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Heat shock proteins as modulators and therapeutic targets of chronic disease: an integrated perspective

Running Title: Heat shock proteins in health and chronic disease

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Abstract

Many heat shock proteins (HSPs) are essential to survival as a consequence of their role as molecular chaperones, and play a critical role in maintaining cellular proteostasis by integrating the fundamental processes of protein folding and degradation. HSPs are arguably amongst the most prominent classes of proteins that have been broadly linked to many human disorders, with changes in their expression profile and/or intracellular/extracellular location now being described as contributing to the pathogenesis of a number of different diseases. Although the concept was initially controversial, it is now widely accepted that HSPs have additional biological functions over and above their role in proteostasis (so called 'protein moonlighting'). Most importantly, these new insights are enlightening our understanding of biological processes in health and disease, and revealing novel and exciting therapeutic opportunities. This theme issue draws on therapeutic insights from established research on HSPs in cancer and other non-communicable disorders, with an emphasis on how the intracellular function of HSPs contrasts with their extracellular properties and function, and interrogates their potential diagnostic and therapeutic value to the prevention, management and treatment of chronic diseases.

1. Introduction

The most extensively studied heat shock proteins (HSPs) are the molecular chaperones that function intracellularly in an ATP-dependent manner and include heat shock protein 60 kDa/heat shock protein 10 kDa (HSP60/HSP10; chaperonins) (HSPD/HSPE); HSP40 (DNAJ), HSP70 (HSPA); HSP90 (HSPC); HSP100; and HSP110 (HSPH) families. The expression of many of these HSPs is regulated by heat shock transcription factors (HSFs), of which HSF1 is the best studied. Increasing evidence now suggests that these molecular chaperones also have biological properties in the extracellular environment which may be independent of their chaperone functions. In addition to ATP, the molecular chaperone activity of the major HSPs is regulated by a cohort of non-substrate accessory proteins, known as co-chaperones. Co-chaperones are a diverse group of chaperone regulatory proteins which are required, to a greater or lesser degree, by certain chaperones. HSP90, for example, has over 20 co-chaperones that fine tune its function and adapt it to the different stages of the protein folding pathway. Some HSP families, such as HSP40, include members having both chaperone and co-chaperone activity.

A particularly lively area relates to the evolving insight into the therapeutic potential of targeting HSPs in cancer, and their value as an exciting class of molecular target. Although HSPs and their transcription factors have been the subject of sustained interest in the field of cancer biology, more recently they have been attracting interest in many other chronic conditions such as diabetes, obesity, autoimmune disease, neurodegeneration, muscular dystrophies, psychiatric disorders and chronic heart failure. These studies are revealing that although increased levels of intracellular HSPs may be beneficial for acute conditions, such increases can be detrimental for certain chronic conditions, as exemplified by acute and chronic heart conditions. The contribution of extracellular HSPs to chronic disease is poorly understood. Increased levels of extracellular HSPs appear to be detrimental by enhancing inflammation pathways, and hence for conditions such as diabetes a reduction in the ratio of extracellular to intracellular HSPs is beneficial. In contrast, extracellular HSPs can also be beneficial to certain autoimmune conditions as a consequence of their ability to engage with, and recruit the immunomodulatory activity of regulatory T cell populations. Although the reported dichotomies in functionality of HSPs would appear to be counter-intuitive and has been the subject of great debate and counter-arguments, one needs to consider the context and the temporal nature of disease and its control. What is clear from current knowledge is that HSPs play important biological roles under physiological, stressful and disease conditions.

The articles in this theme issue highlight how insights (both anticipated and unanticipated) into the biological function of HSPs in cancer have revealed new therapeutic options for the treatment of the disease. The issue also explores how the intracellular function (ATP-rich context) of HSPs contrasts with their extracellular function (ATP-poor context), and their potential diagnostic and therapeutic value to the prevention, management and treatment of chronic diseases. Here we integrate and critique the content of this theme issue, addressing HSP moonlighting in the context of their contrasting intracellular and extracellular roles.

2. Heat shock proteins and protein moonlighting

Although the finding that exposure to a non-physiological temperature (37°C *versus* 26°C) induced a new puffing pattern in the polytene chromosomes of Drosophila [1] was interesting, the author could not have anticipated the significance and broad reach of this finding, especially given that the 'biological relevance of the findings were unclear' and it proved difficult to publish the findings. However, over 50 years later, we continue to appreciate the importance of this heat shock response (HSR) to the maintenance of cellular homeostasis and protection against a multitude of physical, chemical and biological stressors that exist in the environment [2].

As the protein folding paradigm and molecular chaperone functions of HSPs were developing in the late 1980s and 1990s, it became apparent that some of these proteins were also present on the surface of cells or in the extracellular fluids. This contradicted the established dogma that these proteins were exclusively intracellular and so it took time for the data to be accepted, the findings to gain traction with the scientific community and for this new field of extracellular HSPs to be accepted and become established. Interest in the biological role(s) and functions of these proteins grew, as did interest into the potential capacity of extracellular HSPs to influence biology and physiology. As discussed in this issue, it was shown that the treatment of cells with purified HSPs resulted in cell activation similar to that induced by pro-inflammatory cytokines. Despite controversy surrounding the possibility that at least some of the pro-inflammatory effects of HSPs might be due to contaminants of the preparations that have been used [3, 4], there is also a wealth of evidence from a number of settings which argues against this concept [5].

A new paradigm has arisen that at least some HSPs are secreted proteins [6] with pro- (HSP60, HSP70, HSP90) or anti-inflammatory (HSP10, thioredoxin, HSP27, BiP) actions of importance in human diseases such as cancer, coronary heart disease, diabetes and rheumatoid arthritis [7], to name but a few. In addition to having direct effects on cells, HSPs can bind peptides and present them to T cells to modulate immune responses, and this might have implications in a number of disease settings, including cancer [8]. It has become apparent that HSP70 can be present in a membrane expressed form. The significant diagnostic, therapeutic and imaging potential of this finding, and the progress which has been made in exploiting membrane HSP70-based theranostics (i.e. combining diagnostic and therapeutic capabilities into a single agent; a key element of Precision Medicine) for the management and treatment of patients with cancer, is considered in detail in this issue [9].

Taken together, the findings that HSPs can be present in the extracellular and cell-associated compartments have led to the establishment of a new paradigm which designates these proteins as 'moonlighting proteins' (proteins with more than one function) that have the capacity to 'escape' from cells and interact with different cell types to elicit a range of biological effects. These proteins can even act as receptors for inflammatory mediators called 'inflammogens' [10]. Support for this new paradigm comes from a number of studies that are highlighted in this issue [11], and a large number of studies that have, and continue to reveal, the presence of a number of HSPs in the bodily fluids of humans and animals [12]. The first two contributions in this issue provide a critical overview of extracellular HSPs [11] and the biology of protein moonlighting [13].

3. Intracellular *versus* extracellular heat shock proteins in cancer

The initiation, progression and metastasis of cancer have all been shown to be accompanied by multiple cellular insults arising from both intracellular and extracellular sources. Internal to the cancer cell, the high expression of oncogenic proteins (many of which are mutated), altered cellular metabolism, aneuploidy and genomic instability all contribute to its characteristic stressed phenotype. Moreover, during cancer development, cells are exposed to altered extracellular conditions that can include hypoxic, acidotic, mechanical and nutrient deprived microenvironments, further stimulating the cancer cell to engage highly conserved survival pathways such as the HSR. Consistent with the knowledge that cancer cells are exposed, both internally and externally, to major proteotoxic insults that challenge cellular homeostasis and survival, it is not surprising that cancers constitutively express high levels of HSP family members. In fact, tumour cells have become to be regarded as addicted to HSPs (e.g. HSP90) as well as their transcriptional regulators (e.g. HSF1).

Increased expression of many HSPs, including HSP27 (HSPB1), HSP72 (HSPA1A, HSPA1B) and HSP90 (HSP90AA1, HSP90B1), have been shown in a wide variety of cancer types such as breast, prostate, lung and melanoma, and are associated with poor patient outcomes. Moreover, HSF1, the master regulator of the HSR has also been shown to be increased in expression and constitutively activated in many cancers. The parallel molecular, genetic and pharmacological investigations that have been performed in relation to HSPs and their signalling and transcriptional regulation, has further confirmed their importance to the growth and progression of many tumour types (reviewed in this issue [14]). For example, the work in targeting and developing HSP90 inhibitors has confirmed the importance of HSP90 to cancer signalling and oncogene driven growth (reviewed in this issue [15]). In a similar manner, the HSR has been shown to be an integral part of the oncogenic network, working through the actions of HSF1 to maintain cancer cell survival and function (reviewed in this issue [16]). Interestingly, it has been shown that within the oncogenic context, the expression of HSF1 is indispensable for the growth and survival of cancer cells, while its loss in non-transformed cells has little to no effect [17].

HSF1 and many of the HSPs have been shown to play fundamental roles in many aspects of the cancer cell phenotype associated with the hallmarks of cancer [18] including sustained proliferative signalling, evading growth suppression, replicative immortality, angiogenesis, resisting cell death and supporting invasion and metastasis [19]. Moreover, they are also involved in a number of the more recently identified hallmarks of cancer such as the deregulation of cellular energetics, genome instability, avoiding immune destruction and enabling tumour-promoting inflammation. The wide-ranging actions of the HSPs and HSF1 are not limited to the cancer cells themselves, but have also been shown to play important roles for accessory cell function within the tumour microenvironment such as the cancer associated fibroblasts (CAFs) and tumour associated macrophages (TAMs), ultimately contributing to cancer cell growth and progression [20].

Although it was originally proposed that the actions of HSPs were primarily intracellular to cancer cells and other cells of the tumour microenvironment, it is now evident that their presence and functionality are also very important to many molecules and processes external to the cell. For example, HSP90α (HSP90AA1) is known to exist outside the cell, termed as eHSP90, and has been shown to interact with a number of client proteins, including matrix metalloproteinase 2 (MMP2) through which it enhances the migration and invasion of cancer cells (reviewed in this issue [14, 15]). It has been shown that the functions of extracellular HSPs can have both anti-tumour or pro-tumour effects, ranging from anti-tumour or pro-tumour immunomodulation (HSP90, HSP72, HSC70, HSP60, HSP27), suppression or promotion of tumour cell proliferation (GRP78, HSP20, HSP27), as well as promotion of cancer cell invasion (HSP90, GRP75, HSP27) and angiogenesis (HSC70) [21-26]. Moreover, co-chaperones of HSP90, such as the HSP70/HSP90 organising protein (HOP), HSP40 and p23 have also been shown to be extracellular, and similar to their role

internal to the cell, are in complex with HSP90 to elicit extracellular functions such as MMP-2 activation and cancer cell invasion and migration [23, 27].

Our increasing knowledge of the unique roles of HSPs and their co-chaperones external to the cell is leading to novel approaches for the therapeutic targeting of cancers. For example, cell surface HSP70 is currently being used as a target of novel therapies that include nanoparticle-based treatments for cancer, and cell-impermeable HSP90 inhibitors are being examined as to their efficacy in inhibiting cancer migration and invasion (reviewed in this issue [9]). Therefore, our increased understanding of the actions of extracellular HSPs will not only lead us to a better understanding of the biology of cancer and its progression, but will also reveal further therapeutic opportunities for the treatment of advanced cancers.

4. Intracellular *versus* extracellular heat shock proteins in chronic diseases

Much of the research into the function of HSPs in chronic disease has been focussed on cancer. However, it is also clear that HSPs are involved in many other chronic conditions, from neurological and muscle-wasting disorders to obesity and post-traumatic stress. This range of chaperonopathies highlights the important and central role which these proteins play in maintenance of correct cellular function.

Findings from experimental, pharmacological or exercise studies on changes to HSP72 expression levels suggest that the manipulation of the extracellular to intracellular ratio of HSP levels represents a useful avenue for the prevention and treatment of diabetes (reviewed in this issue [28]). For example, there is evidence that exercise promotes the release of extracellular HSP72 from certain human cells (brain, [29]; epithelium, [30]; immune system, [31]; muscle and adipose tissue, [32]). However, long-term exercise promotes a decrease in extracellular HSP72 and an increase in intracellular skeletal muscle HSP72 [28]. In fact, it is now apparent that the balance of extracellular (pro-inflammatory) *versus* intracellular (anti-inflammatory) HSP72 appears to be a determining factor for the extent of tissue inflammation and hence the pathology associated with diabetes. It is hypothesised that interventions that lower the extracellular to intracellular HSP72 ratio are potentially beneficial in the context of diabetes progression [33]. Hence, carefully constructed exercise regimes that favourably modulate this HSP72 ratio may serve as powerful therapeutic interventions for the prevention and management of diabetes. However, more detailed studies on extracellular HSPs and the effects of exercise are needed, particularly the contribution of different tissues to extracellular HSP expression levels, and the biochemical and physiological mechanisms of action of these HSPs.

HSPs, and HSP72 in particular, also play an important role in muscle function and are potential therapeutic agents for muscle wasting conditions (reviewed in this issue [34]). HSP90, HSP72, and HSP27 all have a pro-myogenic role in muscle development, albeit via distinct mechanisms. HSPs are also differentially expressed in the muscle progenitor pool that differentiates to give rise to new muscle tissue [34]. HSP72 is the most widely studied HSP in this context and is required for muscle repair after acute injury. Both intracellular and extracellular HSP72 contribute to this process, with extracellular HSP72 functioning primarily via the activation of the immune response. Interestingly, many of the effects of HSP72 knockout on muscle regeneration involve the immune response, which suggests that, given that extracellular HSP72 arises from intracellular HSP72, the extracellular functions of HSP72 are more important in this context. Indeed, injection of extracellular HSP72 has been shown to ameliorate many of the effects of muscle injury in HSP72 null mice [35]. With respect to disease, over-expression of intracellular HSP72 had a positive effect and led to improvements in body strength and endurance, diaphragm health, normalised muscle force and reduced markers of muscle damage in a mouse model of Duchenne muscular dystrophy [36]. HSP72 also has a positive effect on muscle function in the context of muscle immobilisation, suggesting that over-expression of this protein may be a therapeutic approach for a range of muscle wasting conditions. It is likely that at least some of the

described functions of HSP72 in these conditions are attributed to the extracellular function, but this has not been demonstrated definitively.

In addition to a role in muscle-related immune responses, experimental models have provided evidence that both intracellular and extracellular HSPs also have a protective function in autoimmune diseases (reviewed in this issue [37]). The application of exogenous extracellular recombinant HSPs and the experimental co-induction of endogenous intracellular HSPs have been shown to lead to production of disease protective regulatory T (Treg) cells [37, 38]. This has stimulated research into the development of therapeutic HSP-based peptide vaccines for the restoration of immune tolerance in inflammatory diseases.

There is emerging evidence for increased expression of extracellular HSP70, HSP90, and certain associated co-chaperones (e.g. BAG-3) in heart failure, and that their functions are complementary and independent of their intracellular isoforms. The important therapeutic and diagnostic considerations of these findings are reviewed in this issue [39]. Current findings suggest that therapeutic strategies involving the increase of HSP levels may be applicable in the context of acute heart conditions (e.g. acute myocardial infarction/ischemic reperfusion injury), but not chronic heart conditions (e.g. hypertension). Indeed, the pharmacological enhancement of intracellular HSP function has been shown to provide protection against experimental myocardial infarction [40]. With respect to chronic heart conditions, extracellular and intracellular HSPs exert different effects. For example, a decrease in the expression of intracellular HSP70 promotes cardiomyocyte hypertrophy and dysfunction while protecting mice from cardiac fibrosis, whereas inhibition of extracellular HSP70 has been shown to improve hypertension-induced hypertrophy and fibrosis [41]. In the context of chronic heart disease, there are some parallels in the findings for extracellular HSP90 and extracellular HSP70. For example, the decrease in fibronectin levels, collagen production and the associated TGFβ signalling pathway via the inhibition of extracellular HSP90 [42, 43] has implications for the fibrosis-related pathology of chronic heart conditions. Although there is great promise for extracellular HSP70 and HSP90 as diagnostic markers of chronic heart disease, a deeper understanding of the mechanism(s) of action of extracellular HSP70 and HSP90 and its co-chaperones is required before effective prevention and treatment can be achieved.

HSPs are also important in the context of neurodegeneration and neurological dysfunction leading to psychiatric diseases. HSP40s are the largest and most diverse of the HSPs and changes in different HSP40 isoforms all give rise to different, but related forms of neurodegeneration (reviewed in this issue [44]). Although these HSP40 isoforms share structural features such as the J domain, they also contain a number of unique functional domains (particularly since most of the isoforms associated with disease are the more diverse type III HSP40/DNAJC). The redundancy between isoforms in some contexts can also explain why it is possible to ameliorate the disease consequences of a mutation or deficiency of one isoform via over-expression of another. For example, overexpression of DNAJA1 can suppress aggregation of polyQ ataxin associated with neurodegeneration [45]. Interestingly, there are no neurological disorders associated with mutations in type I HSP40s like DNAJA1, presumably because many of these proteins are essential and loss of function cannot therefore be tolerated. With respect to psychiatric disorders, the co-chaperone FKBP51, acting via HSP90, is both a causative agent and biomarker for various forms of the disease (reviewed in this issue [46]). Increased levels of FKBP51 lead to glucocorticoid resistance by retarding the recruitment of glucocorticoid receptor (GR) to the nucleus and perturbing signalling via the hypothalamic-pituitary-adrenal (HPA) axis that culminates in a poor stress coping phenotype [46]. Specific single nucleotide polymorphisms that result in methylation changes which alter levels of FKBP51 may be a risk or prognostic factor for anxiety or suicide risk [47, 48]. This suggests that modulation of FKBP51 levels may be a relevant therapeutic strategy. However, in the context of both HSP40-related neurodegeneration and FKBP51-related psychiatric disorders, we have limited

understanding of the relative contribution of intracellular *versus* extracellular forms of the relevant HSPs due to a paucity of data. Certainly, it is known that both HSP70 and HSP90 are extracellular and therefore it is at least theoretically possible that co-chaperones of these two proteins (HSP40 and FKBP51) also exist in functional extracellular forms. In these examples, what we do know is that disease is usually associated with a change in the levels of a particular HSP. For example, mutations or deletions in the HSP40 isoform DNAJC29 is one of the most common causes of ataxia [49]. In some instances, the change in HSP levels are associated with missense mutations, deletions or splicing changes, while in other cases levels change in response to the environment (such as age-induced increases in FKBP51 levels which are associated with psychiatric disorders).

5. Conclusion

Fundamental insights into how HSPs give rise to disease will be an important component of therapeutic targeting of these proteins. However, many knowledge gaps remain and need to be addressed. Importantly, with cancer and autoimmune disease being the exceptions, there is limited insight into the role played by extracellular HSPs in chronic diseases such as neurodegeneration or psychiatric disorders. In addition, while much is known about the mechanism of action of specific intracellular HSP networks, such as the HSP90-HOP-HSP70 or HSP70-HSP40 complexes, the genesis and function of these HSP complexes in the extracellular milieu is poorly understood and raises many fundamental questions that need to be answered before therapeutic applications can be properly developed. Like the HSPs they regulate, co-chaperones like HOP appear to also be secreted via exosomes [50]. However, it is not known if HOP is secreted together with HSP90 and HSP70 as a functional complex, or if it is secreted separately and then forms a complex with the HSPs [51]. Therefore, the major questions that need to be answered for these extracellular HSP complexes and many other extracellular HSPs include the following:

1. What is the origin of extracellular HSPs, and which isoforms are structurally and functionally distinct from their intracellular counterparts, and which isoforms are derived from their intracellular counterparts?
2. Which isoforms of extracellular HSPs are encoded by separate genes and which are encoded by splice variants of the same gene?
3. Are there receptors associated with extracellular HSPs?
4. As a general principle, is the ratio of extracellular to intracellular HSP levels important for cellular and physiological homeostasis?
5. What stimuli, mechanisms and pathways are required for the secretion of extracellular HSPs?
6. Do extracellular (exosomal) HSPs function as molecular chaperones, is their activity regulated by extracellular co-chaperones and what defines extracellular client proteins?

While there is much work to be done before we can more fully define the true biological role, therapeutic potential and significance of extracellular HSPs, we can draw inspiration from Hippocrates who stated: ‘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’.

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