Heat shock proteins: Stress proteins with Janus-like properties in cancer

STUART K. CALDERWOOD¹ & DANIEL R. CIOCCA²

¹Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA and ²Institute of Experimental Medicine and Biology of Cuyo (CRICYT-CONICET) and Argentine Foundation for Cancer Research (FAIC), Mendoza, Argentina

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Abstract
Heat shock proteins (HSPs) were first identified as stress proteins that confer resistance to physical stresses such as elevated temperatures in all cellular organisms. HSPs are rapidly elevated after stress and confer a temperature resistant phenotype. Temperature resistance is dependent on the ability of HSPs to function as molecular chaperones and prevent aggregation and on the capacity of Hsp27 and Hsp70 to act as wide spectrum inhibitors of the cell death pathways. HSP expression becomes deregulated in cancer leading to elevated expression. Elevated HSP expression promotes cancer by inhibiting programmed cell death (Hsp27, Hsp70) and by promoting autonomous growth (Hsp90) and leads to resistance to chemotherapy and hyperthermia. Tumor HSPs have another property that can be exploited in therapy. They are immunogenic and can be used to form the basis of anticancer vaccines. Elevation in HSP levels may thus have competing effects in tumor growth, being required for tumor cell survival but conferring a hazard for cancer cells due to their immunogenic properties. This dichotomy is also reflected by the approaches used to target HSP in therapy. Pharmacological approaches are being employed to inhibit activity or expression of tumor HSP. Immunological approaches aim at increasing HSP levels in cells and tissues with the aim of increasing tumor antigen presentation to the immune system.

Keywords: Heat shock protein, cancer, apoptosis, tumor, immunity, thermotolerance

Introduction
With the recent finding that the human genome is unexpectedly compact – with far fewer than the anticipated 100,000 genes, it would appear that genes may be required to encode multiple protein isoforms and proteins may be called on to carry out more than one function. Indeed, globular proteins likely display a number of interaction domains on their surface which are potential interaction or catalytic sites, and it may be that the names given to such proteins only reflect the functions attributed when they were first discovered [1]. Such versatile properties are becoming evident to those of us who investigate the functions of heat shock proteins.

Study of HSP functions was initiated by the finding of a new gene expression pattern, through a happy accident involving the overheating of a Drosophila salivary gland preparation on a microscope stage [2]. This was first reported as: ‘A new puffing pattern induced by temperature shock and DNP in Drosophila’ in 1962 [2]. However, it took another 10–15 years before the first Drosophila HSP mRNA were isolated [3]. Around this time however, (1978) HSPs were discovered in mammalian tissue culture cells, in Escherichia coli, in yeast, and in plants [4–9]. The heat shock field emerged as a major study area in experimental biology at the 1982 meeting ‘Heat Shock: From Bacteria To Man’ held at the Cold Spring Harbor Laboratory [3]. At this time however, the functions of the HSP remained mysterious and the details of regulation of HSP gene expression were only beginning to emerge. All that was known was that the proteins possess ‘homeostatic activity’ and are to this day associated with resistance to heat shock and other stresses.
However, in the early 1980s, the concept began to emerge that the HSP belonged to a new kind of protein which functions to modify the structure of other proteins. Most notably the functions of the E coli genes DNA-K (Hsp70), DNA-J (Hsp40) and GroEL (Hsp60) were determine genetically in a study of $\lambda$ phage replication and biochemical analysis of the clathrin coated pits at the membranes of mammalian cells hinted at a new function for Hsp70 [10, 11]. The HSPs were apparently required to fold the proteins involved in $\lambda$ page replication and to disassemble the proteins involved in the huge lattice structures of the clathrin coats with the aid of ATP hydrolysis. Hsp70 was described as an ‘unfolding ATPase’, a protein that employs the energy derived from ATP and an intrinsic ATPase activity, to influence quaternary interactions between proteins [11]. HSPs thus became known as ‘molecular chaperones’ due to their role in associating with other proteins and among other things, discouraging promiscuous interactions, and this name has been retained to the present time [10].

During this period (1980–1990) huge strides were also made in elucidating the processes underlying transcriptional regulation of HSP genes. In eukaryotes, a cis-acting element (the heat shock element, or HSE) that confers heat shock regulation on genes was discovered first in Drosophila and then strikingly similar sequences were found in yeast, avian and mammalian cells [12]. Discovery of the HSE sequence was then instrumental in the isolation of the transcription factors (heat shock factor, or HSF) that respond to heat shock, bind to the HSE and activate HSP gene transcription [13]. An excellent review describes the early studies on the refinement of the canonical HSE sequence and the early studies on HSF regulation [14].

In recent years HSPs have been assigned a more empowered role than as chaperones and are now envisaged as central regulators of cell metabolism. This new concept was heralded by the finding that Hsp90 is required for glucocorticoid receptor activity and that, in the absence of Hsp90, GR fails to mature or HSF) that respond to heat shock, bind to the HSE and activate HSP gene transcription [13].

Heat shock proteins mediate resistance to hyperthermia

The discovery of a powerful mechanism for hyperthermia resistance (thermotolerance) coincided with the discovery of HSPs and it was soon shown that the onset of thermotolerance coincides quite closely with HSP synthesis [18]. A causal relationship was later shown [19–21]. Thermotolerance is a potent and long-lived form of heat resistance and, for instance a relatively minor pre-exposure to heat such as 15 min at 43°C can lead to profound loss of heat sensitivity [18]. This has had a major impact on how hyperthermia is scheduled when used in combination with radiotherapy, and heating doses have typically been given on a weekly basis to avoid complications due to thermotolerance [22]. The discovery that the HSPs have molecular chaperone activity led to the hypothesis that thermotolerance involves the increased ability to refold heat denatured proteins in cells with elevated HSP levels [23, 24]. Intracellular HSPs were envisaged as limiting protein unfolding during hyperthermia and refolding protein aggregates during the recovery from heat shock. There is a considerable body of evidence in favor of this hypothesis, which considers that a subset of thermally sensitive proteins may confer temperature sensitivity on the cell. Different HSPs appear to chaperone different subsets of proteins [25, 26]. Most of these thermotolerance studies have utilized the clonogenic cell survival assay to quantitate tumor cell inactivation, as this is the most therapeutically relevant parameter.

Heat shock proteins inhibit programmed cell death

With the increasing understanding of molecular pathways of cell death that began in the later 1980s, it rapidly became evident that HSPs can act directly to inhibit cell death pathways. Hsp70 was shown to inhibit death induced by the stress kinase c-jun kinase [27, 28]. It has recently been
shown that Hsp27 and Hsp70 can both inhibit caspase-dependent apoptosis pathways. Elevated levels of these HSPs interact directly with intermediates in the death pathways and prevent progression through to the ‘execution stage’ of the cellular death sentence. A number of independent death pathways exist and the HSP appear to act on these through contrasting mechanisms. For instance, Hsp70 inhibits a caspase-independent form of death by interacting with the lysosomal membrane and preventing the activity of hydrolytic lysosomal enzymes [29].

The levels of heat shock proteins are elevated in many types of cancer

The levels of Hsp70 and Hsp27 are elevated in a wide spectrum of human cancers and mediate tumorigenesis through mechanisms involving inhibition of PCD, an essential trait in cancer [30–32]. Elevated HSP gene transcription in cancer is coupled to some of the basic oncogenic pathways. A primary mechanism for HSP regulation in normal cells involves the tumor repressor p53 and the related protein p63. These proteins repress HSP transcription through binding sites for the transcription factor NF-Y present within HSP promoters [33, 34].

During transformation, p53 mutation (a genetic change associated with over 45% of cancers at many organ sites) reverses this effect and leads to enhanced Hsp70 transcription through loss of Hsp70 promoter repression [35–38]. Alterations in p63 are closely associated with Hsp70 expression in cancer and expression of the isoform ΔNp63α, a dominant negative inhibitor of wild-type p63 up-regulates Hsp70 and Hsp40 levels in head and neck cancer [33]. In addition to reversal of repression by p53 family proteins, induction of tumor HSP also involves positive effects on transcription through the signaling circuitry of the heat shock response pathway [39]. During the heat shock response massive levels of HSP gene expression occur through interaction of HSF1 with the HSE elements present in all HSP promoters [10, 14, 40]. It was shown recently shown that the tumorigenic factor heregulin-β1 binds to the cell surface of breast cancer cells and leads to increased HSP expression, enhanced survival and transformation through induced stabilization of HSF1 [31]. Heregulin activates HSF1 through a signaling pathway involving activation of HER2 and PI-3 kinase at the cell surface and leads to expression of HSP genes through the HSE elements in their promoters [39]. As PI-3 kinase is a key enzyme in malignant progression, particularly through its activation by PTEN mutation and induction of c-Myc, this may be an important mechanism for HSP elevation in cancer [41]. In addition, the proto-oncogene c-Myc, which is activated by heregulin and HER2 also positively regulates HSP transcription through activation of HSE [34]. Indeed, c-Myc activates the Hsp90A promoter and inhibition of this activation decreases the transforming effects of c-Myc expression [42]. HSF1 also plays a number of additional roles in the malignant phenotype, including the override of cell cycle checkpoints leading to tumor aneuploidy and enhanced metastasis which may involve non-HSP dependent effects of HSF1 [43, 44]. In addition, Hsp90 is the intrinsic repressor of HSF1 under non-stress conditions and one can envisage a mechanism for HSP elevation that includes increased sequestration of Hsp90 by unstable mutated tumor proteins, de-repression of HSF1 resulting in expression of HSP [45]. In addition, Hsp27 transcription is activated by factors in addition to HSF1 including the POU domain protein Brn3a [46–48]. Overall therefore, elevated expression of HSP occurs through relief of repression by p53, which is inactivated in many cancers, and through positive regulation by oncogenic signaling pathways that lead to activation of HSP promoters.

It also seems possible that HSP expression could be induced by the micro-environmental stress
imposed by the tumor milieu [49]. However, little information is available to encourage this suggestion and the available data indicate that another transcriptional stress response, the unfolded protein response rather than HSP expression, is activated by the tumor milieu, and growth of tumor cells as xenografts leads to the inhibition rather than enhanced expression of HSP in cells growing in tumors [50].

The levels of Hsp70 and Hsp27 are elevated in a wide spectrum of human cancers and mediate tumorigenesis through involving inhibition of PCD (described above), an essential event in cancer progression [52–54]. The enhanced activity of a number of oncogenes, most notably c-Myc or Ras, induce PCD pathways including apoptosis and autophagy, and therefore a number of systems involved in PCD regulation including the p53 and Bcl-2 family mediated networks are subverted, and inactivation of these networks are important steps in the emergence of cancer cells [55, 56]. The relationship of Hsp27 and Hsp70 to the p53 and Bcl-2 pathways is currently not clear, although each can function independently in countering death signals [51, 57]. It has been shown that the blockage of cell death through apoptosis and autophagy can lead to a proportion of such cells dying through default by necrosis [58]. This form of death may be less efficient than other death pathways, permitting enhanced growth [56]. In addition, necrosis arising from inhibition of PCD and ischemia in the poorly perfused tumor core is not opposed by Hsp70 overexpression and leads to the release of cell contents into the tumor milieu and initiation of an inflammatory environment in the vicinity of the tumor that favors angiogenesis, tumor cell invasion and metastasis [56, 58, 59].

The effects of HSP on the anabolic pathways leading to self-sufficiency in growth signals are mediated largely through Hsp90. This molecular chaperone is essential for the stability of the fragile structures of many of the receptors, protein kinases and transcription factors that lie along the pathways of normal cellular growth [61]. Hsp90 is required to maintain signaling proteins in active conformation that can be rapidly triggered by growth signals [16, 61]. This chaperone may thus be viewed as a facilitator of the rapid and fluid responses to extracellular signals required, particularly in development and cell renewal [61, 62]. Transformation involves the overexpression or mutation of many of these Hsp90-dependent signaling molecules and Hsp90 is increasingly required to maintain such proteins in active conformation. Hsp90 is essential for the stability and activity of over 200 Hsp90 clients [61]. Hsp90 performs these molecular chaperone functions as the dominant component of a high Mr chaperone machine, a large complex incorporating five core proteins found in all complexes: HSP90 itself, the scaffold protein Hop, the p23 protein which mediates substrate choice and a Hsp70/Hsp40 complex that mediates formation of the Hsp90-substrate complexes [62]. Hsp90 complexes are, however, heterogeneous and steroid hormone receptors, for instance, contain in addition to the core proteins the immunophilins FKBP51, FKBP52 and CyP40 necessary for receptor function [62]. Pharmacological targeting of Hsp90 using specific chemical inhibitors leads to the degradation of the client proteins and inhibition of tumor growth through G1 arrest, morphological and functional differentiation and activation of apoptosis [61] (Figure 2). This strongly implicates Hsp90 as a key component required for ‘self sufficiency in growth signals’. However, overexpression of Hsp90 in tumor cells compared to normal tissues has been observed only in sporadic cases [30, 42]. More subtle alterations are also involved. For example, the splice variant Hsp90N which lacks an ATP binding site is observed in some cancers and mediates transformation [63, 64]. In addition, the co-chaperone cdc37 which is essential for the function of a subset of growth-related, Hsp90 binding protein kinases in normal cells is an oncogene in itself when overexpressed in prostate carcinoma [65]. Cdc37 binding to protein kinases and cyclophilin binding to nuclear receptors are mutually exclusive interactions, pointing to the

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**Figure 2.** Hsp90 inhibitors can preferentially reduce growth signaling in malignant cells. In normal cells, growth requires input from receptors coupled to extracellular signaling molecules. Input signals are amplified by coupled signaling enzymes (●), leading to a powerful effector signal (●). Arrows indicate that each intermediate in the cascade regulates the next. In malignant cells, signaling becomes autonomous. Autonomy can be induced by overexpression of signaling enzymes (●), or their mutation (●●). Overexpressed or mutant signaling molecules require binding to Hsp90 (●) to deter aggregation. Hsp90 inhibitors lead to degradation of Hsp90 client signaling enzymes and loss of autonomous growth.
existence of unique classes of individual Hsp90 complexes that could be targeted in therapy. Changes in the abundance and composition of Hsp90 complexes in cancer increase the chaperoning reservoir needed to foster oncogenic proteins [66–69]. In addition, the increased susceptibility of Hsp90 in tumors to ansamycin family drugs (which target their ATPase domains) reflects the concentration of tumor Hsp90 within the chaperone machine complexes in which it forms a high affinity for the drugs as opposed to ‘free Hsp90’ in unbound form which predominates in normal cells and has low affinity for drugs [69].

**Extracellular HSP; carriers of tumor antigens and vaccine components**

Heat shock proteins have recently been shown to play important roles in the immune system as carriers of tumor antigens and inflammatory agents [70, 71] (Figure 3). Such HSPs are able to form complexes with peptide antigens in the cytoplasm, which can then be secreted and may ultimately participate in immune surveillance. Extracellular Hsp70 interacts with receptors on antigen presenting cells (APC) either during episodes of cell death and lysis in vivo or after treatment with molecular chaperone-based vaccines. Hsp70-peptide complexes are thus able to deliver antigens to major histocompatibility (MHC) class I and II molecules on the APC cell surface and present such tumor antigens to T lymphocytes. HSP-antigen complexes have proven effective in the treatment of rodent tumors in preclinical studies and are now undergoing clinical trial for treatment of human cancer.

The pioneer in molecular chaperone-based anticancer vaccine design has been Pramod Srivastava who has prepared autologous vaccines in mice and in human patients with the aim of directly targeting the unique, often mutated antigens that characterize each individual neoplasm [72–76]. In this approach, HSP.PC (Hsp70 and gp96) are isolated from the patients’ tumors by affinity chromatography and formulations of such purified HSP are then applied in a multi-dose regimen. The aim is for tumor antigens to be cross-presented to the patient’s APC and for the unique mixture of peptides from the individual tumor to induce immunity. These studies are currently undergoing Phase III trial and recent results suggest a trend towards increased response particularly in patients receiving longer courses of vaccination [77]. Success may thus be limited by the amount of tumor available after resection as response to treatment evidently is related to the number of treatments with HSP vaccine [77]. These results may be viewed as encouraging when compared with the recent NCI trials of metastatic cancer treatment in the United States with synthetic peptides, DNA vaccines, dendritic cell vaccines and viruses in which an objective response rate of 2.6% was reported [78]. Factors that limit the effects of vaccines may be structural and relate to the avidity with which individual HSPs bind to peptides, the nature of their peptide repertoires and ability to induce a co-stimulatory response. For instance Hsp70 gene family members Hsp110 and grp170 possess a greatly enhanced ability to bind peptides compared with other HSPs, can bind avidly to larger polypeptides, and are superior agents in cancer vaccine production than smaller HSPs [79, 80]. Recent studies suggest that larger polypeptides are superior to smaller peptides in inducing immunity [81, 82].

Another recent approach emphasizes use of combinations of chaperones with the hope of increasing the repertoire of peptides presented to APC. Indeed the studies of Binder and Srivastava (2005) indicate that Hsp70, gp96, Hsp90 and calreticulin carry between them the whole of the intracellular peptide repertoire required for cross priming T cells against ovalbumin or β-galactosidase. An approach using chaperone-rich lysates has been described in which tumor cell lysates are partially purified by iso-electric focusing to enrich the above chaperones [83]. Such preparations are effective against mouse tumors in prophylactic context.

![Image](image_url)
These studies are supported by a recent publication showing that the effectiveness of an Hsp70-based vaccine derived from tumor-DC fusion cells is partially dependent on co-isolation of Hsp90 [84]. Use of multi-chaperone formulations may thus be indicated. These studies used MUC1, a normal antigen whose expression and processing is altered in cancer. Mice expressing MUC1 became tolerant to this antigen while the Hsp70-based vaccine was able to overcome tolerance and cause tumor rejection, suggesting that non-mutated antigens may be targeted by Hsp70-based vaccines [84].

It has been suggested that there are three requirements for effective cancer immunotherapy: (1) a sufficient number of avid tumor reactive lymphocytes present in the tumor bearing host, (2) these must be capable of reaching the tumor and extravasating, (3) lymphocytes that penetrate the tumor must have appropriate effector mechanisms to destroy the cancer [85]. One innovative approach described recently emphasizes the inflammatory nature of HSP; local inflammation may lead to increased influx of T lymphocytes and enhanced cell killing due to APC maturation. The approach devised by Vile et al. involves targeted killing of normal melanocytes overexpressing HSP70 to generate an antigen-specific CD8+ T lymphocyte response against established melanoma [86, 87]. The rationale behind this unorthodox approach is that the majority of peptides that are presented by melanoma cells and recognized by T cells from patients arise from developmental proteins that are also expressed in normal melanocytes [88]. The existence of shared antigens between the proliferating melanocytes and the melanoma cells suggested that if a CD8+ T cell response could be generated against the dying melanocytes it could also target the tumor cells [86]. This approach requires the specific targeting of proliferating melanocytes using local expression of a cell suicide gene (HSVtk) under the control of a promoter from the tyrosinase gene that is specifically active in this cell population. For tumor rejection, the critical requirement was that killing take place in melanocytes engineered to overexpress Hsp70 [86]. Cell death in the sacrificed population leads to the extracellular release of the Hsp70, modulation of DC function, and generation of a CD8+ T cell response against melanocytic TSTA that eradicates primary and metastatic melanoma [86]. The preclinical studies in mice show the feasibility of this approach and its potential for translation for clinical treatment of malignant melanoma [86]. One further question is whether such an approach could be generalized to the treatment of other tumors at different sites. This approach would require the targeted sacrifice of a population of normal cells with an antigenic repertoire similar to the tumor under treatment. A related approach involves local killing of tumor cells with forced overexpression of Hsp70, obviating the need to find a related normal tissue for priming antitumor immunity [89, 90]. Of course, the question of potential autoimmune destruction of normal tissues, especially as this approach depends on the generation of CD8+ T lymphocytes directed against antigens common to normal cells and tumor cells is a concern with this approach [86]. The targeting of the small fraction of proliferating melanocytes may, however, limit the extent of normal cell targets. In addition, the studies of Vile et al. show that the effects of the treatment are self-regulatory, through the activation of CD4+CD25+ lymphocytes, which inhibited the activity of tumor-specific CD8+ T lymphocytes and rapidly attenuated the response [86].

A further refinement is to combine chaperone-based immunotherapy with low temperature hyperthermia [91]. Fever-range hyperthermia has the advantage of causing HSP induction and release and inducing highly efficient homing of APC and T lymphocytes to tumors and activation [92, 93]. Recent studies show that hyperthermia also causes lymphocyte trafficking across the high endothelial venules through a mechanism involving enhanced IL-6 signaling [94]. This approach therefore is of high promise in combination with HSP-based immunotherapy. Finally, in another novel approach, HSPs and peptide/protein antigens derived from the tumor have been combined with ceramic microparticles to produce an autologous anticancer vaccine. In this case, the ceramic particles were used to purify several HSPs and when injected into mice attracted APC. This therapeutic vaccine is showing encouraging clinical results [95].

**Conclusions**

**Heat shock proteins are stress proteins with Janus-like properties in cancer**

The HSPs play a fairly unambiguous role in mediating the response to stress. Elevated levels of HSP lead to resistance in almost all contexts. However, their role in cancer is less straightforward. Elevated HSP levels may be required in the early stages of cancer to counter the induction of programmed cell death accompanying transformation. There may thus be selection for cells expressing high levels of HSP. Such cells would be expected to be resistant to therapies that function through targeting programmed cell death. In more advanced cancers. There may, however, be immune selection for cells with lower HSP levels in order to escape immune surveillance [96]. More information is required in order to resolve these questions.
This dichotomy is also reflected in approaches to targeting HSP in therapy. Pharmacological approaches aim to inhibit activity or expression of tumor HSP and in the case of Hsp90 this is a highly effective approach. Immunological approaches aim at increasing HSP levels in cells with the aim of increasing tumor antigen presentation to the immune system. These contrasting approaches to therapy therefore mirror the Janus-like properties of the HSP in the development of cancer.

References


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