Extracellular Heat Shock Proteins: Alarmins for the Host Immune System

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Abstract: Heat shock proteins (HSPs) are molecular chaperones that facilitate the proper folding and assembly of nascent polypeptides and assist in the refolding and stabilization of damaged polypeptides. Through these largely intracellular functions, the HSPs maintain homeostasis and assure cell survival. However, a growing body of literature suggests that HSPs have important effects in the extracellular environment as well. Extracellular HSPs are released from damaged or stressed cells and appear to act as local "danger signals" that activate stress response programs in surrounding cells. Importantly, extracellular HSPs have been shown to activate the host innate and adaptive immune response. With this in mind, extracellular HSPs are commonly included in a growing list of a family of proteins known as danger-associated molecular patterns (DAMPs) or alarmins, which trigger an immune response to tissue injury, such as may occur with trauma, ischemia-reperfusion injury, oxidative stress, etc. Extracellular HSPs, including Hsp72 (HSPA), Hsp27 (HSPB1), Hsp90 (HSPC), Hsp60 (HSPD), and Chaperonin/Hsp10 (HSPE) are especially attractrive candidates for DAMPs or alarmins which may be particularly relevant in the pathophysiology of the sepsis syndrome.

Keywords: Heat shock proteins (HSPs), danger-associated molecular patterns (DAMPs), Extracellular HSPs, polypeptides.

INTRODUCTION

Virtually all cells respond to stress through the activation of primitive, evolutionarily conserved genetic programs that maintain homeostasis and assure survival. *Stress adaptation* (also known in the literature as *tolerance*, *desensitization*, *conditioning*, or *reprogramming*) is a common paradigm found throughout nature in which a primary exposure of a cell to a stressful stimulus results in an adaptive (frequently protective) response, such that a second exposure to the same stimulus produces a minimal response. More importantly, this adaptive response is not unique to the original stimulus, in that exposure to a different stressful stimulus is also associated with a minimal response. This particular phenomenon is often called *cross-tolerance* or *cross-adaptation* [1-2]. The heat shock response is one of the more commonly described forms of stress adaptation and was first described nearly 50 years ago [3-4]. The heat shock response, also frequently referred to simply as the *stress response*, is an ancient, highly conserved, endogenous cellular defense mechanism characterized by the rapid upregulation of a specific class of proteins known collectively as heat shock proteins (HSP) or stress proteins [5]. The structure, mode of regulation, and function of HSPs are phylogenetically conserved among different species, and HSPs have been isolated from virtually every class of living organism to date, including both prokaryotes and eukaryotes. These proteins range in molecular weight from 7 kDa to 110 kDa and have been found in virtually every part of the cell, including the nucleus, cytoplasm, and mitochondria [1-2, 6-7]. By convention, the HSPs are grouped and classified into families based upon their molecular weight, e.g. Hsp70 refers to the 70 kDa family of HSPs, though the nomenclature of human HSPs has recently been standardized (Table **1**) [8]. Herein, we will refer to both the traditional and standardized nomenclature in order to avoid confusion. There are also an increasing number of proteins that have well defined cellular functions not directly related to cellular stress, but have been demonstrated to be expressed in response to heat shock and other cellular stresses [9-10]. These so-called *moonlighting proteins* include ubiquitin [11-12]; heme oxygenase [13]; inhibitor of κB, IκBα [14]; endothelial nitric oxide synthase, eNOS [15]; and mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1) [16].

The HSPs are generally thought to maintain cellular homeostasis by acting as molecular chaperones, facilitating the proper folding and assembly of nascent polypeptides and assisting in the refolding and stabilization of damaged peptides [6-7, 17]. Consistent with their function as molecular chaperones, HSPs have traditionally been considered to be exclusively intracellular proteins. However, not long after the HSPs were first discovered, Morton and colleagues [18] discovered a circulating immunosuppressive protein, termed early pregnancy factor (EPF), which was later found to be a mitochondrial HSP known as chaperonin (Cpn)/Hsp10 (HSPE) [19]. Later, Hightower and Guidon

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Table 1. Major Heat Shock Protein Families

Name	Size (kDa)	Localization	Bacterial Homolog	Some Known and Possible Functions
Ubiquitin	8	Cytosol/nucleus	—	Nonlysosomal degradation pathways
HSP 27 (HSPB1)	27	Cytosol/nucleus	—	Regulator of actin cytoskeleton; molecular chaperone; cytoprotection
Heme oxygenase	32	Bound to ER, extends to cytoplasm	—	Degradation of heme to bilirubin; resistance to oxidant stress
HSP 47	47	ER	—	Collagen chaperone
HSP 60 (HSPD)	60	Mitochondria	Gro EL	Molecular chaperone
HSP 70 (HSPA)	72	Cytosol/nucleus	Dna K	Highly stress inducible; involved in cytoprotection against diverse agents
	73	Cytosol/nucleus	—	Constitutively expressed chaperone
HSP 90 (HSPC)	90	Cytosol/nucleus	htpG	Regulation of steroid hormone activity
HSP 110	110	Nucleolus/cytosol	Clp family	Protects nucleoli from stress

[20] demonstrated that Hsp70 (HSPA) was released into the extracellular fluid by cultured rat embryo cells in response to thermal stress. Release of Hsp70 (HSPA) occurred in the absence of cell death and was not inhibited by either monensin or colchicine, suggesting that release occurred via a non-classical secretory pathway (see below). More importantly, release was inhibited when these proteins were synthesized in the presence of the lysine analogue aminoethyl cysteine, suggesting that proper folding of Hsp70 (HSPA) was necessary for secretion [20]. Since that time, there has been a virtual explosion of literature on the biology of extracellular HSPs. Perhaps even more intriguing is the relatively recent recognition that extracellular HSPs possess the ability to stimulate many cells of the innate and adaptive immune systems [21]. Herein, we will briefly review the current roles that extracellular HSPs play in both the innate and adaptive immune responses. We will focus primarily upon host-derived HSPs, rather than bacterial-derived HSPs. Moreover, we will focus primarily upon five specific families of HSPs – Hsp70 (HSPA), Hsp27 (HSPB1), Hsp90 (HSPC), Hsp60 (HSPD), and Hsp10 (HSPE) as the extracellular roles of these HSP families are the best characterized to date.

DANGER SIGNALS, ALARMINS, AND DAMPS

The host immune response has evolved over time to protect organisms from infection, injury, and subsequent death. Danger signals [22-24] are molecules that alert and activate the host immune response – these danger signals may come from either the internal or the external environments and activate cells of either the innate or adaptive immune response. External or exogenous danger signals have traditionally been called pathogen-associated molecular patterns (PAMPs) and include pathogen-derived proteins, nucleic acids, and lipids such as lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acid, CpG DNA, and flagellin. These PAMPs are recognized by a surprisingly limited number of highly conserved pattern recognition receptors (PRRs), which include the Toll-like receptors (TLRs) and the nucleotide-binding oligomerization domain (NOD) receptors. Perhaps it is more than just a mere coincidence that these same PRR appear to recognize endogenous danger signals as well [25] – hence the term, danger-associated molecular pattern or DAMP [26]. Endogenous danger signals may be secreted by damaged cells or released rather non-specifically by necrotic cells to act in an autocrine, paracrine, or endocrine manner, thereby alerting the host to the presence of tissue injury. Oppenheim was the first to coin the term *alarmin* for these endogenous DAMPs [27]. In summary then, exogenous DAMPs (LPS, CpG DNA, etc) usually trigger an immune response to an infectious insult (e.g. bacteremia), while endogenous DAMPs or alarmins trigger an immune response to tissue injury, such as may occur with trauma, ischemia-reperfusion injury, oxidative stress, etc. Several candidate alarmins have been described, including high mobility group box 1 protein (HMGB1) [28-33], uric acid [34-39], and extracellular HSPs [6, 26, 33, 40-43], among others.

Several properties would suggest that extracellular HSPs are biologically plausible and likely candidates to serve as alarmins [44-46]. First, collectively, the HSPs are the most abundant intracellular proteins, representing up to 10% of the total protein in the cell. For example, 1 g of tissue contains approximately 2.5 mg of HSPs. The lysis of 10^5 to 10^6 cells (approximately 1 mg) would result in the release of approximately 2 μg HSP, translating to a local concentration of 1-2 mg/mL at the tissue level [47]. Second, several of the HSPs are markedly induced (up to 15% of the total

Table 2. "Leaderless" Proteins Involved in the Host Immune Response

PROTEIN	EXTRACELLULAR FUNCTION	INTRACELLULAR FUNCTION
IL-1α	Pro-inflammatory cytokine	Activator of transcription
IL-1β	Pro-inflammatory cytokine	-
IL-18	Pro-inflammatory cytokine	-
Caspase I (ICE)	?	IL-1/IL-18 converting enzyme
HMGB1	Pro-inflammatory cytokine	Chromatin component
IL-16	Pro-inflammatory cytokine	-
MIF	Pro-inflammatory cytokine	Transcription factor modulator

intracellular protein content) in response to a diverse range of cellular insults, including increased temperature, oxidative stress, glucose deprivation, chemical exposure, I/R injury, ultraviolet radiation, and infectious agents such as LPS. Third, HSPs are ancient, highly conserved molecules that have been identified in virtually every organism, both prokaryotic and eukaryotic, that have been examined to date. In comparison, LPS, an important "exogenous" danger signal, appeared relatively late on the evolutionary time-scale and is much less ubiquitous, being unique to only gram-negative bacteria. It is tempting to speculate that the programmed response to the exogenous "danger signal", LPS, is modeled on the more primitive programmed response to the endogenous "danger signal" [47]. Fourth, HSPs are highly immunomodulatory and have the capacity to mediate the induction of peptide-specific immunity. For example, as molecular chaperones, HSPs bind to many peptides derived from the cells from which they are isolated. HSP-peptide complexes elicit potent T cell responses against the chaperoned peptide as well as the cell type from which the chaperoned peptide is derived, including tumors and viruses, and vaccination with HSP-tumor peptide complexes as an immunotherapy for cancer is an active area of investigation [44-45, 47-50]. Similarly, HSP-pathogen-derived peptide complexes have the capacity to elicit a pathogen-specific immune response [45]. Finally, HSPs themselves, especially members of the Hsp70 (HSPA) and Hsp60 (HSPD) families, have the capacity to activate the host innate immune response, resulting in dendritic cell activation and maturation, activation of complement, and release of proinflammatory cytokines [51-62].

POTENTIAL MECHANISMS OF RELEASE OF THE HEAT SHOCK PROTEINS

One argument that is frequently cited against the purported role for extracellular HSPs as endogenous danger signals has been the lack of any clearly defined mechanism to explain their release into the extracellular environment [63]. Collectively, the HSPs lack the classic N-terminus leader sequence necessary for the canonical protein secretory pathway [64]. Of interest, several additional proteins involved in the host immune response similarly lack the classic N-terminus leader sequence and are secreted through non-classical pathways (Table **2**) [65]. What many investigators fail to recognize, however, is that there are diverse secretory pathways utilized by both prokaryotic and eukaryotic organisms, many of which have only recently been elucidated [7, 66-69]. While HSPs undoubtedly are released during necrotic cell death, most studies suggest that this is not the major mechanism of release [20, 64, 70-73]. For example, several studies have shown that release of HSPs generally occurs in the absence of significant cell death [20, 64, 70-76]. Perhaps the best evidence comes from an experiment in which serum Hsp72 (HSPA) concentrations were measured in Sprague-Dawley rats following exposure to a cat. Rats were physically separated from the cat using a clear Plexi-glass shield. In this experiment, cat exposure induced a significant increase in both corticosterone and extracellular Hsp72 (HSPA) production. This response was not observed in adrenalectomized rats [77]. Finally, there are several studies demonstrating increased serum Hsp72 (HSPA) levels in patients following exercise [78-79]. Collectively, these studies strongly suggest that HSP are released via a specific, though as yet undefined, secretory mechanism.

Hsp72 (HSPA) is perhaps the best and most widely studied extracellular heat shock protein and was first reported in cultured rat embryo cells following exposure to increased temperature in the late 1980's [80]. The mechanism of release appeared to be specific, in that Hsp72 (HSPA) release could not be reproduced by induction of cell lysis through exposure to non-ionic detergents. However, the mechanism did not appear to involve classic secretory pathways either, as it was not inhibited by either colchicine or monensin, both of which inhibit this pathway. Finally, Hsp72 (HSPA) synthesized in the presence of a lysine amino acid analogue (aminoethyl cysteine) was not released from these cells, suggesting that the altered protein structure prevented interaction with an as yet unidentified, but specific secretory mechanism [80]. Several groups, including ours [70, 80-86] have shown that viable cells release Hsp72 (HSPA) in a specific and inhibitable manner. Monensin and brefeldin A are inhibitors of the classic endoplasmic reticulum (ER)/Golgi protein transport and secretory pathways. We have shown that Hsp72 (HSPA) release from THP-1 cells is not inhibited by either monensin or brefeldin A [70]. In this particular study, release of extracellular Hsp72 (HSPA) was early and sustained up to 24 hours following heat shock and did not require new protein synthesis. Others have shown that Hsp72 (HSPA) release

from peripheral blood monocytes (PBMC) is inhibited by brefeldin A, but not monensin [84, 86]. Hsp72 (HSPA) release has also inhibited by methylamine and methyl-β-cyclodextrin, both of which inhibit protein secretion via lysosomal pathways [86]. Recent studies suggest that Hsp72 (HSPA) is actively released via an exosome-dependent, non-classical protein secretory pathway [74, 76, 87].

THE IMMUNOMODULATORY EFFECTS OF EXTRACELLULAR HEAT SHOCK PROTEINS

Heat Shock Protein 72 (HSPA)

Extracellular Hsp72 (HSPA) is a highly immunomodulatory protein with effects on both the innate and adaptive immune responses [6-7, 17]. In this regard, extracellular Hsp72 (HSPA) appears to specifically interact with a wide myriad of cell surface receptors, including TLR2, TLR4, LOX-1, CD91, CD94 (C-type lectin), CD40 and chemokine receptor CCR5 [45-46, 61, 88-90]. These studies have used a variety of approaches to demonstrate physical interactions between Hsp72 and the purported receptor protein and are reviewed further in an excellent discussion by Binder and colleagues [91]. Hsp72 (HSPA), consistent with its role as a "chaperokine" appears to stimulate pro-inflammatory gene expression in macrophage and monocyte cell lines via both TLR-2 and TLR-4 [51, 53-54]. In addition, we have shown that extracellular Hsp72 (HSPA) stimulates pro-inflammatory gene expression in cultured bronchial epithelial cells [92], hepatocytes [93], neutrophils [71], and cardiomyocytes (*Wheeler, unpublished data*). Moreover, intratracheal administration of extracellular Hsp72 (HSPA) induced pro-inflammatory gene expression in mice, primarily through TLR4 and NF-κB activation [92]. Our group has also demonstrated that extracellular Hsp72 (HSPA) induces the endotoxin tolerance phenotype in THP-1 cells. Preconditioning with low-dose Hsp72 (HSPA) reprogrammed the subsequent response to LPS, such that LPS-mediated TNF-α gene expression was markedly abrogated [70, 94]. Subsequent experiments have demonstrated that these effects are not exclusive to TLR4 ligands, but that Hsp72 (HSPA) preconditioning also reprograms the response to TLR2 ligands, such as Pam3CysK, lipoteichoic acid, peptidoglycan, heat-killed *Staphylococcus aureus* (HKSA), and Hsp72 (HSPA) itself (*Wheeler, unpublished data*).

Some of the immunomodulatory effects of extracellular Hsp72 (HSPA) and other heat shock proteins have been ascribed to the presence of bacterial contaminants in the recombinant protein [95-100]. However, extensive and elegantly performed experimental controls strongly suggest that it is indeed the heat shock protein itself that is responsible for these effects. Typically, these controls include (i) measuring the LPS content of the recombinant protein via *Limulus* amebocyte lysate assay (LAL) and adding this concentration to control; (ii) directly inhibiting LPS activity with concomitant treatment with polymyxin B, lipid A, or lipid IVa treatment; (iii) heat denaturation or trypsin pretreatment of the recombinant protein to confim loss of immunomodulatory activity; and (iv) comparing immunomodulatory activity of the recombinant protein to irrelevant polypeptide sequences or similar sized proteins [101]. In addition, Hsp72 (HSPA) purified from liver cell lysates induced secretion of IL-1β, TNF-α, and IL-12 by murine macrophages [52]. Similarly, we have shown that endogenously released Hsp72 (HSPA) induces cellular reprogramming in THP-1 cells [70]. Neutralizing antibodies directed against extracellular Hsp72 (HSPA) have been shown to inhibit these pro-inflammatory effects [29]. Finally, an important consideration is whether extracellular Hsp72 (HSPA) chaperones small amounts of LPS and other PAMPs to their respective cell surface receptors, thereby augmenting stimulation of the innate immune response [101]. With this in mind, the effects of Hsp72 (HSPA) on the innate immune response in the absence of bacterial contaminants may be moot.

Extracellular Hsp72 (HSPA) has additional effects on the innate immune response. For example, extracellular Hsp72 (HSPA) both directly [102-103] and indirectly (by stimulating increased chemokine synthesis) [71, 92] induces neutrophil and dendritic cell chemotaxis. In addition, extracellular Hsp72 (HSPA) appears to play an important role in priming dendritic cells, macrophages, monocytes, and natural killer cells [40, 46, 63, 101]. To this end, extracellular Hsp72 (HSPA) enhances synthesis of pro-inflammatory cytokines and upregulates cell surface expression of MHC class II and other important co-stimulatory molecules on dendritic cells [51, 53-56]. Extracellular Hsp72 (HSPA) also directly stimulates macrophage phagocytic activity [104-106]. Extracellular Hsp72 (HSPA) induces natural killer (NK) cell activity [107-110]. NK cells play an important role in both the innate and adaptive immune responses. These unique cells exert cytotoxicity through both direct effects (via perforin production) and via mediation of antibody-dependent cytotoxicity.

Extracellular Hsp72 (HSPA) also exerts important effects on the adaptive immune response. For example, Hsp72 is able to chaperone a large variety of immunomodulatory peptides derived from tumors, virally-infected cells, etc and deliver them to antigen presenting cells. These chaperone/peptide complexes subsequently generate peptide-specific T-lymphocyte-mediated responses both *in vitro* and *in vivo*. Several groups are currently attempting to exploit this unique facet of heat shock protein biology for vaccine development and cancer immunotherapy [40, 46, 49, 111-113].

A few studies have been able to demonstrate uptake of extracellular Hsp72 (HSPA). More importantly, uptake of extracellular Hsp72 (HSPA) appears to confer some degree of stress tolerance [85, 114-117]. While the data are far from complete, these studies at least suggest a potential autocrine or paracrine role for extracellular Hsp72 (HSPA), further supporting the concept that heat shock proteins are endogenous danger signals or alarmins.

Extracellular Hsp72 (HSPA) has been found in the blood, cerebrospinal fluid, and bronchoalveolar lavage fluid of critically ill children and adults with a variety of inflammatory disease states. For example, increased extracellular Hsp72 levels following cardiopulmonary bypass have been detected in both adults [118-119] and children (*Wheeler, unpublished data*). Increased extracellular Hsp72 (HSPA) levels correlate with poor outcome in

critically ill patients with liver disease [120], coronary artery disease [121-124], pre-eclampsia [125], sickle cell disease vaso-occlusive crisis [126], diabetic ketoacidosis [127], and septic shock [128]. Extracellular Hsp72 (HSPA) levels in the cerebrospinal fluid (CSF) of children with traumatic brain injury also appear to correlate with poor outcome [129]. Finally, extracellular Hsp72 (HSPA) has been found in the pulmonary edema fluid of adults with acute lung injury [130] and the urine of children following renal transplantation [131]. Given the signaling properties recently ascribed to Hsp72 (HSPA), these data suggest that the release of Hsp72 (HSPA) could potentiate an already active host immune response, thereby leading to poor outcome [132]. Alternatively, extracellular Hsp72 (HSPA) could serve an as yet undefined cytoprotective function at lower levels as a normal response to infection or stress, and once a certain critical threshold is attained, it could potentiate the dysregulated inflammatory response that subsequently results in multiple organ failure. Recent experimental data [133] and the finding that extracellular Hsp72 (HSPA) levels > 15 ng/mL correlated with improved outcome following multiple trauma in adults [134] support this concept. As such, it is tempting to speculate that lower levels of extracellular Hsp72 (HSPA) "cool down" the host inflammatory response, while higher levels further stimulate and/or augment the host inflammatory response. These important questions remain an active focus of investigation in many laboratories, including our own.

Hsp27 (HSPB1)

Hsp27 (HSPB1) is a member of the so-called small heat shock protein family (HSPB). Within quiescent cells, it assists in the degradation and removal of damaged proteins and also slows the rate of actin microfilament polymerization [135]. Like several members of the heat shock protein family, Hsp27 (HSPB1) is highly stress inducible. Following cell stress, Hsp27 (HSPB1) is phosporylated and loses the ability to slow the rate of actin polymerization, resulting in stabilization of the cellular cytoskeleton. Additionally, Hsp27 (HSPB1) has been shown to protect cells against apoptosis through the prevention of downstream caspase activation [136].

Hsp27 (HSPB1) is released from peripheral blood monocytes following treatment with TNF-α [137]. Extracellular Hsp27 (HSPB1) appears to possess anti-inflammatory properties, with the target cell type determining the functionality. However, the receptor through which extracellular Hsp27 (HSPB1) inhibits inflammation is as yet poorly defined. Hsp27 (HSPB1) inhibits neutrophil apoptosis and potentially allows for exaggerated tissue destruction during sepsis, trauma, and acute lung injury [138]. In stark contrast to the exogenous danger signal, LPS, Hsp27 (HSPB1) inhibits neutrophil apoptosis without increasing pro-inflammatory cytokine production [138]. Hsp27 (HSPB1) induces expression of the anti-inflammatory cytokine, IL-10, in monocytes, primarily through the p38 protein kinase pathway, with only a mild increase in TNF-α levels [139-140]. Hsp27 (HSPB1) also inhibits monocyte differentiation into mature dendritic cells or macrophages[141].

These anti-inflammatory effects may be exploited by tumor cells, as the dampening of the host immune response may allow tumor progression and metastasis [142]. For example, increased serum levels of Hsp27 (HSPB1) have been associated with worse outcome in women with breast, ovarian, and uterine cancer [143]. In contrast, antibodies to Hsp27 (HSPB1) have been identified in the blood of women with breast cancer and other gynecologic cancers [144-145]. Those women with the highest levels of anti-Hsp27 serum antibodies seemed to have improved breast cancer survival [146]. Increased tumor expression of Hsp27 (HSPB1) is associated with shorter cancer free periods and advanced cancer staging, possibly due to the inhibition of tumor cell apoptosis [147]. Other studies have contradicted these findings, with no demonstrated association between serum Hsp27 (HSPB1) levels and cancer survival [147-148]. Hsp27 (HSPB1) also appears to be released from some brain tumor cells to dampen the immune response, inhibit apoptosis, and allow for tumor growth, progression, and metastasis [149]. The anti-inflammatory properties of Hsp27 (HSPB1) may be potentially exploited for the treatment of inflammatory disease processes. For example, Hsp27 (HSPB1) administration improves motor neuron survival for up to one week following nerve transection in neonatal mice [150].

In addition to playing a role in tumor growth and progression, extracellular Hsp27 (HSPB1) appears to play a role in the pathophysiology of cardiovascular disease. For example, Hsp27 (HSPB1) blocks lipid uptake by competitive inhibition of a low-density lipoprotein (LDL) scavenger receptor-A and decreases atherosclerotic plaque formation *in vitro* [140]. Clinically, extracellular Hsp27 (HSPB1) levels may be a reasonable biomarker for atherosclerosis disease progression [151-152]. Several studies have demonstrated an inverse association between Hsp27 (HSPB1) levels and atherosclerosis [151, 153-155]. However, a prospective study of 255 healthy women showed an inverse association with age but not serum Hsp27 (HSPB1) levels and future major cardiovascular events [154].

Higher levels of Hsp27 (HSPB1) have also been shown to protect mouse hearts from reperfusion injury and myocardial infarction[156]. In a small adult study, the expression of Hsp27 in transplanted hearts in acute rejection was elevated and thought to be a self-protective mechanism[157]. Further corroborating this theory, the vessels of transplanted hearts with higher phosphorylated levels of Hsp27 (HSPB1) upon biopsy demonstrated less cardiac allograft vasculopathy when compared to transplanted heart vessels with vasculopathy [158]. Similarly, and perhaps more relevant to the present discussion, there have been a few studies showing that elevated levels of Hsp27 (HSPB1) and/or decreased levels of autoantibodies to Hsp27 (HSPB1) correlate with improved outcome in patients with acute coronary syndrome [153, 159-160].

Hsp90 (HSPC)

Hsp90 (HSPC) is one of the most abundant proteins inside the cell. Hsp90 (HSPC) plays an important role in maintaining normal homeostasis and acts as a scaffolding protein for several key enzyme and signal transduction systems [161-163]. So far, however, there have been

comparatively few reports describing Hsp90 (HSPC) in the extracellular environment. We have shown that increased plasma Hsp90 (HSPC) levels correlate with worse outcome in critically ill children with septic shock (*Wheeler, unpublished data*). In addition, extracellular Hsp90 (HSPC) induces IL-8 gene expression in cultured vascular smooth muscle cells in a TLR4- and NF-B-dependent manner [164]. Finally, a human recombinant antibody directed against yeast-derived Hsp90 (HSPC) has been used to treat critically ill patients with invasive candidiasis . In this study, recombinant antibody to Hsp90 plus lipid-associated amphotericin B produced significant clinical and culture-confirmed improvement in outcome for patients with invasive candidiasis [165-168]. Given these data, it is very likely that future studies will demonstrate functional roles for extracellular Hsp90 (HSPC).

Hsp60 (HSPD)

Hsp60 (HSPD) is a key mitochondrial chaperone that forms a complex with the chaperonin (Cpn)/Hsp10 (HSPE) [169-170]. Extracellular human Hsp60 (HSPD) appears to be involved in many autoimmune and inflammatory processes within the body. Some of these processes are thought to be triggered by a number of infectious agents through molecular mimicry, in which the initial host immune response to microbial-derived Hsp60 (HSPD) leads to a subsequent cross reaction to the host-derived Hsp60 (HSPD). For example, both *Chlamydia pneumoniae* Hsp60 (HSPD) and the host human Hsp60 (HSPD) have been detected in atheromas [171]. Both of these molecules were shown to have the ability to activate host macrophages and endothelial cells contributing to the progression of atherosclerosis [171]. For the purposes of this review however, only research involving isolated human Hsp60 will be discussed.

Circulating antibodies to human Hsp60 (HSPD) appear to play a role in the pathophysiology of a number of vasculopathies and related illnesses. For example, in an animal model of atherosclerosis, auto-antibodies to Hsp60 were shown to be involved in disease progression [172]. Increased circulating levels of anti-Hsp60 (HSPD) antibodies have been found in patients with carotid artery atherosclerosis [173]. Elevated Hsp60 (HSPD) antibodies were also found in patients with other vasculopathies, such as borderline hypertension [174]. Increased circulating anti-Hsp60 (HSPD) antibody titers have also been found in patients with coronary artery disease [175] and correlate with the presence and severity of disease after adjusting for traditional risk factors [176]. Interestingly, the antibody titers in coronary artery disease patients decreased after an acute myocardial infarction [175]. The authors speculated that a soluble form of Hsp60 (HSPD) must be released from the ischemic myocardial tissue to complex with the anti-Hsp60 (HSPD) antibodies [175]. This hypothesis was confirmed by several independent groups showing increased levels of Hsp60 (HSPD) following acute myocardial infarction [177-178]. Similarly, increased circulating levels of both Hsp60 (HSPD) and anti-Hsp60 antibodies have been associated with an increased risk of developing coronary artery disease [178] and appear to correlate with increased severity of disease, even controlling for all other risk factors [179]. In a follow-up surveillance study enrolling healthy civil service workers, increased plasma Hsp60 (HSPD) levels correlated with increased plasma TNF-α, psychological distress (primarily in women), low socioeconomic status, and social isolation [180], resulting in an increased propensity for coronary artery disease.

Xu and colleagues discovered an association between elevated circulating levels of Hsp60 (HSPD) and the progression and severity of carotid artery atherosclerosis [181]. This association was further investigated prospectively. Patients with a sustained elevation of serum Hsp60 (HSPD) were found to be at risk for the development of early atherosclerosis [182]. High levels were also found in patients with borderline hypertension which was again associated with the development of early cardiovascular disease [183]. However, when Hsp60 (HSPD) was measured in patients with chronic hypertension and chronic vascular disease, these levels were found to be similar to healthy controls [121, 184], potentially suggesting increased binding to anti-Hsp60 (HSPD) antibodies.

Extracellular Hsp60 (HSPD) plays a role in the pathophysiology of many autoimmune diseases [185]. For example, elevated IgG antibodies to Hsp60 (HSPD) are found in the sera of patients with rheumatic autoimmune diseases [186]. Host-derived Hsp60 (HSPD) appears to play a role in the regulation of autoimmune arthritis in rats [187-188]. An antigen-specific T-cell response to Hsp60 (HSPD) was associated with improved outcome in patients with juvenile idiopathic arthritis (JIA) [189-190], possibly through increased IL-10 production by regulatory T-cells [191]. Armed with this data, vaccines against Hsp60 (HSPD) have shown promising results in models of arthritis and cyclophosphamide-accelerated diabetes [192-194].

It is becoming clearer that extracellular Hsp60 (HSPD) plays a role in inflammation and the body's immune response. To this end, we have shown that increased circulating Hsp60 (HSPD) levels correlates with poor outcome in critically ill children with septic shock [195]. Similar to extracellular Hsp72 (HSPA), Hsp60 (HSPD) appears to act primarily via the TLR4 pathway [196-197], though activation of the TLR2 pathway has also been observed [196]. Hsp60 (HSPD) activates human macrophages and dendritic cells to produce Th1 inflammatory cytokines such as TNFα and interferon (IFN)-γ [198-199]. However, determining the specific cytokine profile may be dose dependent as both Th1 and Th2 cytokine profiles resulted when dendritic cells were treated with lower concentrations of Hsp60 (HSPD) [199]. Similar to LPS and extracellular Hsp72 (HSPA), Hsp60 (HSPD) may also be able to promote inflammation initially and then induce a state of tolerance [200-201], akin to the endotoxin tolerance phenotype. Again, the potential effects of contamination of the recombinant Hsp60 (HSPD) protein with bacterial products must be considered and assessed with the use of adequate controls (see discussion above).

Hsp60 (HSPD) also appears to activate the adaptive immune response. For example, Hsp60 (HSPD) can inhibit pro-inflammatory cytokine production and shift to an anti-inflammatory Th2 profile in T lymphocytes [202]. However,

other studies have shown that Hsp60 (HSPD) increases IFN-γ production in T lymphocytes [203]. Similarly, extracellular Hsp60 (HSPD) has been shown to activate B-cells in a mixed Th1 and Th2 manner with B-cells producing both IFN-γ and IL-10 [204].

Chaperonin (Cpn)/Hsp10 (HSPE)

As discussed briefly above, Cpn/Hsp10 (HSPE) was the first heat shock protein to be isolated outside the cell. In this case, Cpn/Hsp10 (HSPE) was isolated from the serum of pregnant females as early pregnancy factor (EPF) [19, 205]. EPF was originally believed to be essential for the initiation and maintenance of the developing embryo during early pregnancy and was later found to be homologous to rat Cpn/Hsp10 (HSPE). EPF appears to have growth factor qualities [206], as well as anti-inflammatory properties which are necessary for protecting the embryo from the mother's own immune system [207-208]. Cpn/Hsp10 (HSPE) is released into circulation by dividing primitive cancer cells [206]. Cpn/Hsp10 (HSPE) forms a complex with the mitochondrial co-chaperone, Hsp60 (HSPD) and assists with the proper folding of mitochondrial proteins, as well as the reactivation of denatured proteins[170].

Relevant to the present discussion, Cpn/Hsp10 (HSPE) appears to modulate the innate immune system through interactions with monocytes, lymphocytes, and natural killer cells [208]. Disease specific animal models have produced compelling data describing the immunosuppressive activities of Cpn/Hsp10 (HSPE). For example, it was shown to decrease the expression of leukocyte trafficking adhesion molecules and the immune response in an animal model of multiple sclerosis and delayed-type hypersensitivity [209]. The inflammatory response was also suppressed in animal models of allograft transplantation, resulting in increased graft survival [210-211]. The *in vitro* administration of recombinant Cpn/Hsp10 (HSPE) appeared to inhibit LPS-induced NF-κB activation, thereby decreasing inflammatory cytokine and chemokine production, as well [211].

EXTRACELLULAR HEAT SHOCK PROTEINS AND SEPSIS

Our understanding of the relative contribution of intracellular versus extracellular HSPs in the pathophysiology of the sepsis syndrome is far from complete. For example, numerous *in vitro* and *in vivo* studies suggest that augmenting the intracellular expression of HSPs is beneficial to the host [5-7, 17, 212-213]. Indeed, the discovery of innovative methods of augmenting intracellular HSP expression in the clinical setting remains an active area of focus for several laboratories, including our own. On the other hand, there is compelling evidence that extracellular HSPs augment the host innate and adaptive immune response (discussed above). The immunomodulatory effects (i.e. either stimulatory or inhibitory) of extracellular HSPs are largely dependent upon contextual factors. Rather than viewing intracellular and extracellular HSP expression as two independent and mutually exclusive processes, we hypothesize that moderate levels of stress, such that would occur in critically ill patients with sepsis, increase both intracellular HSP expression and extracellular release of HSP, likely through some as yet undefined active, secretory mechanism. These two events result in enhanced protection for both the host cell, as well as cells in the immediate surroundings through either active uptake (and enhanced protection through increased intracellular HSP expression) or activation of stress response programs. Conversely, greater levels of stress further augment intracellular HSP expression and cause necrotic cell death, resulting in the release of large quantities of HSPs and causing an overzealous activation of the host inflammatory response through their immunostimulatory effects.

CONCLUSION

The stress response is characterized by a rapid increase in both the intracellular expression and subsequent release of a unique group of proteins, known as heat shock proteins. While intracellular HSPs predominantly down-regulate the host inflammatory response, extracellular HSPs may increase or decrease the host inflammatory response. The extracellular HSPs are now included in a growing list of so-called danger-associated molecular patterns (DAMPs), or alarmins. Further studies are necessary to better characterize the effects of these special proteins on the host inflammatory response during sepsis.

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