain homeostasis in all physiological systems and it might be that this results, in part at least, from a parallel and progressive decline in the ability to produce heat shock proteins. If this is so, an attenuated heat shock protein response could contribute to the increased susceptibility to environmental challenges and the more prevalent morbidity and mortality seen in aged individuals (Richardson and Holbrook, 1996). In vitro studies have shown that Hsp70 expression in heat-stressed lung cells, hepatocytes and liver, splenocytes, myocardium and

mononuclear cells is reduced with increasing age, as is the induction of Hsp70 expression in response to ischemia and mitogenic stimulation (Faassen, 1989). Hsp70 gene expression declines during normal aging in human retina (Bernstein, 2000), and heat shock-induced Hsp70 expression is decreased in senescent and late-passage cells, both of which suggest that the process of aging itself might be associated with reduced Hsp70 production. Although currently uncertain, possible mechanisms underlying an attenuated stress response during aging might include a reduced availability of HSF or age-associated increases in abnormal or denatured proteins that could interfere with HSF binding to HSEs (Munro and Pelham, 1985). Alternatively, age-related decreases in the capacity of HSF to undergo the oligomerisation that is essential for binding to HSEs might be involved.

Heat shock protein release: Heat shock proteins are typically regarded as intracellular molecules; however, it is now apparent that heat shock proteins can be released into the extracellular compartment. It was reported in the late 1980s that heat shock proteins could be released from cultured rat embryo cells. Heat treatment of the cells broadened the spectrum of proteins released, from a small set of proteins including Hsc70 to a larger set including Hsp70 and Hsp110. It was suggested that the release of heat shock proteins might have resulted from changes in pH and gas tension, disruption of the diffusion layer at the cell surface or mechanical stresses associated with in vitro manipulations. The release of heat shock proteins did not appear to be mediated via the common secretory pathway, as it was not blocked by the inhibitors colchicine and monensin. Nor did it result from cell lysis, as exposure of cells to low concentrations of non-ionic detergents indicated that Hsp70 is not readily released from damaged cells. Instead, a selective mechanism has been suggested. Evidence cited in favour of this is the fact that Hsp70 synthesised in the presence of the lysine amino acid analogue amino ethyl cysteine was not released from cells, probably due to an altered structure or function preventing its correct interaction with the specific release mechanism. The precise mechanism(s) by which heat shock proteins are actively released by viable cells has yet to be elucidated. Subsequent studies have shown heat shock proteins to be released from a variety of cells including cultured human islet cells (Child, 1995), rat glial cells and a human neuroblastoma cell line, as well as cultured vascular smooth muscle cells exposed to reactive oxygen species. In these studies, release did not appear to be a result of cellular necrosis. Myocardial injury induces Hsp60 release from rat hearts in organ culture; however, this most probably resulted from myocardial necrosis. A selective release of heat shock proteins from necrotic, but not apoptotic, cells has also been described. The physiological basis for heat shock protein release from intact (non-necrotic) cells has yet to be fully understood. Glia–axon transfer proteins, which include Hsp70, Hsc70 and Hsp100, are transferred from adjacent glial cells to the squid giant axon, and heat shock protein release might be an altruistic response on the part of one cell for the protection of adjacent cells. Hsp60 and Hsp70 can be detected in the serum of normal individuals (Pockley *et al*., 1998), and in keeping with a reduced capacity to generate stress responses with aging, serum levels of heat shock proteins also decline with age. Elevated levels of heat shock proteins have been observed in subjects with borderline hypertension, as well as in patients with peripheral and renal vascular disease. It is interesting to note that, in the borderline hypertension study, levels of Hsp60 correlated with the presence of atherosclerosis, and a similar finding in a population-based study of clinically normal subjects has been reported (Xu, 2000) [56]. Hsp70 release into the serum following myocardial infarction has also been reported in patients who have experienced preceding angina. Although it is clear that heat shock proteins are present and can be released into the peripheral circulation in response to several conditions, the physiological role of these proteins has yet to be defined. The identification of heat shock proteins and antibodies directed against heat shock proteins in normal individuals (Xu, 2000) [56] indicates that their presence is not limited to disease. The emerging evidence that stress proteins can interact with cell-surface receptors and elicit a range of biological activities including the down regulation of autoimmune disease (see below) suggests that they might be involved in regulating immunity to the ubiquitously expressed and highly conserved heat shock proteins, and in the maintenance of the 'normal' state.

Heat shock proteins as immunomodulators and intercellular signalling molecules: Heat shock proteins have been shown to have a number of immunological effects (Fig. 2).

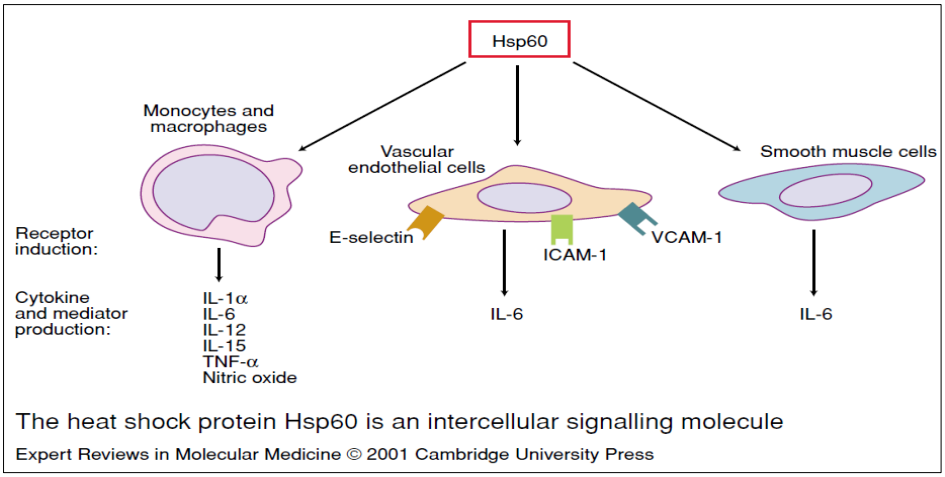

Fig 2: The heat shock protein Hsp60 as an intercellular signalling molecule. Hsp60 has been shown to have several immunological effects, including the induction of pro-inflammatory cytokine secretion from, and adhesion molecule expression on, a number of myeloid and vascular cell types, including smooth muscle cells. Abbreviations: ICAM-1, intercellular adhesion molecule 1; IL, interleukin; TNF-_, tumour necrosis factor; VCAM-1, vascular cell adhesion molecule.

Bacterial and mycobacterial heat shock proteins induce pro-inflammatory cytokine expression (Galdiero *et al*., 1997) [16], and bacterial heat shock proteins induce intercellular cell adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) expression on human vascular endothelial cells (Galdiero *et al*., 1997) [16]. Chlamydial and human Hsp60 activate human vascular endothelial cells to express E-selectin, ICAM-1 and VCAM-1, and activate vascular endothelial cells, smooth muscle cells and macrophages to secrete interleukin 6 (IL-6). With kinetics similar to those induced by lipopolysaccharide (LPS), mammalian Hsp60 has also been demonstrated to induce a rapid release of tumour necrosis factor and nitric oxide from macrophages, as well as the expression of IL-12 and IL-15. Evidence that heat shock proteins can elicit a range of biological and pro-inflammatory effects has stimulated interest in finding cell-surface receptors for these molecules. The existence of specific receptors for heat shock proteins was initially confirmed in studies demonstrating that the presentation of heat shock protein-associated peptides by major histocompatibility complex (MHC) class I molecules required receptor mediated endocytosis (Singh-Jasuja, 2000) [43], and the identities of receptors for heat shock proteins are now becoming apparent.

Human Hsp60 activates human peripheral blood mononuclear cells and monocytes through the CD14 antigen, using the signalling pathway also utilised by LPS (Fig. 3). Signalling is also mediated by the Toll-like receptor 4, which is an important mediator of innate immunity and LPS signalling in murine cells (Hoshino, 1999). Hsp70 has also been shown to bind with high affinity to human monocytes and the CD14 molecule is involved in Hsp70-induced activation. There appears to be a CD14-dependent interaction leading to intracellular calcium fluxes and the induction of pro-inflammatory cytokines (IL-1, IL-6, TNF), and a CD14-independent, but calcium-dependent, response that leads to production (Fig. 3). The cell-surface receptor for gp96 on dendritic cells (DCs) is downregulated as they undergo maturation. This receptor has now been identified as the CD91 molecule (which is also known as the Alpha 2-macroglobulin receptor or the LDL-related protein), to which it binds directly. In addition to gp96, the CD91 molecule has since been shown to be a common receptor for Hsp70, Hsp90 and calreticulin, all of which can mediate the induction of peptide-specific immunity as summarised below. It is now clear that heat shock proteins have an intercellular signalling role as well as a chaperone function, and Asea and colleagues have coined the term ‘chaperokine’ for these ubiquitously expressed and versatile families of molecules.

The potential therapeutic value of heat shock proteins: Some of the earliest evidence that heat shock proteins might have a therapeutic potential arose from the observations that exogenous members of the Hsp70 family protect spinal sensory neurons from axotomy-induced death and cultured aortic cells from heat stress (Johnson *et al*., 1990) [21]. Subsequent work demonstrated that exogenous Hsp70 could also protect rabbit arterial smooth muscle cells subjected to serum deprivation, by a mechanism that involved cell association but not internalisation. There are a number of indications in which heat shock proteins might be of therapeutic value. To date, the majority of studies have focused on either their capacity to regulate inflammatory responses in autoimmune disease or their ability to induce peptide-specific immune responses against tumours and pathogenic organisms.

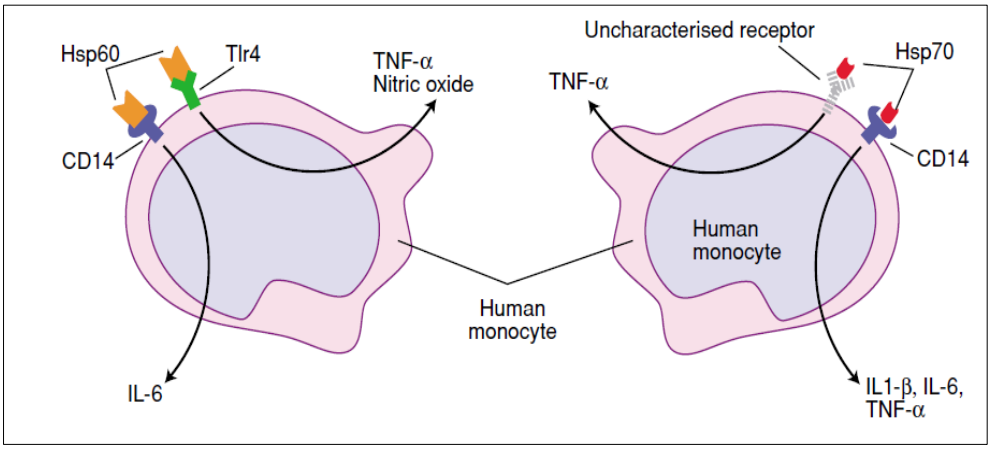

Fig 3: The heat shock proteins Hsp60 and Hsp70 induce pro-inflammatory cytokine secretion from monocytes. Hsp60 induces several pro-inflammatory cytokines, and the signalling pathways responsible are yet to be fully clarified. It induces the secretion of interleukin 6 (IL-6) from human monocytes via signalling through CD14 and p38 mitogen-activated protein kinase, and binds to the Toll-like receptor 4 complex (Tlr4), for which CD14 is a co-receptor, to induce the expression of TNF-_ and nitric oxide. It also induces the expression of a range of cytokines, including IL-12 and IL-15, through as yet uncharacterized pathways. Hsp70 acts through a CD14-dependent pathway to stimulate IL-1_, IL-6 and TNF-_ production, and also a CD14-independent pathway that leads to TNF-_ production, suggesting that CD14 is also a co-receptor for an as yet uncharacterised Hsp70 receptor. Both Hsp70 pathways are calcium dependent.

Modulation of inflammatory disease: Despite the association of heat shock protein expression and heat shock protein reactivity with autoimmunity, several observations question the proposition that self-heat shock protein reactivity has a direct pro-inflammatory role in autoimmune disease. Although the literature on the subject is less comprehensive, the situation might also be the same in the case of transplantation. The normal T-cell repertoire includes cells reactive against autologous heat shock proteins (Ramage, 1999). Although heat shock proteins have been considered to be intracellular proteins and therefore normally shielded from self-reactive T cells, it is now known that they are released from a variety of normal cells in culture, expressed on the cell surface and present in the peripheral circulation of normal individuals (Xu, 2000) [56]. As discussed below, it appears that T-cell reactivity to self-heat shock proteins is a protective

phenotype, and it is interesting to note that peripheral blood T-cell responsiveness to self- and non-self-heat shock proteins segregates with their expression of CD45 isotypes. Human Hsp60 activates CD45RA+RO−(naive) T cells, bacterial-specific peptides activate CD45RA−RO+ (memory) T cells and bacterial Hsp60 activates both CD45RA+RO+ and CD45RA−RO+ T cells. The observation that both types of T-cell subset are activated by bacterial Hsp60 indicates that T cells can recognise and respond to conserved (self) epitopes on the whole bacterial molecule. What is currently not known is the cytokine secreting profiles of cells responding to the different heat shock proteins or specific peptides derived from them. This is of particular importance given the evidence from autoimmune disease that self-heat shock protein reactivity appears to induce a regulatory phenotype, whereas reactivity to non-self induces a proinflammatory phenotype, as is discussed below.

Autoimmune disease: In contrast to their proposed capacity to promote pathogenic processes such as autoimmune disease, T-cell reactivity to heat shock proteins can also protect against disease, as demonstrated by the capacity of Hsp60 and Hsp70 to downregulate autoimmune disease (Anderton and van Eden, 1996). An insight into the possible mechanisms by which self-heat shock proteins might modulate autoimmune disease has come from the work of de Graeff-Meeder and co-workers. In patients with juvenile chronic arthritis, in whom the disease follows a relapsing–remitting rather than progressive course, the presence of circulating T cells responsive to human (self) Hsp60 was beneficial. These T cells were of the regulatory T helper 2 (Th2) phenotype, whereas T cells reactive with the 65 kDa mycobacterial antigen Hsp65 displayed the inflammatory Th1 phenotype and their presence correlated with disease severity. It has also been shown that stimulation of T cells from the synovial fluid of RA patients with human, but not bacterial, Hsp60 can stimulate regulatory responses (van Roon, 1997). The apparent capacity of self-heat shock protein to modulate autoimmune disease in the clinical situation confirms data indicating that protection by Hsp60 in experimental autoimmune disease appears to be elicited by autoreactive T cells recognising specific sequences of self-stress proteins. The ability of Hsp60 peptides to modulate adjuvant arthritis appears to reside in the capacity of induced regulatory T cells to produce IL-10, as well as IL-4 and interferon (IFN). Members of the Hsp70 family can also elicit protection from autoimmune disease, and Hsp71 from *Mycobacterium tuberculosis* can modulate experimental rat arthritis. In a similar way to Hsp60, the capacity of peptides from mycobacterial Hsp70 to protect against the subsequent induction of adjuvant arthritis in Lewis rats appears to be mediated via the production of suppressive cytokines, including IL-10 (Wendling, 2000).

Allograft immunity*:* The capacity of heat shock proteins to modify allograft rejection responses is less well defined; however, immunising recipient animals with self-Hsp60, or Hsp60 peptides that have the capacity to shift Hsp60 reactivity from a Th1 to a Th2 phenotype, can delay murine skin allograft rejection. These studies indicate that rather than being pro-inflammatory, self-Hsp T-cell reactivity could be part of a normal immunoregulatory T-cell response that has the potential to control inflammatory disease.

Induction of peptide-specific immunity: In addition to their capacity to downregulate pro-inflammatory conditions the potential value of heat shock proteins for inducing protective immunity has been explored by several groups, primarily in the areas of tumour immunity and infectious disease.

Tumour immunity: It has been known for some time that heat shock proteins bind peptide (Young, 1993) and that heat shock proteins purified from cells chaperone a large number of peptides derived from the cells from which they are isolated – the so-called 'antigenic repertoire' of that cell. Early studies showed that fractionated tumour cell lysates have the capacity to reduce tumour cell growth in mice. Since then, it has been well established that immunisation of mice with Hsp70, Hsp90 and gp96 isolated from murine tumour cells induces anti-tumour immunity and tumour-specific cytolytic T cells, and that the immunity results from tumour derived peptides associated with the heat shock protein rather than from the heat shock proteins themselves. More recently, it has been reported that calreticulin, Hsp110 and grp170 can also be used in heat shock protein-based cancer immunotherapy. The finding that the immunological properties of heat shock proteins and the capacity of Hsp70 and gp96 to induce tumour protection as shown in rodent models are also observed in amphibians (*Xenopus*) indicates the evolutionary conserved nature of these functions, and strongly supports the successful translation of these strategies into the clinical environment. In that regard, preliminary clinical trials have demonstrated the induction of cancer-specific CD8+ T-cell responses in 6/12 patients immunised with gp96–peptide complexes prepared from their own tumour. Clearly, the capacity of tumour derived heat shock proteins to induce specific and protective immunity might have profound effects on the treatment and management of patients with malignant disease. However, the immunological effects of heat shock proteins purified from tumour cells have a dual nature. The induction of immunity to methylcholanthrene-induced fibrosarcoma by the administration of gp96 purified from the tumour displays a consistent dose restriction: two Intradermal administrations of <1 μg gp96
is ineffective; two doses of 1 μg induce immunity and provide optimal protection against tumour growth; and two doses of 10 μg do not protect (Ref. 155). The lack of protection at high doses of tumour-derived gp96 is an active, antigen specific down regulation of tumour-specific immunity that can be adoptively transferred by CD4+ T cells purified from animals treated with high doses of tumour-derived gp96 (Chandawarkar *et al*., 1999). These findings are exciting as they suggest that immunisation with heat shock proteins that are chaperoning clinically relevant peptides might be an effective strategy for down regulating several diseases including autoimmunity. In that regard, gp96 purified from liver and pancreas of C57/B6 mice has been shown to elicit protection from autoimmune damage in NOD mice that is long term and can be adoptively transferred. The mechanisms by which heat shock proteins can mediate peptide-specific immunity are yet to be clearly defined. Antigen-presenting cells (APCs) such as DCs and monocytes play a key role, as they have been shown to internalise heat shock proteins spontaneously by receptor mediated endocytosis via the CD91 receptor, and direct chaperoned proteins/peptides into the intracellular pathway for MHC class I-restricted presentation to CD8+ T cells, concomitant with the induction of DC maturation and cytokine secretion (Fig. 4).

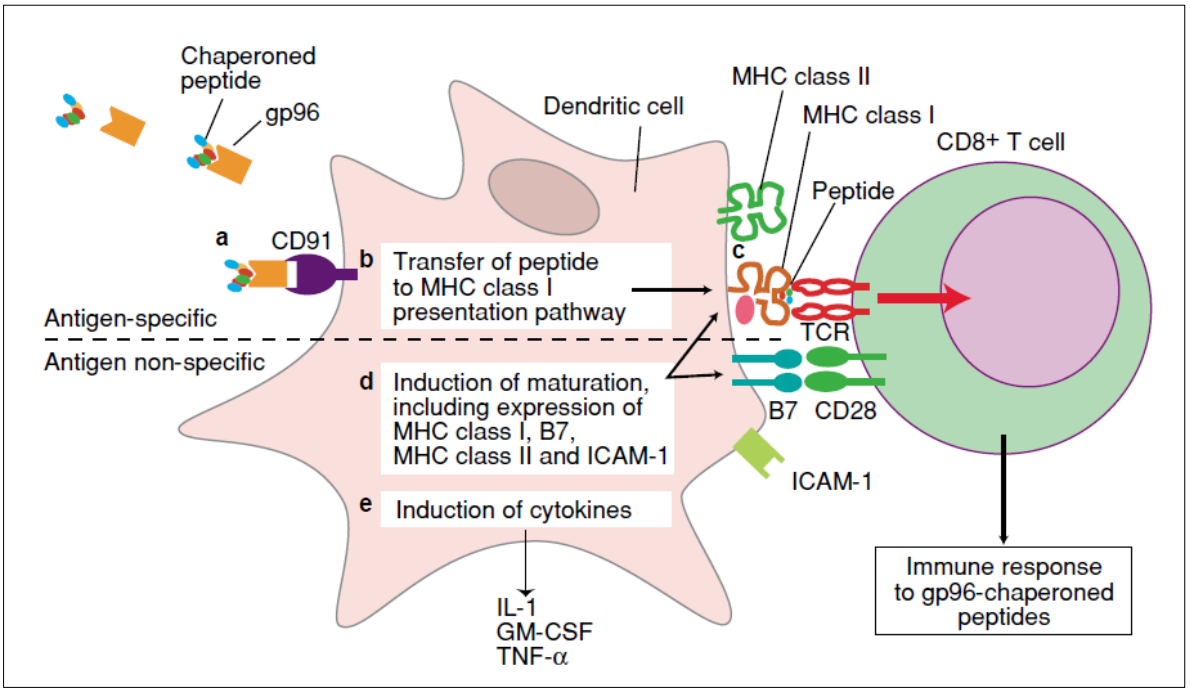

Fig 4: The heat shock protein gp96 delivers antigenic peptides and maturation signals to antigen-presenting cells, and induces release of cytokines. (a) gp96–peptide complexes bind to CD91 and are taken up by dendritic cells via receptor-mediated endocytosis. (b) Peptides carried on gp96 are thus delivered to the major histocompatibility complex (MHC) class I presentation pathway and are (c) represented on the cell surface in association with MHC class I antigens for recognition by antigen-specific CD8+ T cells via the T-cell receptor (TCR) and associated molecules. (d) gp96 also delivers maturation signals to the dendritic cells, and induces the expression of MHC antigens, co-stimulatory molecules such as B7 (which binds to CD28), and intercellular adhesion molecule 1 (ICAM-1). This, combined with (e) the induction of proinflammatory cytokines, promotes the generation of immune responses to gp96-chaperoned peptides. Abbreviations: GM-CSF, granulocyte–macrophage colony-stimulating factor; IL-1, interleukin 1, TNF-tumour necrosis factor.

It is interesting to note that Alpha 2-macroglobulin, the originally described ligand for CD91, is also able to channel exogenous antigens into the endogenous pathway of antigen presentation via the same receptor. The mechanism by which high doses of heat shock protein can induce immunoregulation is also unclear. In addition to inducing DC maturation and the expression of antigen presenting and co-stimulatory molecules (Singh-Jasuja, 2000) [43], gp96 promotes the accumulation of DCs into the draining lymph node. It might be that larger quantities of gp96 lead to a greater APC-mediated cytokine signal and the preferential induction of regulatory CD4+ T cells.

Induction of immunity to infectious agents: - The capacity of heat shock proteins to chaperone antigenic repertoires and induce specific immunity to them has led to studies evaluating whether the administration of heat shock proteins from virally transformed cells, or cells infected by pathogenic organisms, would induce specific immunity. This has been shown to be the case, and specific immunity has been induced by the administration of heat shock proteins isolated from SV40-transformed and influenza-infected cells (Blachere, 1993). Peptide-specific cytolytic T cells and protective anti-viral immunity can also be induced by immunising mice with a mixture of gp96 or Hsp70 reconstituted with specific cytotoxic T lymphocyte epitopes from SV40, influenza virus and lymphocytic choriomeningitis virus. An alternative approach is to covalently link appropriate antigens to heat shock proteins; indeed, the immunisation of mice with the human immunodeficiency virus 1 (HIV-1) p24 protein covalently linked to mycobacterial Hsp70 elicits antibody, cytokine and lymphocyte proliferative responses (Suzue and Young, 1996). Covalent linking is not necessary for the induction of immunity, as non-covalently bound MHC class II influenza virus peptide can also induce immune reactivity to the Hsp70-binding peptide (Roman and Moreno, 1996) [38].

Conclusion

There are clearly many aspects of heat shock protein biology that remain puzzling. On the one hand, reactivity to heat shock proteins appears to be associated with several pathological disease states, yet, on the other hand, heat shock proteins are ubiquitously expressed and reactivity to self-derived molecules can confer protection against a number of pro-inflammatory conditions. Future work will need to translate the experimental data on the capacity of heat shock proteins to induce tumour protection and immunity to infectious agents into the clinical environment and more fully evaluate the mechanisms by which these effects are induced and regulated. The observations that heat shock proteins can be released and that they can directly or indirectly elicit potent immunoregulatory activities give a new perspective on the roles of heat shock proteins and anti-heat shock protein reactivity in autoimmunity, transplantation, vascular disease and other conditions. It is the qualitative nature of the response to heat shock proteins rather than its presence per se that is important, and future experimental and clinical studies attempting to associate heat shock proteins with disease pathogenesis need to be designed to address these issues. It is also important to define definitively the specificity of any responses, so that the outcome can be attributed to self- or nonself-reactivity. By doing this, the contribution of infective agents to pathogenic processes such as autoimmunity and vascular disease can be truly evaluated. Heat shock proteins are extremely versatile and potent molecules, the importance of which to biological processes is highlighted by the high

degree to which their structure and function are phylogenetically conserved. Our knowledge of the physiological role of heat shock proteins is currently limited; however, a better understanding of their function and thereby the acquisition of the capacity to harness their power might lead to their use as therapeutic agents and revolutionise clinical practice in a number of areas.

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