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Extracellular cell stress (heat shock) proteins—immune responses and disease: an overview

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Extracellular cell stress proteins are highly conserved phylogenetically and have been shown to act as powerful signalling agonists and receptors for selected ligands in several different settings. They also act as immunostimulatory 'danger signals' for the innate and adaptive immune systems. Other studies have shown that cell stress proteins and the induction of immune reactivity to self-cell stress proteins can attenuate disease processes. Some proteins (e.g. Hsp60, Hsp70, gp96) exhibit both inflammatory and anti-inflammatory properties, depending on the context in which they encounter responding immune cells. The burgeoning literature reporting the presence of stress proteins in a range of biological fluids in healthy individuals/non-diseased settings, the association of extracellular stress protein levels with a plethora of clinical and pathological conditions and the selective expression of a membrane form of Hsp70 on cancer cells now supports the concept that extracellular cell stress proteins are involved in maintaining/regulating organismal homeostasis and in disease processes and phenotype. Cell stress proteins, therefore, form a biologically complex extracellular cell stress protein network having diverse biological, homeostatic and immunomodulatory properties, the understanding of which offers exciting opportunities for delivering novel approaches to predict, identify, diagnose, manage and treat disease.

This article is part of the theme issue 'Heat shock proteins as modulators and therapeutic targets of chronic disease: an integrated perspective'.

1. Background

> Chance favours the prepared mind.
>
> —Louis Pasteur (1822–1895)

The presence of additional new 'puffs' in the polytene chromosomes of cultured *Drosophila* larva which were induced following their incubation at an inadvertently high temperature and observed by Ferruccio Ritossa (25 February 1936–9 January 2014) in the early 1960s was unexpected and puzzling. He realized the potential importance of this first evidence that stress can influence gene transcription and induce the synthesis of new proteins, yet found it surprisingly difficult to publish this discovery. It was eventually published in *Experientia* [1,2].

Ritossa's findings were extended and expanded upon during the next decade, and by the mid-to-late 1960s, it was clear that exposure of cells containing polytene chromosomes to a variety of environmental stressors resulted in the transcription of novel genes and, presumably, in the synthesis of specific proteins. However, it was not until the 1970s when Tissières at the University of Geneva and other investigators in this area [3,4] applied the newly developed technique of sodium dodecyl sulfate (SDS)–PAGE to reveal the appearance of new protein bands having distinct molecular masses in salivary glands after the application of heat shock. It was also noted that cellular levels of some proteins that were present before the application of elevated temperature either decreased or disappeared after treatment. Here was the first evidence for the existence of heat shock proteins (HSPs) or cell stress proteins, and it was then that these terms were coined.

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However, it is now clear that a range of different stressors, other than heat, such as viral infection, cytokines, oxidative stress, ionizing and UV irradiation, glucose deprivation or exposure to toxins and certain metals, also induce the expression of such proteins. A more descriptively correct term for these proteins is therefore 'cell stress' proteins [5].

The fact that research on the heat shock response was predominantly undertaken in *Drosophila* during the 1960s and 1970s led to the expectation that this response was specific to insects or even to *Drosophila* itself. However, observations that the heat shock response was present in chicken fibroblasts [6], *Escherichia coli* [7], yeast [8] and plants [9] indicated that the heat shock/cell stress response is a universal phenomenon. The cloning of the *Drosophila* genes encoding HSPs and the sequencing of many of the relevant genes by the late 1970s/early 1980s revealed the evolutionary relationships between the response and the proteins involved (e.g. [10]).

The relationships between stress-induced gene transcription and the roles of cell stress proteins in protein folding and the management of the intracellular environment took many years to be understood and consolidate [3,11,12]. Larry Hightower [13], a pioneer in studying the physiological role of cell stress proteins, first suggested that, as many of the stressors were protein chaotropes (agents able to denature proteins), then the most obvious function of this stress response was to manage and deal with improperly folded proteins within the cell. This hypothesis was tested using a simple experimental protocol which determined the influence of direct microinjection of native or denatured proteins into frog oocytes on the induction of the stress response. Only denatured proteins induced the response, thereby establishing the link between protein unfolding within the cell and the induction of the cell stress response [11].

By the late 1980s, it had been recognized that cellular proteins require help with their folding in some instances, and that this was facilitated via the actions of families of proteins termed 'molecular chaperones' [12], the accepted definition of which is 'a large and diverse group of proteins that share the property of assisting the non-covalent assembly/disassembly of other macromolecular structures, but which are not permanent components of these structures when these are performing their normal biological functions' [14]. Molecular chaperones fulfil essential cellular 'housekeeping' and cytoprotective functions, and thereby ensure correct functionality. They also enable cells to cope with the plethora of insults and stresses that exist in the complex and dynamic intracellular environment (table 1).

Since these early studies, a growing number of proteins that are involved in protein folding and in the cell stress response have been identified in all three of life's Kingdoms. Several families of the molecular chaperones include large numbers of proteins. For example, the Hsp40 family contains 50 members. The fact that the number of human molecular chaperones and protein-folding catalysts is probably in the region of 150 proteins underlies the enormous complexity of the cell stress response. The cell stress protein families can be subdivided into molecular chaperones, which aid protein folding without changing the client protein in any way, protein-folding catalysts such as protein disulfide isomerases, which catalyse SH-:-S-S- interconversions, and peptidyl prolyl isomerases, which catalyse *cis*:*trans* isomerization of prolines and thus induce chemical changes in their client proteins [19]. The protein-folding catalysts can also be involved in redox interactions, and this phenomenon of oxidation and reduction both within and outside the cell is now recognized as being a major modulator of biological behaviour (e.g. [20]). To confuse matters further, molecular chaperones and protein-folding catalysts can either be proteins whose genes are induced by stress, or be constitutively expressed proteins whose genes fail to be modulated by stress—only the former are classed as cell stress proteins.

Discontinuous PAGE gels enable accurate molecular masses to be identified, and informed the nomenclature for the heat shock (cell stress) proteins (e.g. Hsp60, Hsp70). However, despite the publication of nomenclature guidelines [17], the literature remains unclear, especially in the case of the 70 kDa family of molecules. The human *HSP70* (gene) family consists of at least eight members, only three of which show stress-inducible expression [18]. Of the 13 protein members of the family, two closely linked genes, referred to as Hsp70-1, are the major stress-induced members [18]. Although some evidence implicates Hsp70-2 in human cancer, the cytosolic, stress-induced Hsp70-1 is the predominant form that is overexpressed in cancer [18]. It is, therefore, likely that it is this form of the molecule which is being measured in the studies that have been reported to date. However, it is important that the identity of the analyte being reported upon is verified using information on the specificity of the antibodies that are being used in the assays.

As indicated above, it is now apparent that proteins can have multiple functions, the manifestations of which are dictated by the context in which they are generated and encountered. For instance, can proteins such as stress proteins exhibit distinct profiles of physiological activities when in the intracellular and extracellular environments? If so, and this indeed appears to be the case, then this would argue against the concept of 'one protein, one function'. Although the concept of 'one protein, one function' is not universally accepted, Campbell & Scanes [21] first proposed the term 'protein moonlighting' to describe the capacity of certain proteins to exhibit more than one biological function, specifically the apparent immunological functions of 'endocrine peptides'. A number of prokaryote and eukaryote proteins have been shown to exhibit 'moonlighting functions', and this concept has been expanded upon by a number of proponents [22–25]. The concept of protein moonlighting is discussed in detail by Constance Jeffery elsewhere in this issue [26].

However, it should be noted that many biologically important molecules—if not all of them—express more than one function, and the implication that a protein has only one bona fide function and that the other functions are secondary, if not superfluous, might not necessarily be the case. Another counterview to moonlighting functions of HSP (stress) in the immunological context (see below) is that these proteins might not have evolved a second function at all. Rather, it was the immune system that evolved to recognize and respond to these proteins on the basis of changed accessibility, rather than changes in physiological function.

2. Cell stress proteins are released into the extracellular environment

The concept that stress proteins can be released from cells in the absence of necrosis was highlighted by Hightower & Guidon in 1989. In this study, heat treatment was shown to increase the profile of proteins that were released from cultured rat

Table 1. Mammalian cell stress response proteins, and their intracellular localization and function. 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. Adapted from [15,16]. Further information on the nomenclature and individual family members has been published elsewhere [17,18].

major family, and members	intracellular localization	intracellular function
small Hsps		
αB-crystallin	cytoplasm	cytoskeletal stabilization
Hsp27	cytoplasm/nucleus	actin dynamics
haeme oxygenase, Hsp32	cytoplasm	haeme catabolism, antioxidant properties
Hsp60 or chaperonins		
Hsp60	mitochondria	both bind to partially folded polypeptides and assist correct folding
TCP-1	cytoplasm	assembly of multimeric complexes
Hsp70		
Hsp70 (inducible)	cytoplasm/nucleus	all bind to extended polypeptides
Hsc70 (cognate)	cytoplasm/peroxisome	all prevent aggregation of unfolded peptides
Grp78/BiP	ER	all dissociate some oligomers
mtHsp70/Grp75	mitochondria	ATP binding
		ATPase activity
		Hsp70 is involved in the regulation of HSF1 activity and the repression of heat shock protein gene transcription
Hsp90		
Hsp90 (α and β)	cytoplasm	all bind to other proteins
Grp94/gp96/Hsp100	ER	all regulate protein activity
		all prevent aggregation of re-folded peptide
		correct assembly and folding of newly synthesized protein
		Hsp90 appears to be involved in maintaining the HSF1 monomeric state in non-stressful conditions. Represents 1–2% of total protein
Hsp110		
Hsp110 (human)	nucleolus/cytoplasm	thermal tolerance
Apg-1 (mouse)	cytoplasm	protein refolding
Hsp105	cytoplasm	

embryo cells, from a small profile that included the constitutively expressed member of the 70 kDa family of molecules, Hsc70, to also include its inducible counterpart, Hsp70 and Hsp110 [27]. Although protein release occurred in the absence of any overt level of cellular necrosis (and was therefore likely to be an 'active' physiological process), it was not mediated via the common secretory pathway, as inhibitors of this pathway (colchicine, monensin) did not block it [27]. These findings, aligned with the slightly earlier study from Tytell *et al*. [28] in 1986, who reported the transfer of glia–axon transfer proteins (including Hsp70, Hsc70 and Hsp100) from adjacent glial cells into the squid giant axon. This response was proposed to reflect a mechanism that enabled glial cells to protect adjacent neuronal cells that exhibit a deficient ability to generate a protective response to stress.

These initial findings, and the subsequent studies reporting the presence of Hsp60 and Hsp70 in the peripheral circulation of healthy individuals by Pockley *et al*. in the late 1990s [29,30], were received with scepticism by the biological and biochemical communities, as it was unclear how these proteins could be released from viable cells, given that they do not express the typical N-terminal signal peptide sequences that enable secretion. However, this argument is not a strong one, as 'non-classical' secretion of proteins lacking such sequences has been observed for several proteins, including fibroblast growth factors 1 and 2 (FGF-1,2), interleukin-1 (IL-1) and high mobility group box 1 (HMGB-1). The mechanisms underlying non-classical secretion pathways have been reviewed elsewhere [31]. Cell stress proteins have now been reported to be released from a wide range of cells including insulin-secreting β cells, rat cortical astrocytes, a human neuroblastoma cell line, a human keratinocyte-derived cell line, cultured vascular smooth muscle cells and a broad profile of tumour cells including murine and human prostate cancer cells and B cells (reviewed in [32]), and to exist in the circulation in a number of healthy and diseased states (see below).

Extracellular Hsp70 exists either as a free protein, as a protein in association with lipid vesicles such as exosomes [33,34] and lysosomal endosomes [35] or in the context of cholesterol-rich microdomains [36]. Vesicular transport [37] and ubiquitination-triggered transport [38] have also been proposed. Recent studies have demonstrated that the minority of extracellular Hsp70 is 'free' Hsp70, and this is mostly derived from dying cells [39].

Exosomes are small membrane vesicles that form within late endocytic compartments called multi-vesicular bodies (MVBs). They are distinct to apoptotic vesicles in that they differ in their mode of production and protein composition [40]. The fusion of MVBs with the plasma membrane leads to the release of exosomes into the extracellular space. Various haematopoietic and non-haematopoietic cell types secrete exosomes, including reticulocytes, B and T lymphocytes, mast cells, platelets, macrophages, alveolar lung cells, tumour cells, intestinal epithelial cells and professional antigen-presenting cells (APCs) such as dendritic cells (DCs), with the function of exosomes in different physiological processes depending on their origin [41]. DC- and tumour-derived exosomes are enriched in Hsp70, Hsc70 and Hsp90 [42,43], and exosomes released from reticulocytes also contain Hsp70 [44]. Exosomal release of stress proteins and the role of exosomal-associated HSPs in cancer have been reviewed in the literature [45,46] and by Gabriele Multhoff elsewhere in this issue [47].

3. Cell stress proteins as immunomodulatory mediators

Although probably not fully appreciated at the time, the concept that stress proteins can exist in the peripheral circulation had been established in 1977 in a study that reported the presence of a protein (early pregnancy factor (EPF)) in the serum of women in the first trimester of pregnancy [48]. This protein was demonstrated to have immunosuppressive properties two years later [49] and was identified as being HSP 10 (Hsp10) in 1994 [50]. It has also been shown that Hsp10, a 10 kDa monomer that caps the Hsp60 oligomer and facilitates protein folding [51] is also present at low levels in non-pregnant individuals [52]. Hsp10 inhibits the secretion of several inflammatory mediators [53], and its immunosuppressive properties can attenuate a variety of human inflammatory diseases [54–57]. The finding that circulating levels of Hsp10 in patients with periodontal disease are lower than those in matched, disease-free, controls and that levels only return to normal after effective therapy suggest the control of circulating Hsp10 levels by localized inflammation [52]. Hsp10 therefore appears to be a homeostatic controller of inflammation, in addition to being an integral component of the intracellular molecular chaperone machinery.

With regard to Hsp60 and Hsp70, the discovery of these proteins in the peripheral circulation of overtly normal individuals led to a certain degree of confusion, as these proteins were considered as being pro-inflammatory molecules when present in the extracellular environment. Indeed, one of the major issues for investigators studying the immunobiology of extracellular stress proteins is the apparently contradictory evidence that indicates both pro- and anti-inflammatory roles for these proteins. The problem is that the immunological properties of these proteins continue to be discussed in isolation and it is essential that a more systems biology approach to extracellular HSPs is adopted in order to better reflect their physiological context and roles. Although many studies indicate pro-inflammatory properties for Hsp60 and Hsp70 in their interactions with monocytes, macrophages and DCs [58–62], it has been speculated that at least some of these inflammatory effects result from the presence of contaminating endotoxin in the recombinant protein preparations, especially those that have been generated using bacterial expression systems [63–66]. However, much evidence argues against this being the universal explanation for these effects, as has been reviewed elsewhere [25,67]. It is, therefore, essential to ensure that reagents and experimental design(s) are beyond question when it comes to undertaking experiments in this area. The influence of HSPs on immune responses in a number of contexts has been reviewed elsewhere [15,68,69] and in this issue.

In contrast with their reported pro-inflammatory properties, a body of literature indicates that Hsp60 and Hsp70 can have profound anti-inflammatory effects. Relatively historic data have reported that the induction of T cell reactivity to self-Hsp60 and self-Hsp70 promotes the development of Th2 type $CD4^+$ T cells producing the regulatory cytokines IL-4 and IL-10 and downregulates disease in a number of experimental models of inflammatory disease [70–74]. It has also been shown that DNA vaccines encoding for these proteins inhibit experimental arthritis and diabetes [74,75]. The recognition of conserved (self) epitopes on these highly conserved molecules dominantly downregulates the inflammatory capacity of the non-conserved (non-self) epitopes [76]. Human Hsp60 can act as a co-stimulator and activator of $CD4^+CD25^+$ regulatory T cell populations by interacting with Toll-like receptor 2 (TLR2) [77] and the treatment of such cells with Hsp60 enhances their ability to regulate the $CD8^+$ T cell populations via direct cell–cell contact and the secretion of the immunoregulatory cytokines IL-10 and TGF-β [77]. The anti-inflammatory potential of Hsp60 and Hsp60-derived peptides has also been demonstrated in studies that have used these to modulate the rejection of murine skin allografts [78,79] and autoimmune disease—the latter is discussed elsewhere [80–82] and by Willem van Eden in this issue [83]. It, therefore, appears that the net outcome of any immune response is dependent on the relative strengths of these antagonistic events (reviewed in [68]). The interactions of Hsp60 with the innate and adaptive immune systems and their immunoregulatory consequences have been reviewed and considered by Quintana & Cohen [84].

4. Extracellular cell stress proteins in health and disease

The initial identification of Hsp60 and Hsp70 in the peripheral circulation [29,30] stimulated interest in this area and the development of a range of 'in-house' and commercial enzyme immunoassays for measuring stress proteins in extracellular compartments. Most commercially available enzyme immunoassays for cell stress proteins are optimized for free Hsp70 in buffer, but not for Hsp70 in the serum, plasma or other body fluids, and so it is essential that investigators are aware of the limitations of the assays they use. It is also a matter of debate as to whether liposomal cell stress proteins can be detected using the standard detergents that are typically included in commercial enzyme immunoassay kits. Notwithstanding the above, these studies have led to many reports associating circulating levels of cell stress proteins with healthy and diseased states (table 2), including cancer (table 3). An immediate issue relating to these studies is the need to ensure that the commercial assay kits and the 'in-house' assays that have been used have been properly validated for the analysis of the relevant analytes in the biological fluid that is under investigation [132]. Such information is not

Table 2. Circulating cell stress proteins in disease.

	condition	key findings	reference
Hsp10	*periodontitis*	lower plasma levels in periodontal disease and treatment increases these. Post-treatment levels correlate with markers of clinical improvement	[52]
Hsp27	*renal disease*	elevated serum and urine levels in chronic kidney disease	[85]
	autoimmunity	serum levels may be a novel marker for diabetic neuropathy in patients with Type 1 diabetes	[86]
	chronic heart failure	soluble Hsp27 is a novel candidate biomarker for diagnosing CHF with preserved ejection fraction	[87]
Hsp60	*stress*	association between elevated levels of Hsp60, low socioeconomic status and social isolation in males and females, and with psychological distress in women	[88]
	cardiovascular disease	elevated serum levels in patients with renal and peripheral vascular disease and individuals with borderline hypertension. Serum levels in individuals with hypertension are similar to normotensive controls	[89–92]
		elevated levels present in coronary eluates after myocardial infarction	[93]
		serum levels increase with accumulating features of the metabolic syndrome in postmenopausal women	[94]
		endothelium-dependent vasodilator function is impaired in children with detectable levels of serum Hsp60. Circulating Hsp60, or factors that stimulate the expression and systemic release of Hsp60, may contribute to the initiation of arterial disease in early life	[95]
		association between higher levels of plasma Hsp60 in subjects with clinically manifest cardiovascular disease and those with a history of myocardial infarction in diabetes mellitus	[96]
	infections	plasma Hsp60 levels are elevated in HIV-infected patients. Although levels reduce after anti-retroviral therapy, they remain higher than uninfected controls. Hsp60 levels correlate with viral load, $CD4^+$ T cell counts, and circulating soluble CD14 and lipopolysaccharide levels	[97]
	periodontitis	a larger proportion of patients with periodontal disease exhibit intermediate levels of plasma Hsp60 than controls. Treatment has no influence on levels	[52]
		atherogenic dyslipidaemia and elevated circulating Hsp60 levels are linked and associated with periodontal pathology	[98]
	autoimmunity	serum Hsp60 levels correlate with time required for remission from flare-ups in patients with juvenile idiopathic arthritis	[99]
Hsp70	*surgery/trauma*	plasma Hsp70 levels markedly increase in patients undergoing liver resection and are associated with post-operative infection, hepatic ischaemic time and the degree of post-operative organ dysfunction	[100]
		Hsp70 is released into the circulation following coronary artery bypass grafting	[101]
	cardiovascular disease	elevated serum levels in patients with renal and peripheral vascular disease and individuals with borderline hypertension. By contrast, serum levels in hypertension are similar to normotensive controls	[89–91]
		low serum levels at baseline predict the development of atherosclerosis in individuals with established hypertension	[102]
		increased serum levels associated with low risk of coronary artery disease	[103]
		increased circulating levels may be associated with the progression of atrial fibrillation and its recurrence after catheter ablation	[104]
		serum levels correlate with the severity of atherosclerosis in patients with carotid artery disease and chronic lower limb ischaemia. Putative role for circulating Hsp70 in the development of arterial calcification	[105]
	infections	serum levels positively associated with the degree of inflammation in an elderly population living in a remote area in Cameroon, where infection and parasitosis are endemic	[106]
		positive correlations between serum levels and inflammatory markers	[107]
		serum Hsp70 levels in patients with chronic hepatitis are higher than controls, but lower than in patients with liver cancer	[108]

(*Continued*.)

Table 2. (*Continued.*)

	condition	key findings	reference
	pregnancy	serum levels are lower in normal human pregnancy, but elevated in transient hypertension of pregnancy, in pre-eclampsia and in superimposed pre-eclampsia. Increased serum levels reflect systemic inflammation, oxidative stress and hepatocellular injury in pre-eclampsia	[109–111]
	asthma	induced sputum and plasma Hsp70 levels could serve as a useful marker for assessing airway obstruction in patients with asthma	[112]
	renal disease	elevated urinary Hsp70 levels in stages 4 and 5 chronic kidney disease	[85]
	diabetes	serum levels are increased in Type 1 and Type 2 diabetes	[113–118]
		serum levels are increased and correlate with HbA1c values in women with gestational diabetes mellitus	
	autoimmunity	plasma Hsp70 levels are high in patients with Type I diabetes	[119,120]
BiP	*periodontitis*	lower circulating levels of BiP (grp78) in periodontal disease as compared to controls. Treatment has no influence on levels	[52]
grp94	*autoimmunity*	plasma grp94 (gp96) levels are high in patients with Type I diabetes	[119,120]

always apparent, and differences in the levels of HSPs in the circulation which are measured by commercial and 'in-house' enzyme immunoassays have been reported [133]. Serum and plasma are complex and 'matrix'-related effects can influence measurements in biological samples. Furthermore, a clinical method comparison study has revealed that commercially available HSP27 assays are not equally useful for differentiating patients with non-small cell lung carcinoma (NSCLC) from healthy controls [134].

Reports on relationships between circulating stress protein levels and the clinical and physiological status of an individual (tables 2 and 3) are providing insight into the role of these proteins in the maintenance of a healthy state and/or the induction, progression and resolution of diseased states. As an example, the positive association between plasma Hsp60 levels in patients with cardiovascular disease and those with a history of myocardial infarction in diabetes mellitus implicates extracellular Hsp60 in the cardiovascular pathology which is associated with diabetes [96]. Lower levels of serum Hsp70 in normal human pregnancy could provide insight into the maintenance of immune tolerance in pregnancy [109]. Although studies have associated levels of Hsp70 in infection-related inflammation, the variability of the measured levels cannot distinguish patients from healthy subjects in this context [107]. However, the variability in levels, as measured using current approaches, does not currently allow measurement of these analytes to be used as a robust discriminatory approach for the identification of disease.

Given that a number of studies have reported relationships between circulating antibodies and their corresponding antigens in the peripheral circulation and the presence, severity and progression of disease, one should consider the potential involvement of circulating immune complexes in this context. This is a commonly overlooked parameter in studies that have investigated such relationships, and this might, in some instances at least, result from potential problems that are associated with measuring the presence of antibodies in a sample that includes its cognate antigen. Our own personal experience is that HSPs and HSP antibodies coexist in the peripheral circulation [29,30,89,90,102]. Although the presence of circulating anti-cell stress protein antibodies in the peripheral circulation might impact the measurements that are made, we have not found this to be the case, in that we have not observed any direct correlation between measured levels of circulating HSPs and anti-HSP antibody levels, at least using the assays that were available at the time [29,30,89,90,102,135]. Notwithstanding this, the presence/differential presence of immune complexes has the potential to influence the inflammatory status of an individual in that the interaction of APCs with soluble immune complexes has been shown to reduce their production of the Th1-biasing cytokine IL-12 to enhance their production of the regulatory cytokine IL-10 and, consequently, to induce a Th2-like (immunoregulatory) adaptive immune T cell response [136]. By contrast, a more recent study has reported that grp94 in complexes with IgG, which is a soluble diagnostic marker of gastrointestinal tumours, exert immune-stimulating activity on peripheral blood immune cells, as demonstrated by the triggering of inflammatory cytokine secretion [137]. It is clear that more studies aimed at understanding the relationship(s) between circulating cell stress proteins in their free and lipid-associated forms and their corresponding antibodies is required.

5. Extracellular cell stress proteins in cancer

The presence of circulating cell stress proteins in cancer, and relationships with tumour volume and therapeutic response [128,129] have been reported upon in a number of studies (table 3). Importantly, Multhoff and co-workers [108] have demonstrated that serum Hsp70 levels in patients with liver cancer are significantly higher than those that are measured in a control group of individuals without liver disease, and (importantly) are also higher than in individuals with chronic hepatitis. The same study showed that serum Hsp70 levels in a subgroup of patients with liver cirrhosis who subsequently developed liver cancer were higher than those in individuals with liver cirrhosis alone [108]. Dutta *et al*. [130] have reported that serum Hsp70 levels are significantly elevated in patients with pancreatic cancer, compared with both healthy controls and individuals with chronic pancreatitis. These findings demonstrate the capacity of serum Hsp70 levels to distinguish

Table 3. Extracellular cell stress proteins in cancer. LipHsp70, liposomal Hsp70.

	tumour	key findings	reference
Hsp27	*ovarian*	serum Hsp27 levels are increased in epithelial ovarian cancer and correlate with peritoneal metastases. Serum Hsp27 levels may be used as a potential additional indicator for peritoneal metastases and the response of patients to treatment	[121]
	breast	significant differences in the profiles of Annexin V^+, $CD66^+$, $BCRP1^+$ and $Hsp27^+$ microparticles are present in breast cancer patients with lymph node metastases, as assessed using flow cytometry	[122]
	lung	serum levels of Hsp27 are significantly elevated in patients with non-small cell lung cancer diagnosed at an early or at an advanced stage and can distinguish between early and advanced stage disease	[123]
Hsp70	*leukaemia*	levels of plasma Hsp70 reflect overall tumour load and patients with higher levels of plasma Hsp70 have significantly shorter survival in acute myeloid leukaemia and acute lymphoblastic leukaemia circulating Hsp70 might, therefore, be a biomarker for poor prognosis?	[124]
		plasma Hsp70 levels above the median in chronic myeloid leukaemia are associated with a higher rate of progression to the accelerated/blast phase, and a tendency towards shorter survival. Plasma Hsp70 could be a potential marker for predicting disease progression in patients with chronic phase in chronic myeloid leukaemia	[125]
	colorectal	serum levels of Hsp70 and mortalin are independent variables, and high serum levels of mortalin (mitochondrial Hsp70, grp75, HSPA9*)* are a risk factor for shorter survival patients with colorectal cancer. The concurrence of high serum Hsp70 and mortalin levels is associated with rapid disease progression	[126]
		serum Hsp70 levels have potential as a stage-independent prognostic marker in colorectal cancer without distant metastasis	[127]
	head and neck	plasma Hsp70 levels are significantly higher in mice bearing membrane Hsp70-positive FaDu human squamous cell carcinomas of the head and neck, and these correlate with tumour volume. Radiation-induced tumour regression is associated with significantly decreased Hsp70 levels, and these return to those of control animals after complete remission	[128]
		serum Hsp70 levels are significantly higher and associated with tumour volume in patients with squamous cell carcinoma of the head and neck. Following surgery and radiotherapy, Hsp70 levels fell without tumour relapse in the follow-up period. Hsp70 is, therefore, a potential tumour biomarker for monitoring the clinical outcome of radiotherapy. High levels associated with high levels of membrane Hsp70 expression on tumour cells	[129]
	liver	serum Hsp70 levels in patients with liver cancer are significantly higher than a control group without liver disease, and individuals with chronic hepatitis. A subgroup of patients with cirrhosis who subsequently developed liver cancer exhibited higher serum Hsp70 levels than those patients with cirrhosis that did not progress to cancer	[108]
	pancreatic	plasma Hsp70 levels are significantly higher in mice bearing membrane Hsp70-positive spontaneous pancreatic ductal adenocarcinomas, and levels correlated with tumour volume. Radiation-induced tumour regression was associated with significantly decreased Hsp70 levels, and levels returned to those of controls after complete remission	[128]
		serum Hsp70 levels are significantly increased in patients and may be useful as an additional biomarker for the detection of pancreatic cancer	[130]
	lung	serum levels of Hsp70 are significantly elevated in patients with non-small cell lung cancer diagnosed at an early or at an advanced stage when compared with healthy control groups	[123]
LipHsp70		circulating lipHsp70 levels in patients with head and neck, lung, colorectal, pancreatic cancer, haematological malignancies and especially glioblastoma are significantly higher than those in healthy human volunteers	[39]

(*Continued*.)

Table 3. (*Continued.*)

tumour	key findings	reference
membrane Hsp70	membrane Hsp70 expression correlates with an improved overall survival in patients with colon and gastric carcinomas, whereas it is negatively associated with survival in patients with lower rectal and squamous cell carcinoma	[131]
Hsp90	the baseline serum HSP90 levels of melanoma patients are significantly higher than those of the control subjects, but are not associated with clinical variables or survival	

between inflammatory events/disease and cancer, and suggest that circulating Hsp70 might indeed be of value as a biomarker in cancer.

Hsp90 inhibitors are being evaluated for the treatment of cancers such as myeloma, breast, prostate and lung cancer, melanoma, gastrointestinal stromal tumours and acute myeloid leukaemia. Although the activity of Hsp90 inhibitors is currently assessed based on Hsp70 induction in peripheral blood mononuclear cells using western blot analysis, this approach is laborious, only semi-quantitative and difficult to implement in the clinic [138]. Serum Hsp70 measurements are now being used to monitor responses to Hsp90 inhibitors in the clinical setting, especially when access to tumour tissue is not possible [138].

6. Therapeutic potential and biological role of extracellular cell stress proteins

The concept that extracellular cell stress proteins could have therapeutic potential originally arose from studies into cross-reactive immunity to human Hsp60 by Irun Cohen's group in Israel, which found that T cells cross-reactive with Hsp60-induced diabetes in mice. Curiously, the administration of *Mycobacterium tuberculosis* Hsp65 protein could either induce diabetes or prevent it [139]. Analysis of the Hsp65 sequence (epitopes) recognized by T cells identified peptide 437–460 as a major T cell recognition epitope. The same sequence was identical in mouse Hsp60, apart from K for T at position 455. Crucially, it was found that immunization of non-obese diabetic (NOD) mice with this peptide (termed p277) inhibited the induction of diabetes [140]. Some 20 years later, the evaluation of this peptide in a phase III clinical trial showed evidence of clinical benefit [141].

The mitochondrion and bacterial cytosol contains Hsp60 and the co-chaperone, Hsp10, which acts as a 'lid' to the Hsp60 folding chamber. This 10 kDa protein, which normally forms a heptameric structure, was identified as a circulating immunosuppressive factor that was required for inhibiting immunity to the implanted embryo (termed 'EPF'), in 1977 [48,49]. The potential role of this factor in the maintenance of pregnancy was confirmed by studies demonstrating that pregnant mice treated with anti-EPF antibodies failed to maintain their pregnancy [142]. It was not until 1994 that EPF was identified as Hsp10. A number of years later, Hsp10 was shown to inhibit experimental immunological models such as adjuvant arthritis (in this case, the protein was *M. tuberculosis* Hsp10 [143]) and experimental autoimmune encephalomyelitis (EAE) [144]. Short-term administration of *M. tuberculosis* Hsp10 has also been shown to inhibit experimental allergic asthma in mice [145].

The findings that recombinant Hsp10 inhibited LPS-induced inflammatory changes in macrophages and in mice exposed to LPS [53] led the Brisbane-based biopharmaceutical company, CBio Ltd, to attempt the commercialization of human Hsp10 as a therapeutic, and several small-scale clinical trials of a modified human Hsp10 (termed XToll) in a small number of conditions were undertaken. In a randomized, double-blind study of 23 rheumatoid arthritis patients, the intravenous administration of Hsp10 (5, 7.5 and 10 mg twice a week) induced either clinical benefit or disease remission in a significant number of individuals, with only one serious adverse event being reported [54]. Another small randomized, double-blind study demonstrated that the administration of XToll (Hsp10, 5, 7.5 and 10 mg) to 24 patients with plaque psoriasis over a 12-week dosing regimen of two doses per week reduced disease parameters [56]. Experimental findings that Hsp10 inhibited allergic encephalomyelitis prompted a double-blind randomized, placebo-controlled, phase II trial in 50 patients with multiple sclerosis. Although the Hsp10 was well tolerated, apart from showing a decreased circulating leucocyte cytokine synthetic capacity, the changes in clinical parameters were not significant [57]. Large peptides are not natural candidates for drug therapy and it is known that XToll, which is a modified Hsp10, induces antibodies in patients [56]. The two alternatives for this cell stress protein as a therapeutic are either to: (i) couple it with an Fc receptor of with polyethylene glycol; or (ii) generate active peptide fragments. The anti-arthritic actions of synthetic *M. tuberculosis* Hsp10 were found to reside in the N-terminus [146]. It is, therefore, possible that smaller fragments of Hsp10 may retain activity and could be the basis for developing non-peptidic isosteres of the Hsp10 peptide.

Another stress protein, which was originally considered as being an autoantigen that drove the progression of autoimmune disease, has subsequently been characterized as being a potent immunomodulatory molecule with clinical potential. Glucose-regulated protein 78 (grp78, binding immunoglobulin protein, BiP) [147] is essential for the assembly of immunoglobulin molecules [148], and is required for the translocation of nascent polypeptides across the endoplasmic reticulum membrane and protecting cells against ER stress [149]. It can also be expressed on the cell surface and acts as regulator of coagulation [150] and cell proliferation [151,152]. It has also been shown to be a potent immunoregulator [147].

BiP is present in the circulation of healthy individuals and at lower levels in patients with rheumatoid arthritis [147]. It is also found in synovial and oviductal fluid [153,154]. In contrast with the stress proteins that have been considered above, the secretion of BiP is likely to be via a classic route as it possesses the C-terminus ER retention signal (lysine, aspartic acid,

glutamic acid, leucine (KDEL) amino acid sequence), which is common to proteins that reside in the ER. The multiple activities of BiP and its potential as a therapeutic agent for the management of inflammatory disease have been eloquently and comprehensively studied elsewhere [155]. With respect to therapeutic potential, a phase I/IIA clinical trial in 24 patients with rheumatoid arthritis who received a 1 h infusion of BiP (1, 5 or 15 mg) and who were followed up for 12 weeks has reported evidence of remission in patients receiving 5 and 15 mg doses [156].

Returning to the 70 kDa family, the constitutive member Hsc70 appears to play a role in, arguably, the most important biological process for the survival of species, namely reproduction. Proteomic analysis of porcine oviductal fluid has revealed that epithelial cells in the oviductal lumen secrete several molecules in response to the presence of spermatozoa, most notable of which are HSPs (stress) [157]. HSPs have also been identified in soluble fractions of pig and cow oviductal apical plasma membranes (sAPM) and in the human apical epithelium [158–160]. These are potentially important findings, as the oviduct and oviductal sperm storage play key roles in reproduction by providing a secure reservoir in which spermatozoa can attain full fertilizing properties. Hsc70 appears to interact with components of the sperm cell surface membrane [158,159], and exposure to Hsc70 prolongs the survival of boar, bull and ram sperm [158,161]. Studies have now shown that a recombinant form of Hsc70 (HSPA8) rapidly promotes the viability of uncapacitated spermatozoa, the ability of spermatozoa to bind to oviductal epithelial cells, enhances the performance of *in vitro* fertilization procedures, and decreases sperm mitochondrial activity [162]. The repair of membrane damage is mediated via increase in sperm membrane fluidity. The ability of HSPA8 to influence membrane stability and fluidity, alongside its conserved nature among mammalian species, supports the idea that this protein protects sperm survival through membrane repair mechanisms [162]. Ongoing studies are elucidating the mechanisms that are involved in these protective effects and their potential impact on reproductive success and potential.

7. Therapeutic potential and biological role of a typically intracellular cell stress protein

Although this article has focused on those proteins that are known to be secreted by cells and therefore to be present under normal circumstances in biological fluids, several cell stress proteins that are not as well established as being in the extracellular environment under normal conditions have also been shown to have contrasting effects. A good example of such proteins is glucose-regulated protein 94 (gp96, HSPC4). Gp96 is a 94–96 kDa member of the Hsp90 family of molecular chaperones/stress proteins which resides within the lumen of the endoplasmic reticulum. In addition to being an intracellular chaperone [163,164], the administration of tumour-derived gp96 has been shown to induce tumour-specific cytolytic T cells and a protective tumour-specific immunity, the specificity of which is defined by peptides that are associated with the administered gp96 [165–167]. By contrast, no protective effect is observed when high doses (2×10 μg intradermally) of tumour-derived gp96 are administered to mice [166]. Furthermore, appropriate doses of gp96 purified from normal liver can suppress the onset of diabetes in NOD mice and myelin basic protein- or proteolipid protein–induced autoimmune encephalomyelitis (EAE) in SJL mice [168], as well as prolonging the survival of murine skin allografts [169] and rat cardiac allografts [170]. The mechanisms that underlie these effects were originally proposed to involve the induction, activation and/or recruitment of as yet unidentified immunoregulatory T cell populations [168,169]. In our hands, gp96 could not be shown to be an activator of DCs, but did appear to activate $CD3^+$ T cells *in vitro* [171], and lead to a state of peripheral T cell hyporesponsiveness following *in vivo* administration to rats bearing cardiac transplants [170]. More recent work provides a better insight into the mechanisms via which gp96 elicits dichotomous immune responses by providing evidence that low and high doses of gp96 preferentially engage conventional and plasmacytoid dendritic cells (pDCs), respectively, via CD91. Global DNMT-dependent epigenetic modifications modify protein expression within these APCs leading to an upregulation of neuropilin1 by pDCs which enables long-term interactions with Treg cells, thereby enhancing suppression of Th1 anti-tumour immunity [172].

The administration of autologous tumour-derived peptides bound to gp96 (HSPPC-96) induces individual tumour-specific immunity in patients with high-grade glioma [173] and has been shown to be safe for the treatment of patients with recurrent glioblastoma multiforme (GBM) in an open-label, single-arm, phase II study of 41 adult patients with surgically resectable recurrent GBM who were treated after gross total resection [174]. In the case of patients with newly diagnosed GBM, the addition of HSPPC-96 (Prophage^{TM}) to standard radiotherapy and temozolomide chemotherapy in a phase II, single-arm multi-centre trial involving 46 patients has been shown to have the potential to improve survival [175]. That the expression of PD-L1 on circulating myeloid cells impacts on systemic immunity suggests that the inhibition of such immunological 'checkpoint' pathways could further enhance the efficacy of this approach [175].

8. Membrane Hsp70: a 'third' form of the 70 kDa cell stress protein with diagnostic, therapeutic and imaging potential

Gabriele Multhoff discovered the selective expression of a membrane form of Hsp70, the major stress-inducible member of the 70 kDa HSP family, on the plasma membrane of tumour cells (but not normal tissue) using a unique monoclonal antibody (mAb, cmHsp70.1) [176–178]. The expression of membrane Hsp70 has now been detected on a broad panel of cancer cell lines, and the density of membrane Hsp70 expression on cancer cells is increased by treatments such as radio(chemo)therapy [179]. An ongoing screening programme of over 1300 patients with various solid tumours in the Multhoff laboratory is revealing that more than 50% of all patients bear a membrane Hsp70–positive tumour. Membrane Hsp70 is also highly expressed on metastatic disease [180], and its expression is associated with an unfavourable prognosis and a reduced overall survival in patients with rectal carcinoma and squamous cell carcinoma [131]. Membrane Hsp70 expression is therefore a universal, selective tumour-specific marker of 'aggressive' disease.

Tumour cells that express Hsp70 on their plasma membrane secrete exosomes that express Hsp70 on the surface of their plasma membranes [33]. Given that the protein composition in the exosomal lumen reflects that of the cytosol of the respective cell, it would be expected that exosomes derived from normal cells contain low levels of Hsp70, whereas exosomes from tumour cells having a high cytosolic Hsp70 contain high levels of Hsp70 in their lumen and also present it on their lipid surface [181]. This concept has been confirmed, at least in part, by studies that have reported serum Hsp70 levels to be associated with a high membrane Hsp70 expression on tumours in patients with squamous cell carcinoma of the head and neck [129].

Aligned with these studies has been the development of an enzyme immunoassay that detects liposomal Hsp70 (lipHsp70) in serum and plasma [39]. This assay was conceived and developed based on the evidence that Hsp70 membrane-positive tumour cells actively release Hsp70 in exosome-like lipid vesicles and that most commercial Hsp70 enzyme-linked immunosorbent assays (ELISAs) are not validated for the detection of liposomal Hsp70 in serum. The assay exhibits a high level of precision, and a greater recovery of 'spiked' Hsp70 than its commercially available counterparts. The lipHsp70 ELISA is equally suitable for serum and plasma and the measured Hsp70 concentrations are not influenced by food intake, repeated freezing and thawing of the sample or moderate haemolysis. A comparison of the Hsp70 levels in patients with head and neck, lung, colorectal, pancreatic cancer, GBM or haematological malignancies and healthy human volunteers has revealed significantly higher levels in patients bearing tumours, and especially in those bearing aggressive tumours (e.g. GBM). The lipHsp70 ELISA, therefore, provides a highly sensitive and robust method for measuring liposomal and free Hsp70 in the circulation and could provide a clinically approach for detecting tumours and monitoring therapeutic responses and clinical outcome.

From a functional perspective, Hsp70-positive tumour-derived exosomes stimulate migratory and cytolytic activity of natural killer (NK) cells [33,182] and activate macrophages [183]. In a different context, tumour-derived exosomes expressing surface Hsp72 can restrain tumour immune surveillance by promoting the suppressive functions of myeloid-derived suppressor cells and plasma-derived exosomes expressing Hsp70 have powerful cardioprotective effects in models of cardiac ischaemia–reperfusion injury via a membrane Hsp70/TLR4 communication axis [184].

The significant diagnostic, therapeutic and imaging potential of membrane Hsp70-based 'theranostics'[1] is considered by Gabriele Multhoff elsewhere in this issue [47].

9. Conclusion

Levels of HSP (cell stress) in biological fluids have been associated with a plethora of clinical conditions. These proteins could, therefore, act as indicators, drivers and/or moderators of disease processes and have potential utility as biomarkers of disease. Many, if not all, of the stress proteins that are released from cells under normal physiological conditions possess a range of biological functions, the nature of which depends on the context in which they are encountered. These proteins and networks have the potential to deliver a wealth of valuable, clinically relevant diagnostic and therapeutic approaches. The current challenge is to more fully understand these networks and establish their clinical potential.

> That which drugs fail to cure, the scalpel can cure. That which the scalpel fails to cure, heat can cure. If the heat cannot cure, it must be determined to be incurable
>
> —Hippocrates

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Endnote

[1]Theranostics: combining diagnostic and therapeutic capabilities into a single agent—a key element of precision medicine.

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