Novartis Foundation Symposium 291

THE BIOLOGY OF EXTRACELLULAR MOLECULAR CHAPERONES

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Chair's introduction

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This meeting has two major parts. The first deals with the mechanisms of extracellular chaperones. We may pose the following key questions here:

- What are the extracellular chaperones doing in general, and how do they get out of the cell?
- Is this a nicely regulated process or are we just witnessing the agony of some cells and the product of this?

These are all open questions. The second part of the meeting deals with the effects of extracellular molecular chaperones. This is an area where we probably know much more. However, this field is much wider than the previous one. Our meeting will give us a great opportunity to survey all the possible applications of extracellular chaperones as well as the various therapeutic approaches. This will be an exciting exercise for all of us.

I would like to raise some additional questions, which we might want to discuss over the next few days.

- **Plasma membrane dynamics, membrane rafts and microheterogeneities.** To begin with, I am curious as to whether we have enough knowledge about the dynamics of the plasma membrane system in our experiments. Let's remember the paradigm change that occurred in nuclear transport: first we thought this was a specific transport process, then we believed that it is a relatively free process, where only the retention of proteins in the nucleus is defining which of them is getting out and which of them stays in, and now we have returned again to the specific transport hypothesis. We have to be aware of the importance of rafts and microheterogeneities in the plasma membrane, and their possible roles in the secretion of extracellular chaperones.
- **Personal stress history.** There will be lots of discussion on immunomodulation, and the pro- and anti-inflammatory role of the chaperones outside the cells. We are close to the point where we might start to talk about a personal stress history of patients reflected by molecular chaperones and the antigens

against them, including the prenatal and postnatal period. It is a highly complex picture that is emerging.

- **Personal symbiotic status.** It also important that we should take into account the personal symbiotic status: the plethora of those bacteria that inhabit us, and whether there is some conflict between the two which may lead to serious health problems.

I also have some questions, which may have a lesser importance, but which might turn out to be quite exciting later on.

- **Role of extracellular ATP in the complex formation of extracellular chaperones**. We know a lot about the role of ATP in the function of intracellular chaperones. What about extracellular chaperones? I know that *generally* ATP is not stable outside the cell, but what about its *local* role? How does ATP affect the local complex formation of extracellular chaperones? It is an interesting open question.
- **Chaperones of the extracellular matrix, oligosaccharide-chaperones.** What about the extracellular matrix? Are there special chaperones for certain parts of the extracellular matrix, and how general and necessary is that? Is it just necessary from time to time? There are a lot of oligosaccharides outside the cell: do they need or have chaperones at all? These are questions that could be important but which are seldom addressed.

1962–2007: a cell stress odyssey

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Abstract. The induction of a cellular stress response was first observed in 1962 in a set of serendipitous experiments in *Drosophila melanogaster* larvae, which led to the discovery of a family of intracellular polypeptides known as heat shock proteins (HSPs). These highly conserved proteins are present in both prokaryotic and eukaryotic species, suggesting that they play important roles in fundamental cellular processes. Moreover, these proteins are induced in response to a range of stimuli, implicating HSPs as important modifying factors in an organism's response to a variety of physiological conditions. HSPs were initially regarded as intracellular molecules mediating cytoprotective, regulatory and chaperoning functions. However, the past two decades have seen an explosion of information related to the cell stress response, with a primary focus on molecular chaperones, which are a class of multifunctional intracellular proteins that assist in folding and assembly of other proteins. Stress proteins have also been identified on cell surfaces and in extracellular fluids, and are now viewed as potential immunomodulators, pro-inflammatory signalling molecules, and anti-inflammatory proteins in disease states. This chapter serves as an overview of the rapidly expanding world of cell stress proteins and aims to provide the reader with a foundation for more detailed presentations in subsequent sections of this book.

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In the face of stress, cells utilize a variety of intricate coping mechanisms. One key system involves a broad array of highly conserved stress proteins that have a range of functions aimed at maintaining cellular homeostasis. These molecules, which include a high-profile collection of proteins termed molecular chaperones, play vital roles in mediating polypeptide folding, signalling, chaperoning and cytoprotection inside cells. However, several provocative lines of investigation now implicate these proteins as important players in other realms, including the extracellular milieu, with involvement in critical stress-related processes related to inflammation and immunity. Much of the current work in the field is now aimed

[1]This paper was presented at the symposium by Kevin Kregel, to whom correspondence should be addressed.

at delineating the molecular and cellular mechanisms by which these different protein families function in maintaining homeostasis and modulating stress responses.

Historical overview

The first evidence for a cellular stress response is generally associated with a set of experiments in flies in 1962 (Ritossa 1962). Larvae of *Drosophila melanogaster* were accidentally kept overnight at an elevated temperature and upon inspection the next day, an unusual puffing pattern was noted on salivary gland chromosomes, indicating that the 'heat shock' episode had caused marked changes in the gene expression pattern of the larvae. However, the term 'heat shock protein' (HSP) was not coined until 1974, when specific protein products associated with these genes were identified in flies (Tissieres et al 1974). Over the next three decades, the cellular stress response, as the heat shock response came to be known, has been extensively studied, particularly with regard to stress proteins and their ever-expanding array of functions (Benjamin & McMillan 1998, Hightower 1991, Kregel 2002, Lindquist & Craig 1988, Morimoto et al 1994, Welch 1992).

Relevant to this symposium, the term 'molecular chaperone' appeared in 1987 as a general descriptor of a newly identified group of intracellular proteins (Ellis 1987). These chaperones were made up of several diverse and unrelated families of proteins that were proposed to assist in the correct folding of proteins and function in protein assembly and disassembly. As this field has 'unfolded' since the early 1990s, two observations deserve mention. First, it has been a challenge for investigators to understand and apply the correct terminology as it relates to HSPs and molecular chaperones (Ellis 2005). For the purposes of this chapter and the necessity of presenting a broad, general perspective on the cellular stress response in mammalian systems, it should be appreciated that many chaperones are inducible by stress and assist other proteins in achieving proper folding. Thus, these molecular chaperones are included in the family of heat shock proteins (or cell stress proteins). However, it should also be noted that some molecular chaperones are not stress proteins. Second, it has become very apparent over the past two decades that molecular chaperones have additional functions besides their protein-folding actions. For instance, paradoxical immune functions have been identified for some molecular chaperones, as they are now known to play a role in both autoimmunity and as immunogens in association with challenges such as infection (Henderson & Shamaei-Tousi 2005). Another interesting twist in the stress response story involved the observation that some molecular chaperones are localized to the surface of cells (Ferrarini et al 1992, Multhoff 2002). These reports were followed by studies suggesting that molecular chaperones have actions that mimic pro-inflammatory cytokines as well as anti-inflammatory and immunosup-

pressive properties. In addition, there is now evidence that some molecular chaperones are present in extracellular fluids and function as cell-to-cell signalling molecules (Frostegard & Pockley 2005, Pockley 2003). Taken together, a modern view of the cell stress response must include the notion that various stress-related proteins, including HSPs and molecular chaperones, are multifunctional and have a range of activities both inside and outside the cell.

The classical view of the cell stress response

Stress proteins are present in both prokaryotic and eukaryotic cells and have a high level of conservation, suggesting that they play an important role in fundamental cellular processes. A variety of intracellular and extracellular stimuli can elicit a stress response, with a general profile that includes the activation of stress genes and the inhibition of housekeeping genes. Importantly, the physiological impact of this response can be observed at molecular, cellular and systemic levels. Thus, it has become evident that the regulation and function of stress proteins are key components of the stress response in a physiological setting.

HSP families

The HSPs are a subset of the more broadly defined category of cell stress proteins. As their name indicates, HSPs were identified in cells as the product of genes induced by heat shock. While the initial view of HSPs was that their induction was associated with a heat shock episode, a more modern usage of the HSP term now encompasses a larger class of proteins that have a range of molecular chaperone functions.

A large number of proteins have been discovered within the HSP family, and they are generally divided into groups based on both their size and function (Table 1). These proteins are present in the cytosol, mitochondria, endoplasmic reticulum, peroxisomes and nucleus inside cells, as well as the cellular membrane and extracellular fluids. Specific locations vary depending on the particular protein. One of the most studied families of HSPs is the collectively conserved ATP-dependent Hsp70 family. Expressed in virtually every cell type, up-regulation of Hsp70 can be triggered by exposure to a wide range of environmental conditions, including heat, metabolic stress, hypoxia, oxidative stress, infection, and inflammation.

The classical paradigm

The classical paradigm associated with the heat shock response for a mammalian system is depicted in Fig. 1. Application of a cellular stress such as heating can cause the denaturing of native proteins and aggregation and/or misfolding of

TABLE 1 Nomenclature and examples of intracellular locations and proposed functions of mammalian heat shock protein families

Major protein families and their members	*Cellular location*	*Proposed cellular functions*
Small HSPs		
α-crystallin	Cytoplasm	Chaperone activity; structural control of the cytoskeleton
Hsp27	Cytoplasm, nucleus, cell surface, extracellular	Microfilament stabilization; anti-inflammation
Hsp32 (haem oxygenase)	Cytoplasm	Haem catabolism; antioxidant
Hsp40		
Hsp40	Cytoplasm, nucleus	Protein folding; regulates Hsp70 activity
Hsp47	Endoplasmic reticulum	Processing of pro-collagen
Hsp60 (chaperonins)		
Hsp60	Mitochondria, cell surface, extracellular	Folding of newly synthesized and denatured proteins; protein import; inflammation
TCP1	Cytoplasm	Protein folding; maintenance of actin cytoskeleton
Hsp70		
Hsc70	Cytoplasm, peroxisomes	Constitutively expressed; molecular chaperone; assists with protein translocation
Hsp70	Cytoplasm, nucleus, cell surface, extracelllular	Stress-inducible; protein folding and transport; disassembly of oligomers; inflammation; antigen presentation; natural killer cell activation
mtHsp70 (Grp75)	Mitochondria	Cytoprotection; molecular chaperone; protein import
Grp78(BiP)	Endoplasmic reticulum, cell surface	Cytoprotection; molecular chaperone; anti-inflammation
Hsp90		
Hsp90	Cytoplasm, cell surface, extracellular	Constitutively expressed; molecular chaperone; assists with protein translocation; pro-inflammation; antigen presentation
Grp94/Gp96	Endoplasmic reticulum, extracellular	Protein folding and transport; disassembly of oligomers; pro-inflammation; antigen presentation
Hsp110		
Hsp110	Cytoplasm, nucleolus	Protein folding and assembly; cytoprotection
Ubiquitin	Cytoplasm	Protein degradation
Thioredoxin (TRX)		
TRX1	Cytoplasm, nucleus, extracellular	Inhibit apoptosis; antioxidant
TRX2	Mitochondria	Inhibit apoptosis; antioxidant

nascent polypeptides in the cell. These misfolded and aggregated proteins can either lose or alter their function, in addition to becoming unable to reach their target cellular location. To protect against extensive protein damage caused by heat, the cell invokes a cell stress response. In the simplified scheme depicted in Fig. 1, a stressor activates a heat shock transcription factor, which then translocates to the nucleus and binds to a heat shock element in the promoter region of the HSP gene. This results in rapid induction of HSPs, which function in protein folding

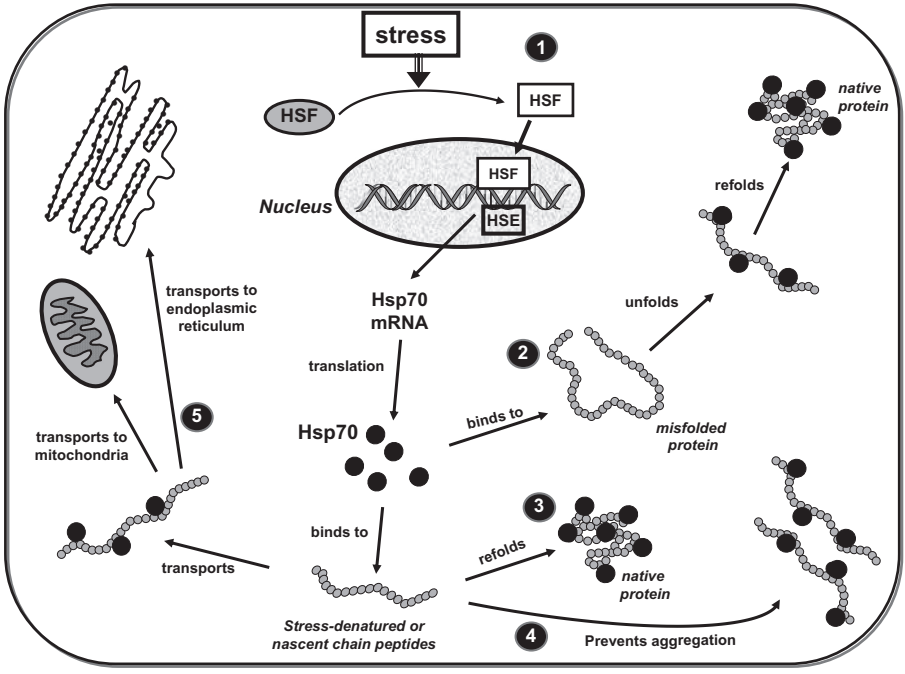

FIG. 1. A schematic representation of the classic cell stress response. This example includes the activation of the inducible form of the 72 kDa heat shock protein (Hsp70), and some of the intracellular roles played by this stress protein are highlighted. (1) A diverse range of physiological signals, including stressors such as hyperthermia, hypoxia, energy depletion, and reactive oxygen species, can activate the heat shock transcription factor (HSF). The activated HSF enters the nucleus and binds to heat shock elements (HSE) in the promoter region of the Hsp70 gene. Hsp70 mRNA is then transcribed and leaves the nucleus for the cytosol, where new Hsp70 is synthesized. (2) In response to a stress, Hsp70 can bind to misfolded proteins in the cytoplasm and assist in the unfolding and subsequent refolding of these proteins to their native form. Hsp70 can also bind to stress-denatured or nascent chain peptides, resulting in (3) refolding of the protein to its native form, or (4) prevention of aggregation of these proteins. (5) Hsp70 can also be bound to stress-denatured or nascent chain peptides, and this complex can then be transported to various organelles, including mitochondria and the endoplasmic reticulum.

and repair, protein transport to various intracellular organelles, cytoprotection and cell signalling. It is important to note that stress proteins also function in non-stressful conditions, assisting in the folding of nascent polypeptides and acting as monitors of the cellular milieu. Thus, these proteins contribute in vital and varied ways to both cellular homeostasis and survival, and also have physiological effects that impact at tissue, organ and systemic levels.

Development of thermotolerance

In addition to their protein-repair roles subsequent to an acute stress, numerous studies have demonstrated that HSP induction can protect against ensuing stressors. In the 1970s and 1980s, it was demonstrated that stimulation of Hsp70 production by a mild stress can confer transient protection against subsequent, more intense stress (Landry et al 1982). This phenomenon was initially termed 'thermotolerance' because it was demonstrated in experiments involving heat stress. In the classic case of induced thermotolerance, exposure to a mild heat shock can induce stress proteins and other cellular changes to make cells more resistant to a subsequent, more severe (i.e. lethal) heat shock. In general, the degree of conferred thermotolerance is considered directly proportional to the level of HSP induction.

The development of thermotolerance consists of a specific series of responses to a moderate level of stress. For instance, within hours of exposure to a pre-conditioning agent, a cell exhibits increased synthesis of Hsp70, which confers a transitory resistance to protein damage and cell death that lasts for hours to a few days. While initial studies regarding the protective effect of Hsp70 pre-conditioning were performed under *in vitro* conditions and focused on heat as a stressor, a succession of studies have shown that resistance to a variety of insults can be developed upon up-regulation of Hsp70 (Kregel 2002). This tolerance phenomenon is also very important *in vivo*, and involves resistance to stressors (e.g. hypoxia, ischaemia/reperfusion, acidosis, ethanol, radiation) applied at the whole-organism level. Indeed, as will be discussed in subsequent sections, additional reports have linked HSPs to a multitude of physiological functions in animals, thus implying that pre-conditioning could be a potential therapy for a vast range of perturbations.

A modern view of the cell stress response

While the classic view of the stress response primarily focused on cellular housekeeping functions such as protein synthesis and transport across biological membranes (Fig. 1), a more modern perspective has developed over the past two decades that includes a primary focus on stress proteins that can be categorized

as molecular chaperones (Fig. 2). These molecular chaperones, which have also been identified within cells and particular organelles, on cell surfaces, and in extracellular fluids, have now been integrated into physiologically relevant settings and viewed as potential immunomodulators, pro-inflammatory and anti-inflammatory signalling molecules, and regulators of cell survival.

Inhibition of apoptotic pathways

One important cellular stress response is the initiation of an apoptotic cascade that can ultimately lead to the death of a cell. Hsp70, along with other molecular

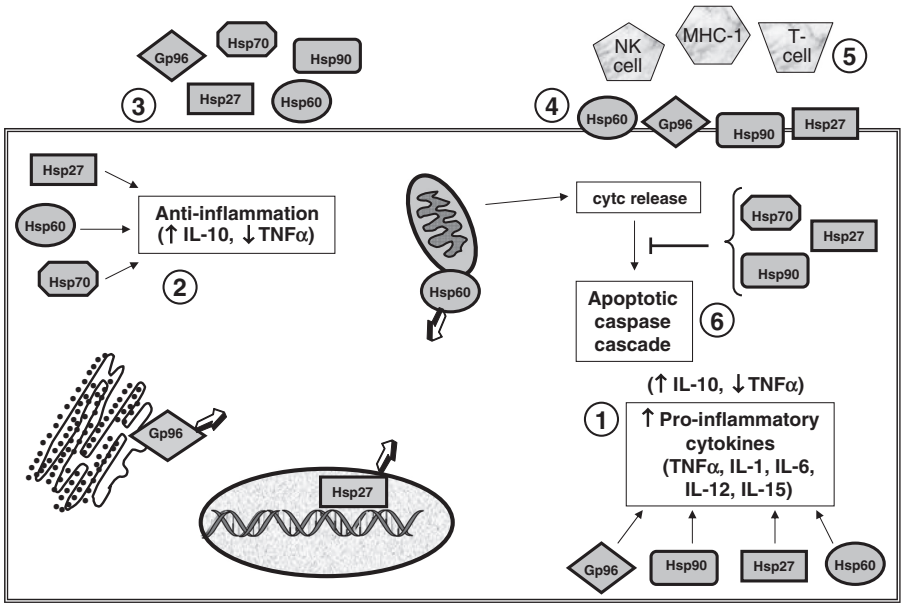

FIG. 2. A modern view of molecular chaperones and the cell stress response. In this schematic, cellular stress leads to the activation of several different molecular chaperones (e.g. Hsp27, Hsp60, Hsp70, Hsp90, and Gp96), which in turn can stimulate many major signalling pathways. In this scenario, responses can involve the separate activation of selected signalling pathways or a co-ordinated response among many pathways. Responses include: (1) stimulation of pro-inflammatory cytokines (e.g. TNFα, IL1 and IL6); (2) modulation of anti-inflammatory effectors (e.g. stimulating IL10 and inhibiting TNFα); (3) extracellular release of molecular chaperones; (4) surface expression of molecular chaperones and antigen presentation; (5) immune cell activation and migration associated with surface expression of molecular chaperones; and (6) inhibition of apoptotic pathways via blockade of cytochrome c (cyt c) induced stimulation of apoptotic caspase cascades. Molecular chaperones can also move from selected organelles (e.g. Hsp27 in the nucleus) to other locations within and outside the cell.

chaperones, is thought to interfere with the apoptotic signalling pathways (Fig. 2), thus leading to cell survival (Garrido et al 2006, Nadeau & Landry 2007). Hsp70 induction also reduces activation of stress kinase c-Jun N-terminal kinase (JNK), a critical activator of the apoptotic death cascade. In turn, reduction of JNK activity suppresses the apoptotic cytochrome c/caspase cascade that can ultimately lead to cell death. These discoveries serve to highlight the fact that chaperone functions initially attributed to Hsp70 were merely the tip of the iceberg, and a multitude of other critical functions involving numerous molecular chaperones are still being unravelled.

Paradoxical inflammatory actions of stress proteins

In addition to their regulatory roles in apoptotic/cell survival pathways, the majority of molecular chaperones are also involved in signalling processes associated with inflammation pathways. Independent of their protein-binding capacities, these chaperones may also have powerful effects on inflammation via direct cytokine-like activity and these pro-inflammatory properties support the concept of their behaviour as 'chaperokines' (Asea et al 2000). However, molecular chaperones also present an apparently paradoxical picture with regard to inflammatory processes, as they can exhibit both pro-inflammatory and anti-inflammatory properties.

Though molecular chaperones play a primary role in both protecting and repairing damaged polypeptides, they are also involved in the induction of an inflammatory response in injured cells. Specifically, the Hsp60, Hsp70, Hsp90 and Gp96 families have potent pro-inflammatory effects that are independent of their ability to bind to proteins (Tsan & Gao 2004). These HSPs have been shown to induce production of several pro-inflammatory cytokines, including tumour necrosis factor (TNF)α, interleukin (IL)1, IL6, IL12 and IL15 (Fig. 2). Additionally, Hsp60 and Hsp70 have been linked to increasing T-lymphocyte production of inflammatory cytokines and macrophage production of TNFα. Finally, elevations in circulating levels of HSPs have been found in patients with some types of cancers, cardiovascular disease, renal disease, and acute conditions such as infections (De et al 2000, Johnson & Fleshner 2006, Panayi et al 2004, Wright et al 2000). These extracellular HSPs may be involved in sending a 'danger signal' to the rest of the organism, stimulating and sustaining a systemic inflammatory response (Multhoff 2002).

Ironically, molecular chaperones have been found to be involved not only in the initiation of inflammation, but also in the suppression of some inflammatory processes (Fig. 2). Both Hsp27 and the HSP known as BiP have been found to induce production of the anti-inflammatory cytokine IL10 (De et al 2000, Panayi et al 2004). Additionally, in sublethal stress conditions, Hsp60 and Hsp70 family

members have been implicated in reducing inflammation by suppressing both TNFα production and promoting IL10 induction (Johnson & Fleshner 2006, Moseley 2000). These responses may protect the cell from further damage due to the inflammatory response and potentially contribute to the aforementioned thermotolerance phenomenon.

Immune responses

While the resolution of these seemingly contradictory reports regarding stress proteins and their impact on inflammation at both cellular and systemic levels will certainly take more investigation, it appears that the extent and mode of chaperone production likely determines the net effect of their influence on the innate inflammatory response. Not only have they been linked to inflammation, but many molecular chaperones have been associated with the adaptive immune response as well. Several molecular chaperones, including Hsp70, Hsp90 and Gp96, have been linked to roles in antigen presentation (Fig. 2), and these proteins are suspected of carrying immunogenic peptides for recognition and activation of the major histocompatability complex class I (MHCI) molecules (Johnson & Fleshner 2006, Pockley 2003, Tsan & Gao 2004). HSPs have also been linked to antigen presentation for cytotoxic T cell activation. Additionally, Hsp70 and others have been found to bind specifically to dendritic cells, which are among the main antigen presenting cells (Asea 2005).

Adding to the complexity of the integrated picture of molecular chaperone function, proteins such as Hsp90 and Hsp70 have been identified on the surface of cancer and virally infected cells, in both *in vitro* and *in vivo* conditions. It has been postulated that these cell surface locations allow chaperones to stimulate the activation and migration of natural killer cells. Additionally, molecular chaperones have been shown to exhibit strong binding to B cells, which are responsible for antibody production (Asea 2005). These data implicate molecular chaperones as potential key players in stimulating immune responses. Indeed, vaccination of mice with Hsp70, Hsp90 or Gp96 has been shown to stimulate immune responses that suppress tumour progression and promote tumour rejection (Tsan & Gao 2004). As evidence accumulates, it is becoming clear that molecular chaperones have functions well beyond the classical understanding of molecular chaperone protein folding.

Potential ramifications of stress protein dysfunction

Ironically, while their primary job is to protect the integrity of various proteins in the cell, stress proteins can also fall victim to the same variety of insults that cause their target proteins to malfunction. Alterations in the structural integrity of

molecular chaperones can disrupt their function and potentially lead to pathological conditions that have been termed 'chaperonopathies' (Macario & Conway de Macario 2005). These chaperonopathies, which are implicated in a wide range of disease states, are postulated to develop through mutations or post-translational defects (Fig. 3). The modification of molecular chaperones at post-translational levels can occur through a variety of mechanisms and result in the loss of chaperone function. In a condition that has been termed 'acquired chaperonopathy,' alteration of these molecular chaperones can lead to an accumulation of protein damage and protein aggregation. This type of pathology has been linked to a number of neurological disorders that are associated with aggregates of various proteins (e.g. Parkinson's, Huntington's and Alzheimer's disease) (Meriin & Sherman 2005).

However, not all chaperonopathies involve structural modification of the chaperones themselves. Many pathological states that have been connected with these proteins are more specifically targeted to gene regulation of chaperone expression. In this scenario, the proteins themselves are structurally normal, but they are improperly expressed, resulting in either elevations or reductions in their levels at intracellular and extracellular locations. The mechanisms responsible for these alterations are unclear, but are postulated to be

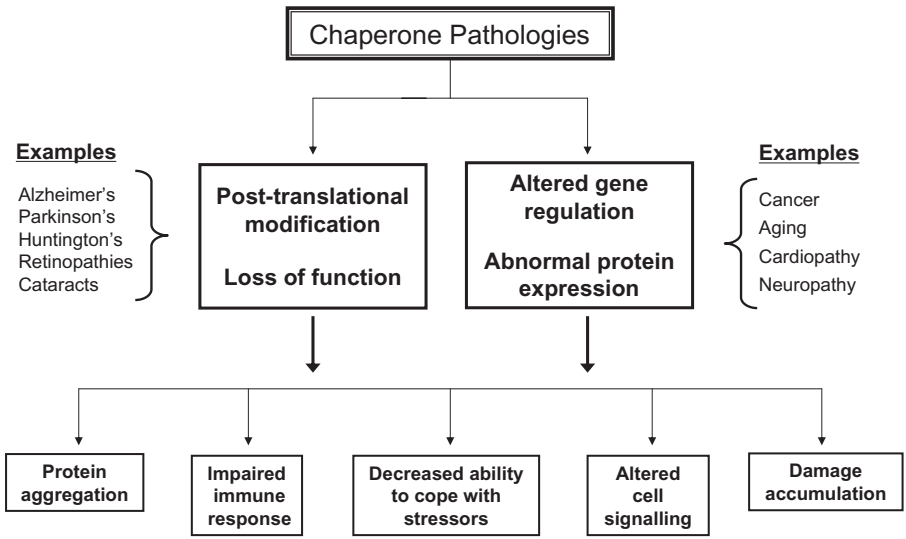

FIG. 3. Examples of chaperone pathologies. Different mechanisms at the cellular level can contribute to modifications in molecular chaperones, resulting in a variety of pathophysiological conditions and functional alterations that are manifested at cellular and systemic levels.

related to possible mutations in heat shock transcription factors. Perhaps most relevant, abnormal expression of HSPs has been associated with several conditions involving physiological decline, including ageing, neurodegenerative disorders and cardiopathies.

Ageing is one of the primary biological processes that has been associated with decreased stress protein expression. In addition, the ability of an organism to upregulate stress proteins in response to stress is altered with advancing age (Arslan et al 2006, Kregel 2002). For instance, our own laboratory has shown an altered HSP expression in response to stress when comparing aged and young animals (Hall et al 2000, Kregel 2002). This blunted cell stress response can have broad physiological ramifications, leading to an accumulation of protein damage, reductions in repair processes, functional declines at cellular and systemic levels, and susceptibility to further insult (Zhang et al 2003).

Loss of chaperone function can also lead to impairment of the immune response (Pockley 2003). As was noted in earlier sections of this chapter, molecular chaperones play various roles in regulating the immune response; therefore, alteration of their function, whether through damage to the protein itself or changes in protein levels due to gene control, could have dire implications for the immune response to a stressor. Atypical levels of several molecular chaperones have also been found in a broad range of tumour cells and cancerous tissues. In fact, tumour cells appear to be dependent on increased HSP expression for survival and growth. It is thought that because these stress proteins play a role in the control of cell growth, and more specifically, the inhibition of apoptosis, they may aid in tumour formation and proliferation (Jolly & Morimoto 2000, Nollen & Morimoto 2002, Soti et al 2005). Overexpression of several molecular chaperones has been found to result in tumour formation, and these provocative observations further implicate altered HSP expression in oncogenesis (Calderwood et al 2006).

Conclusion

Due to the wide range of functions ascribed to molecular chaperones and the myriad conditions associated with stress protein dysfunction (Fig. 3), it is no surprise that these proteins are emerging as potential therapeutic targets in a broad range of fields. Current and future research will examine whether manipulation of molecular chaperones can successfully treat or prevent conditions ranging from ageing to cancer. As the molecular chaperone field moves forward, many opportunities will present themselves to investigators. The integration of disciplines such as genomics, molecular biology, systems biology, and translational medicine will provide scientists with an array of tools to aid in the delineation of diagnoses and treatments of numerous diseases and pathological conditions.