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Extracellular cell stress proteins as biomarkers of human disease

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Abstract

Although heat-shock (cell stress) proteins are commonly considered as being intracellular molecular chaperones that undertake a number of cytoprotective and cellular housekeeping functions, there is now a wealth of evidence to indicate that these proteins can be released by cells via active processes. Many molecular chaperones are secreted, or exist as cell surface proteins which can act as powerful signalling agonists and also as receptors for selected ligands. Levels of heat-shock (cell stress) proteins in biological fluids are now being associated with a plethora of clinical conditions, and these proteins therefore have potential utility as biomarkers of disease and/or response to therapeutic intervention. The present article summarizes current knowledge relating to extracellular cell stress proteins as biomarkers of human disease.

Background

The technician who inadvertently left the temperature of an incubator in Ferruccio Ritossa's (25 February 1936–9 January 2014) laboratory at too high a temperature one late afternoon in the early 1960s could not have anticipated the impact that this would have on biology. The elevated temperature to which *Drosophila* larvae were exposed overnight induced the appearance of new puffs in the polytene chromosomes and these were noticed by Ritossa on the following day. This was the first evidence that stress can influence gene transcription and induce the synthesis of new proteins; however, Ritossa had difficulty publishing these findings [1]. It was not until the early 1970s that the products of these genes were identified by Alfred Tissières and colleagues, and for obvious reasons, these gene products were termed 'heat-shock proteins' (Hsps) [2]. However, it is now known 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, and so a more descriptively correct term for these proteins might be 'cell stress' proteins [3].

It was many years before the relationships between stress-induced gene transcription and the roles of cell stress proteins in protein folding and the management of the intracellular environment were consolidated [2,4,5]. Thus, by the late 1980s, it was recognized that a proportion of cellular proteins require help with their folding and that this was facilitated via the actions of families of proteins termed 'molecular chaperones' [5], 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" [6]. Members of the molecular chaperone families perform essential cellular 'housekeeping' and cytoprotective functions, and enable cells to function correctly and cope with the plethora of insults and stresses that exist in the complex and dynamic intracellular environment (Table 1).

Cell stress proteins are released into the extracellular environment

The initial reports of the presence of Hsp60 and Hsp70 in the peripheral circulation by Pockley et al. in the late 1990s [7,8] were received with scepticism by the biological and biochemical communities, as it was unclear how these proteins could be released from viable cells in the absence of any cellular damage; given that cell stress proteins do not express the typical N-terminal signal peptide sequences that are typically required for secretion. However, 'non-classical' secretion of proteins that lack such sequences has been observed for a number of proteins such as fibroblast growth factors 1 and 2 (FGF-1 and -2), interleukin 1 (IL-1), and high mobility group box 1 (HMGB-1); the mechanisms underlying non-classical secretion pathways have been reviewed elsewhere [9]. The release of cell stress proteins from viable cells with an intact plasma membrane has been attributed to a number of mechanisms, including exosomal release [10,11], via cholesterol-rich microdomains [12] and secretary lysosomal endosomes [13], vesicular

Key words: biomarker, cancer, cell stress protein, enzyme immunoassay, heat-shock protein, molecular chaperone.

Abbreviations: Hsp(s), heat-shock (cell stress) protein(s); NK cell, natural killer cell; mAb, monoclonal antibody; GrB, granzyme B.

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Table 1 | **Mammalian cell stress response proteins, and their intracellular localization and function**

Abbreviations: ER, endoplasmic reticulum; TCP-1, tailless complex polypeptide; Grp, glucose-regulated protein; BiP, immunoglobulin heavy chain-binding protein; mtHsp70, mitochondrial Hsp70; HSF1, heat-shock factor 1; Apg-1, protein kinase essential for autophagy. Data taken from [48,49]. Further information on the nomenclature and individual family members has been published elsewhere [37,38].

Major family and members	Intracellular localization	Intracellular function
Small Hsps		
αB-crystallin	Cytoplasm	Cytoskeletal stabilization
Hsp27	Cytoplasm/nucleus	Actin dynamics
Haem oxygenase, Hsp32	Cytoplasm	Haem catabolism, antioxidant properties
Hsp60 or chaperonins		
Hsp60	Mitochondria	Both: bind to partially folded polypeptides and assist correct folding; assembly of multimeric complexes
TCP-1	Cytoplasm	
Hsp70		Hsp70 is involved in the regulation of HSF1 activity and the repression of Hsp gene transcription
Hsp70 (inducible)	Cytoplasm/nucleus	All: bind to extended polypeptides; prevent aggregation of unfolded peptides; dissociate some oligomers; ATP binding; ATPase activity
Hsc70 (cognate)	Cytoplasm/peroxisome	
Grp78/BiP	ER	
mtHsp70/Grp75	Mitochondria	
Hsp90		Hsp90 appears to be involved in maintaining the HSF1 monomeric state in non-stressful conditions; represents 1–2 % of total protein
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 synthesized protein
Grp94/Gp96/Hsp100	ER	
Hsp110		
Hsp110 (human)	Nucleolus/cytoplasm	Thermal tolerance
Apg-1 (mouse)	Cytoplasm	Protein refolding
Hsp105	Cytoplasm	

transport [14] and ubiquitination-triggered transport [15]. These mechanisms have been reviewed elsewhere [16]. Dying cells have been found to release free Hsp70, however, in much smaller amounts than Hsp70 in liposomal vesicles. Despite the initial scepticism, the precedent for the release of cell stress proteins and their potential role for intercellular signalling networks had already been established by others.

In 1989, Hightower and Guidon [17] reported that heat treatment broadened the spectrum of proteins released from cultured rat embryo cells, from a small set of proteins including the constitutively expressed member of the 70 kDa family of molecules, Hsc70, to include its inducible counterpart, Hsp70, and Hsp110. The release of these proteins was shown not to be mediated via the common secretory pathway, as inhibitors of this pathway (colchicine, monensin) did not block it [17]. Another of the earliest papers documenting a physiological release of cell stress proteins came from Tytell and colleagues in 1986 who reported the transfer of glia–axon transfer proteins, which include Hsp70, Hsc70 and Hsp100, from adjacent glial cells into the squid giant axon [18]. The authors suggested that the release of such proteins might be a mechanism by which glial cells can protect adjacent neuronal cells which exhibit a deficient response to stress.

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, murine and human prostate cancer cells and B cells (reviewed in [19]). With regards to the presence of cell stress proteins in the peripheral circulation, in 1977, a group of Australian scientists identified an immunoregulatory factor in the serum of women in the first trimester of pregnancy [20,21]. However, the molecular nature of this 'early pregnancy factor' was not identified until 1994, when it was shown to be human chaperonin (Cpn)10 (Hsp10) [22].

Cell stress proteins are present in the peripheral circulation in health and disease

The reports of the presence of Hsp60 and Hsp70 in the peripheral circulation [7,8] stimulated new interest in this area and the development of a range of enzyme immunoassays for measuring cell stress proteins in extracellular compartments. It is worth mentioning that most commercially available enzyme immunoassays for cell stress proteins are optimized for free Hsp70 in buffer, but not for Hsp70 in the serum,

Table 2 | **Circulating cell stress proteins as biomarkers of disease**

Stress protein	Condition	Key findings	Reference(s)
Hsp10	Periodontitis	Lower plasma levels in periodontal disease and treatment increases these. Post-treatment levels correlate with markers of clinical improvement	[50]
Hsp27	Renal disease	Elevated serum and urine levels in chronic kidney disease	[51]
	Autoimmunity	Serum levels may be a novel marker for diabetic neuropathy in patients with Type 1 diabetes	[52]
Hsp60	Stress	Association between elevated levels of Hsp60, low socioeconomic status and social isolation in males and females, and with psychological distress in women	[53]
	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	[25,28,54,55]
		Elevated levels present in coronary eluates after myocardial infarction	[56]
		Serum levels increase with accumulating features of the metabolic syndrome in postmenopausal women	[57]
		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	[58]
		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	[29]
	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	[59]
	Periodontitis	A larger proportion of patients with periodontal disease exhibit intermediate levels of plasma Hsp60 compared with controls. Treatment has no influence on levels	[50]
		Atherogenic dyslipidaemia and elevated circulating Hsp60 levels are linked and associated with periodontal pathology	[60]
	Autoimmunity	Serum Hsp60 levels correlate with time required for remission from flare-ups in patients with juvenile idiopathic arthritis	[61]
Hsp70	Surgery/trauma	Plasma Hsp70 levels markedly increase in patients undergoing liver resection and are associated with postoperative infection, hepatic ischaemic time and the degree of postoperative organ dysfunction	[62]
		Hsp70 is released into the circulation following coronary artery bypass grafting	[63]
	Cardiovascular disease	Elevated serum levels in patients with renal and peripheral vascular disease and individuals with borderline hypertension. In contrast, serum levels in hypertension are similar to normotensive controls	[25,28,54]
		Low serum levels at baseline predict the development of atherosclerosis in individuals with established hypertension	[27]
		Increased serum levels associated with low risk of coronary artery disease	[64]
		Increased circulating levels may be associated with the progression of atrial fibrillation and its recurrence after catheter ablation	[65]
		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	[66]
	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	[67]
		Positive correlations between serum levels and inflammatory markers	[31]
		Serum Hsp70 levels in patients with chronic hepatitis are higher than controls, but lower than in patients with liver cancer	[34]

Table 2 | **Continued**

Stress protein	Condition	Key findings	Reference(s)
	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	[30,68,69]
	Asthma	Induced sputum and plasma Hsp70 levels could serve as a useful marker for assessing airway obstruction in patients with asthma	[70]
	Renal Disease	Elevated urinary Hsp70 levels in stage 4 and 5 chronic kidney disease	[51]
	Autoimmunity	Plasma Hsp70 levels are high in patients with Type 1 diabetes	[71,72]
BiP	Periodontitis	Lower circulating levels of BiP (Grp78) in periodontal disease as compared with controls. Treatment has no influence on levels	[50]
Grp94	Autoimmunity	Plasma Grp94 (Gp96) levels are high in patients with Type 1 diabetes	[71,72]

plasma or other body fluids. 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. These have led to a number of reports associating levels of cell stress proteins with a number of conditions and diseases (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 which is under investigation [23]. Such information is not always apparent, and differences in the levels of Hsp70 which is measured by commercial and 'in-house' enzyme immunoassays have been reported [24]. This is an important issue, as serum and plasma are complex and subject to a number of 'matrix'-related effects which might influence accurate measurements in biological samples. One should also be mindful of the presence of circulating anti-cell stress protein antibodies in the peripheral circulation which might also influence the measurements made, although we have not found this to be the case [7,8,25–28]. The analysis of biological fluids has revealed a plethora of relationships between circulating cell stress protein levels and the clinical and physiological status of an individual (Tables 2 and 3), and these studies have the potential to provide new insight into the role of circulating cell stress proteins in the induction, progression and resolution of disease. For example, the 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 suggests that secreted Hsp60 may play a role in the cardiovascular pathology associated with diabetes [29]. The lower serum Hsp70 levels in normal human pregnancy might be suggestive of as yet unknown regulatory mechanisms that are aimed at maintaining immune tolerance in pregnancy [30]. However, further studies are required in order to establish these proteins as robust biomarkers of disease. Although Hsp70 is involved in inflammation of infectious origin, the inter-individual variation in measured levels appears to preclude the use of serum Hsp70 levels as an approach for distinguishing patients from healthy subjects in this context [31].

Circulating cell stress proteins as biomarkers of disease in cancer

There have been a number of particularly interesting and potentially important findings relating to the presence of circulating cell stress proteins in cancer, and a potential relationship between cell stress protein levels with tumour volume and response to treatment [32,33] (Table 3). The capacity of cell stress protein measurements to distinguish inflammatory events from specific disease states has recently been demonstrated. The Multhoff group has reported that serum Hsp70 levels in patients with liver cancer are significantly higher than a control group without liver disease, and (importantly) are also higher than individuals with chronic hepatitis [34]. Furthermore, a subgroup of patients with liver cirrhosis who subsequently developed liver cancer revealed higher serum Hsp70 levels than patients with liver cirrhosis alone [34]. In addition, Dutta et al. [35] 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 data indicate that serum Hsp70 levels can distinguish between inflammatory events/disease and cancer, and that circulating Hsp70 might be of value as a biomarker for cancer.

Another potential use for circulating cell stress proteins as biomarkers relates to the finding that serum Hsp70 measurements could be used to monitor the pharmacological effects of Hsp90 inhibitors in the clinical setting, especially in those situations when access to tumour tissue is not possible [36]. The use of Hsp90 inhibitors for the treatment of cancers such as myeloma, breast, prostate and lung cancer, melanoma, gastrointestinal stromal tumours and acute myeloid leukaemia is currently under great scrutiny. The activity of Hsp90 inhibitors is currently assessed on the basis of Hsp70 induction in peripheral blood mononuclear cells using Western blot analysis. However, this approach is

Table 3 | **Extracellular cell stress proteins as biomarkers of disease in cancer**

Stress protein	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	[73]
	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	[74]
	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 when compared with healthy control groups. Furthermore, levels can distinguish between early and advanced stage disease	[75]
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?	[76]
		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 toward shorter survival. Plasma Hsp70 could be a potential marker for predicting disease progression in patients with chronic phase in chronic myeloid leukaemia	[77]
	Colorectal	Serum levels of Hsp70 and mortalin are independent variables, and high serum levels of mortalin (mitochondrial Hsp70, Grp75, HSPA9) is a risk factor for shorter survival of patients with colorectal cancer. The concurrence of high serum Hsp70 and mortalin levels is associated with rapid disease progression	[78]
		Serum Hsp70 levels have potential as a stage-independent prognostic marker in colorectal cancer without distant metastasis	[79]
	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	[32]
		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 dropped without tumour relapse in the follow-up period. Hsp70 is therefore a potential biomarker for tumours and for monitoring the clinical outcome of radiotherapy. High levels were associated with high levels of membrane Hsp70 expression on tumour cells	[33]
	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	[34]
	Pancreatic	Plasma Hsp70 levels were significantly higher in the blood of mice bearing membrane Hsp70 positive spontaneous pancreatic ductal adenocarcinomas, and these correlated with tumour volume. Radiation-induced tumour regression was associated with significantly decreased Hsp70 levels, and these returned to those of control animals after complete remission	[32]
		Serum Hsp70 levels are significantly increased in patients and may be useful as an additional biomarker for the detection of pancreatic cancer	[35]
	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	[75]
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	[44]

laborious, only semi-quantitative and difficult to implement in the clinic [36].

This is an opportune time to highlight the issue of nomenclature for the cell stress protein families of molecules. Although this has previously been discussed in an article proposing new guidelines for the nomenclature of the human Hsps in detail elsewhere [37], the literature continues to remain 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 [38]. Of the 13 protein members of the family, two closely linked genes, referred to as Hsp70-1 and Hsp70-2, are the major stress-induced members [38]. Although some evidence implicates Hsp70-2 in human cancer, the cytosolic stress-induced Hsp70-1 is the predominant form which is overexpressed in cancer [38]. 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, the precise identity of the analyte being reported upon needs to be verified using information on the specificity of the antibodies that are being used in the assays.

Membrane Hsp70: a 'third' moonlighting function for the 70 kDa cell stress protein

Another potentially important finding is that serum Hsp70 levels are associated with a high expression of a membrane form of Hsp70 on tumours in patients with squamous cell carcinoma of the head and neck [33]. Gabriele Multhoff and colleagues 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) [39–41]. The expression of membrane Hsp70 has now been detected on a broad panel of cancer cell lines, including all of the human and murine breast cancer cells that have been tested (data not shown), and the density of membrane Hsp70 on cancer cells is increased by treatments such as radio(chemo)therapy [42]. An ongoing screening program of over 1000 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 in metastatic disease [43], and its expression is associated with an unfavourable prognosis and a reduced overall survival in patients with rectal carcinoma and squamous cell carcinoma [44]. Membrane Hsp70 expression is therefore a universal, selective tumour-specific marker of 'aggressive' disease. However, the clinical prognosis of patients is better when they bear Hsp70 positive tumours that preferentially metastasize via the liver (colon and gastric carcinoma) [44].

Membrane Hsp70 acts as a target recognition structure for the induction of antibody-dependent cellular cytotoxicity (ADCC) using a unique mAb which is specific for the membrane form of Hsp70 (cmHsp70.1 mAb) [41], and for activated natural killer (NK) cells [45] via its selective internalization of NK cell-derived serine protease granzyme B (GrB) [46]. It has been proposed that the better survival of patients with tumours that metastasize via the liver results from the fact that hepatic NK cells can recognize and kill Hsp70 membrane-positive tumour cells [44]. It has also been shown that the binding of recombinant human GrB to membrane Hsp70 on human and murine cancer cells and its perforin-independent endocytic uptake induces selective killing of cancer cells [43,47]. Membrane Hsp70 therefore has great potential as a tumour targeting molecule and the use of serum Hsp70 as surrogate biomarker for its expression on tumours would greatly assist the development and delivery of new therapeutic strategies that target this unique form of the 70 kDa cell stress protein.

Conclusions

Levels of Hsps 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. Although it continues to remain difficult to distinguish 'cause' from 'effect', it appears that cell stress proteins have potential as diagnostic and predictive biomarkers of disease. The current challenge is to establish the clinical potential of these proteins, and it is possible that cell stress protein profiles rather than levels of individual proteins will be the way forward.

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