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Hsp60 and human aging: *Les liaisons dangereuses*

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1. ABSTRACT

Stressors can cause abnormal intracellular accumulation of Hsp60 and its localization in extramitochondrial sites, secretion, and circulation, with immune system activation. Dysfunction of chaperones associated with their quantitative and qualitative decline with aging (chaperonopathies of aging) characterizes senescence and is a potential causal factor in the physiological deterioration that occurs with it. The role of Hsp60 in aging is not easy to elucidate, because aging is accompanied by pathologies (e.g., cardiovascular and neurodegenerative disorders, osteoporosis, diabetes, cancer, etc.) in which Hsp60 has been implicated but, although those disorders are more frequent in the elderly, they are not unique to them. Therefore, it is difficult to determine what is due to aging and what to an associated disease that can occur regardless of age. Does Hsp60 contribute to the pathogenesis? How and when does Hsp60 interact with the immune system and, thus, contributes to the initiation-progression of the generalized chronic inflammation characteristic of aging? These and related issues are discussed here in the light of reports showing the participation of Hsp60 in aging-associated disorders.

2. INTRODUCTION

Genes for molecular chaperones, and their protein products were identified in the early 1960's and 1970's, respectively (1, 2). After that, the study of chaperones, many of which are heat-shock proteins (Hsps), was very active in prokaryotic and eukaryotic systems, including the bacterial prokaryotes *Escherichia coli* and *Bacillus subtilis*, and the eukaryotes Drosophila, and a variety of mammals, plants and aquatic organisms (3-8). In the early 1990's, a molecular chaperone gene was identified for the first time by cloning and sequencing in a prokaryote of the phylogenetic Domain Archaea (9). In later times, a number of studies have examined the role of molecular chaperones in protein folding inside the cell, and chaperones were considered intracellular proteins (10-12). However, in the last several years evidence for extracellular chaperones has progressively accumulated and, nowadays, there is little doubt that various types of molecular chaperones can reside inside and outside cells with defined functions in both locations (12-15). In most recent times, significant advances have occurred in the understanding of detailed structure-function relationships in chaperones from the prokaryotes Archaea (16-18).

This article has been written for a Special Issue on Frontiers in Molecular Medicine and, consequently, deals with a theme currently at the outer edge of science: the active participation of defective molecular chaperones in pathogenesis. Diseases in which the primary cause, or one of the most important secondary causes, is a defective chaperone have been called chaperonopathies (19, 20). This unifying concept, encompassing a wide range of pathological conditions with diverse signs and symptoms but sharing important features, is the foundation for the outlining of a new area of Medicine. The scientific, medical, and practical advantages of such unifying approach are multiple and have been discussed elsewhere (21).

The main objective of this article is to present some of the chaperonopathies, focusing on the chaperone Hsp60, that affect aging individuals to illustrate how chaperone defects play a role in senescence and aging-related diseases by failing to interact correctly with physiological partners or by interacting with the wrong partner, namely by getting into "dangereuse liaisons."

The literature on chaperones is abundant, so as to satisfy the bibliographic appetite of interested readers who are not specialists in this article's topic we have cited, whenever possible, review articles that summarize key issues and provide a rich list of references to original work. In addition, we have discussed some original data from various laboratories, including ours that we considered to be illustrative of important aspects of chaperone failure, and its consequences, during aging. We have also highlighted some of the critical points that merit further investigation with priority.

3. ESSENTIAL BACKGROUND ON CHAPERONOLY

As it may be realized from the preceding paragraphs, chaperonology is an emerging area of science encompassing the study of molecular chaperones in all their aspects, normal and abnormal, as a unit of related topics pertaining to chaperones in physiology and pathology examined from various angles (22). Many chaperones are heat shock proteins (Hsps) and, in this article, we will use the terms chaperone and Hsp interchangeably. This field is important because defective chaperones can contribute to the pathogenesis of a number of diseases, now referred to as chaperonopathies (19, 20, 23). Chaperonopathies can be genetic or acquired, the latter being quite common (20, 24).

The role of chaperones in human cell physiology changes from embryo/foetal life through adulthood to old age, and they are involved in cell senescence (25-27). A number of studies have shown that the stress-induced levels of chaperones tend to decrease with age (28). Pathological post-translational modifications causing malfunction of chaperones seem to be implicated in the aging process by affecting other molecules and supramolecular structures, cells, and tissues (20). There is some evidence indicating that the age-associated appearance of defective chaperones (chaperonopathies of aging) contributes to the accumulation of defective non-chaperone proteins (proteinopathies of aging) (27, 29-36). This accumulation, in turn, causes a quantitative deficiency of chaperones, which thus become insufficient to deal with the increased demand from proteins in need of assistance for folding, refolding, translocation, etc. (30-32). Alternatively, chaperonopathies and proteinopathies of aging may start independently of one another – perhaps simultaneously – and progress in parallel, in which case the chaperonopathies would have a negative impact on protein homeostasis and, thus, contribute to the aggravation of the proteinopathies (27, 35).

In this article, we will concentrate on Hsp60, the eukaryotic mitochondrial chaperonin that is also found outside mitochondria, and discuss its role in cell senescence and organismal aging.

4. HSP60 MOLECULAR ANATOMY

Chaperonins are a subset of chaperones highly conserved during evolution and with essential roles in cell physiology. They are classified in two groups: Group I, present in bacteria (e.g., GroEL) and eukaryotic organelles (e.g., mitochondrial Hsp60 also called Cpn60), and Group II, found in archaea (e.g., thermosome subunits) and eukaryotic cytoplasm (e.g., CCT or TriC subunits) (37-40).

Hsp60 works in mitochondria together with its co-chaperonin Hsp10 (or Cpn10). In mammalian cells, Hsp60 and Hsp10 are important mitochondrial molecules, playing key roles in both unstressed and stressed cells (41, 42). The human genes for Hsp60 and Hsp10 are localised head-to-head on chromosome 2 and share a bidirectional promoter (43). This probably means that the DNA sequences encoding these chaperonins moved together to the nucleus from a bacterium, according to the endosymbiotic theory (44). Most of what we know about the eukaryotic Hsp60 and Hsp10 structures and functions derives from studies on their prokaryotic homologues, the bacterial GroEL and GroES, respectively, to which they are evolutionarily related.

The typical chaperonin machine in bacteria is formed by 14 GroEL molecules arranged in two stacked heptameric rings delimiting a barrel-like container with an inner cavity large enough to accommodate client polypeptides of up to nearly 60 kDa (40, 45, 46) (Figure 1A). GroES also forms a heptameric ring, which associates with the GroEL barrel at one of its ends, serving as a sort of lid to the GroEL-complex cavity (47). In addition, some information suggests that eukaryotic mitochondrial Hsp60 can form not only the typical two-ringed barrel, but it can also function as a single heptameric ring (48-50) (Figure 1B). Moreover, the majority of the client proteins are affected by the inactivation of Hsp60, but only a small subset of them are affected by the lack of Hsp10, suggesting that in eukaryotic cells Hsp60 and Hsp10 do not always act together as a functional unit (Figure 1C) (51). Unfortunately, the full range of Hsp60-dependent protein substrates in humans has not yet been fully delineated and

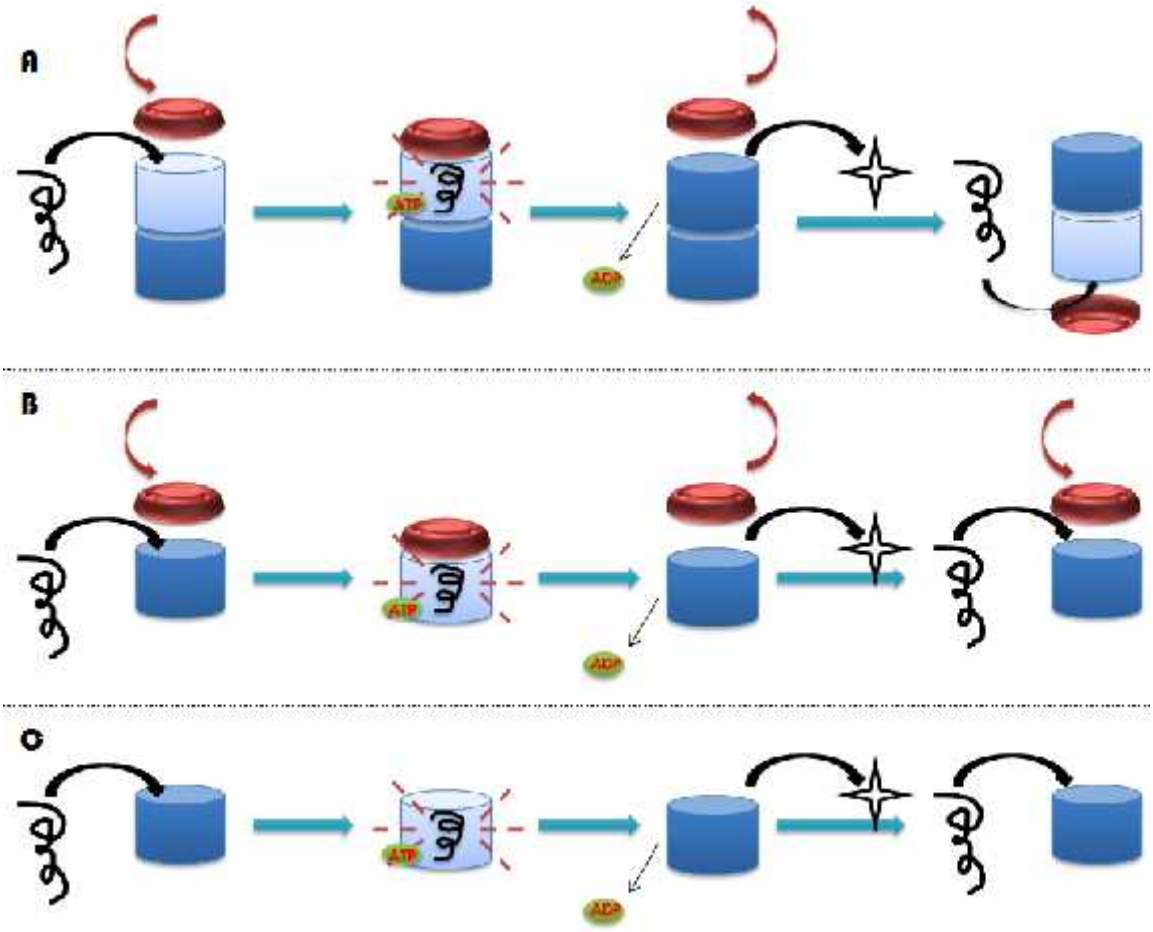

Figure 1. Hsp60 functioning models in human cells. (A) Mammalian mitochondrial Hsp60 forms a double heptameric-ring barrel-like structure with an inner cavity -- the folding chamber -- (blue cylinder), analogously to the prokaryotic homolog GroEL (not shown). A client polypeptide, i.e., a polypeptide in need of assistance for folding (black filament), enters the folding chamber of one of the rings (the light blue cylinder in this figure) in which it will fold to gain the native conformation after the chamber is occluded by the binding of Hsp10 (the homolog of the prokaryotic GroES; represented as a red cap). The process requires energy from ATP hydrolysis. Another chaperoning round begins with the dissociation of Hsp10, the release of the folded protein (four-pointed star) and ADP, and the binding of ATP to the other ring. In addition, there is information suggesting that a similar folding process is carried out by a single Hsp60 heptameric ring with Hsp10 (B), or even by a single ring without participation of Hsp10 (C), but these two possibilities, particularly the last shown in (C) are still controversial.

this lack of information curtails research on the Hsp60 chaperonopathies.

Apart from mitochondria, Hsp60 can be found in other subcellular compartments (e.g., zymogenic granules) as well as in the cytosol and on the cell membrane (52-54). In this context, one of the most interesting issues for investigation is the structure and function of this chaperonin in sites different from the canonical intra-mitochondrial location. For example, some data indicate that cytosolic Hsp60, that is involved in apoptosis activation (55), is sometimes in a monomeric form (56), while other results suggest equilibrium between monomeric and heptameric forms (57). It is likely that both situations are not mutually exclusive. Furthermore, it is also possible that various oligomeric forms of different multiplicities, depending mainly on the Hsp60 concentration in the cytosol, can occur and function.

In what concerns membrane-associated Hsp60, it is now clear that this association is a pre-requisite for its secretion into the extracellular environment by secretory mechanisms involving the lipid rafts/exosomes pathway, in both stressed and tumor cells (54, 58). In the extracellular environment, Hsp60 encounters leukocytes and participates in immune system regulation, as discussed in the following section.

5. EXTRACELLULAR Hsp60 AND IMMUNE SYSTEM ACTIVATION

Hsp60 has been for long time considered an intracellular protein but the reality is that it can also be found in the extracellular milieu as well as in the

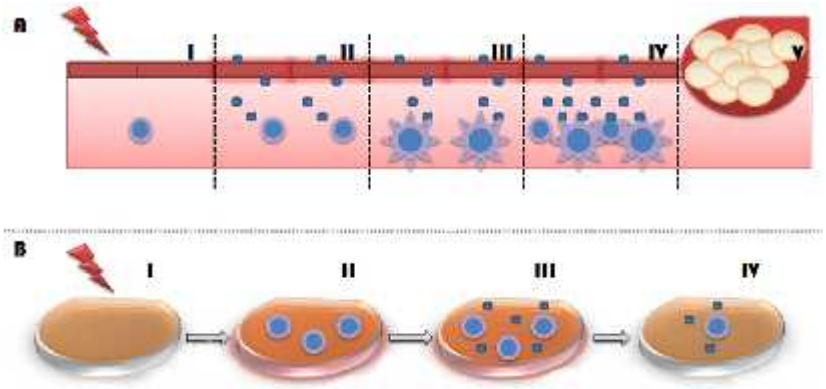

Figure 2. Hsp60 can have both pro- and anti-inflammatory effects. (A) Stress (e.g., hypertension) on vascular endothelial cells (top red thin rectangles) can determine Hsp60 (small blue squares) overexpression, cell membrane localization and secretion (I-II). In turn, Hsp60 can have pro-inflammatory effects on immune cells (lymphocytes, round blue circles with a nucleus; and macrophages, stars with a nucleus; both shown here inside a blood vessel) infiltrating the vessel wall (II-III), which induces, as a feedback effect, a perpetuation of inflammation (III-IV); finally, atherosclerotic lesions develop (IV-V). An incipient atherosclerosis plaque is shown in V (see Ref. 67). (B) In an injured joint (e.g., in rheumatoid arthritis) with inflammation of synovial tissue (I-II) (Hsp60 and lymphocytes are represented as in A, above), Hsp60 peptide administration (III) can exert an anti-inflammatory effect (IV) and contribute to joint lesion improvement (see Ref. 68).

bloodstream and its plasmatic levels seem to be genetically controlled (59). A work on 60 subjects aged between 20 and 96 years of age showed that the serum levels of Hsp60, but not those of anti-Hsp60 auto-antibodies, declined with aging (60). The reason why the antibodies do not decline, and may even increase with age, could be that the immune system reacts not only to the autologous Hsp60 but also to its homolog from microbial pathogens usually present as persistent infections in the elderly (61). Furthermore, it is possible that post-translation modifications occurring in Hsp60 as the age progresses make the autologous chaperonin immunogenic, thus stimulating autoantibody production. Hence, although the number of Hsp60 molecules decreases with age, their immunogenicity-antigenicity would increase.

An open question is how and when (i.e., during fetal development and after birth) Hsp60 is released outside the cell. It has recently been shown that Hsp60 is secreted from normal adult rat cardiomyocytes via the exosomal pathway (58) but, to our knowledge, no other similar observations have been reported regarding normal human cells. We recently demonstrated with cultured cell lines that viable tumor, but not normal cells secrete Hsp60 into the extracellular milieu by the lipid rafts-exosomal pathway (54). The Hsp60 secretion pathway(s) of human senescent cells are currently poorly known.

Independently from the via of release, it is well established that presence of Hsp60 in the extracellular space facilitates the contact with the immune system cells, promoting their activation; for example, we recently showed that Hsp60 is present in macrophages of colon mucosa from patients with ulcerative colitis and its levels decreased after an effective therapy, thus suggesting its role in maintaining mucosal inflammation in these patients (62). Analogously, in another work on bronchial mucosa from chronic obstructive pulmonary disease patients we found increased levels of Hsp60, compared to smoking and non-smoking controls, both in epithelium and lamina propria, and, in the latter, this chaperonin localized into neutrophils (manuscript submitted). In both cases, Hsp60 binding with inflammatory cells may trigger or perpetuate immune system activation and, thus, disease progression. Although it is possible that inflammatory cells in the airways are able to produce their own Hsp60, we cannot exclude that, at least in part, Hsp60 reaches inflammatory cells after release from other cytotypes (e.g., epithelial cells) and interact with receptors localised on the inflammatory-cell surface.

However, there is some controversy concerning whether or not immune-cell activation by receptor-binding Hsp60 (e.g., using toll-like receptors) requires bacterial-derived products such as LPS, flagellin, or lipoprotein (15, 63), a concern that has been ruled out for other Hsps (as Hsp70) by the use of recombinant proteins isolated from insects and of other appropriate controls (64).

From the interstitium, Hsp60 can reach the bloodstream. When in circulation, Hsp60 appears to be a key endogenous inflammatory mediator by causing the release of pro-inflammatory cytokines and nitric oxide by immune competent cells (65). In contrast, other studies have demonstrated that induction of immunity to Hsp60 can attenuate inflammatory diseases (65, 66). It is very difficult, if not impossible, at the present time to always distinguish between situations in which Hsp60 plays a passive role as an autoantigen and situations in which the chaperonin has an active role as a chaperokine (endocrine-like or signalling function), inducing inflammation and/or an immune response to other antigens. What happens and when may depend on a variety of factors, not completely understood. In addition, the role of Hsp60 during inflammation seems to depend on the subtypes of activated lymphocytes infiltrating tissues. For example, in atherosclerosis (ATS) Hsp60 would have pro-inflammatory effects (Figure 2A) (67), whereas in rheumatoid arthritis, it could have an anti-inflammatory action (Figure 2B) (68).

Table 1. Aging-related pathologies in which Hsp60 is believed to play a pathogenetic role

Damaged tissue/organ	Cells involved in physiopathogenesis	Disease	References
Vessels	Endothelial cells, macrophages	Vasculitis, atherosclerosis	84-86, 88, 91
Heart	Myocardiocytes	Myocarditis, infarct, heart failure	18, 58, 59, 80, 87, 89, 90, 92-94, 95-98, 100, 101
	Conducting system cells	Atrial fibrillation	110-112
Brain	Neurons, glia	Neurodegenerative disorders	103-105
Joints	Synoviocytes	Degenerative joint disease, rheumatoid arthritis	66, 107-109
Bone	Osteoblasts, osteoclasts	Osteoporosis	117, 118
Pancreas	Beta-cells	Type II Diabetes	119
Bronchi	Epithelial cells	Chronic obstructive pulmonary disease	120, 121
Eye	Trabecular endothelial-like cells of the iridocorneal angle	Glaucoma	113
Oral cavity	Gingival cells	Periodontitis	115, 116

In any case, given the data available at present, one can assume that the changes in the immune system response observed during aging are correlated, at least partially, to the decline in Hsp60 levels and/or to structural changes in this chaperonin due, for example, to post-translational modifications. Abnormal levels and/or structural damage in Hsp60 will most likely lead to scrambling of its interactions with immune system components and, consequently, to pathogenesis (27). Hence, a future challenge will be to shed more light into the nature and types of interactions between Hsp60 and the immune system during aging.

6. HSP60 IN CELL AGING

Cell aging includes morphological, structural, and biochemical changes as part of a complex biologic program whereby old individuals accumulate senescent cells in their bodies. Aging cells in culture become flat and enlarged, developing extensive vacuolization and, *in vitro* as well as *in vivo*, they show modified secretory pathways and a reduced ability to respond to stressors and to divide, with growth arrest in late stages (69).

Some key components of the senescence process may be regulated by chaperones (70). Analogously to what happens during the normal-dysplasia-carcinoma transition in various anatomical sites (71-75), levels of Hsp60 increase in human skin fibroblasts during replicative senescence (69, 76) (Figure 3), and the rapid increase in the levels of this chaperonin was positively correlated with cell cycle progression (69). A correlation between increased levels of Hsp60 and senescence in human skin fibroblasts was shown to involve interaction between Hsp60 and mtHsp70 (77). These *in vitro* results are in agreement with *in vivo* studies that reported an increased Hsp60 expression in the forearm skin of elderly subjects in comparison with young individuals, while other Hsps did not show differences (78). The authors postulated that the chaperonin-level changes occurring with age are the consequence of the mitochondrial oxidative stress characteristic of cell senescence.

Certain chaperones can inhibit caspase-dependent apoptosis, conferring immortality to the cell (79). Hsp60 is one of these caspase-dependent apoptosis inhibitors (80). Moreover, exogenous Hsp60 produced by a persistent infection with *Chlamydia trachomatis* can also block the anti-apoptotic and the pro-senescence effects of the host's (endogenous) Hsp60, and in turn favour the active proliferation of damaged cells (81). Finally, chronic infection with *C. trachomatis* with host invasion by the bacterial Hsp60 generates anti-chaperonin antibodies that crossreact with the host's counterpart and, thereby, causes a variety of lesions in those locations in which the host's Hsp60 happens to reside (61).

In summary, the pioneering studies discussed in the preceding paragraphs suggest that Hsp60 plays a role in the cell senescence process but the precise molecular mechanisms are not yet fully elucidated and deserve further analysis, because their elucidation will provide useful clues for developing preventive and treatment means applicable to Hsp60 chaperonopathies.

7. HSP60 IN AGING RELATED DESEASES

The number of papers showing the participation of Hsp60 in aging-related disease development is constantly growing. The involvement of Hsp60 in the pathogenesis of aging-related diseases has been studied with a number of approaches, both *in vivo* and *in vitro*, and at tissue, cellular, and subcellular levels, as summarized in Table 1. Some of these papers are mainly based on observational studies and a cause-effect relationship has not been yet properly investigated. Hsp60 in aging individuals seems to have variable roles, which most likely depend on conditions in the cell, tissue, and organism affected. Hsp60 may metamorphose from a cell-protecting molecule to a dangerous one, thus being the Proteus of human disease pathogenesis. This is possibly due to largely unknown mechanisms that regulate its gene expression and to variables in the translation process as well as to post-translational modifications. In addition, the roles of Hsp60 may be influenced by its interactions with other intracellular proteins and other processes, including secretion into the extracellular space with the chaperonin becoming an extracellular chaperone, signal molecule, autoantigen, or cytokine-like or endocrine-like molecule.

The involvement of Hsp60 in age-related tumor pathogenesis has been extensively discussed elsewhere (82, 83). In the following subsections we will briefly discuss other widespread aging-related pathological conditions that reduce significantly the quality and extent of life in Western countries.

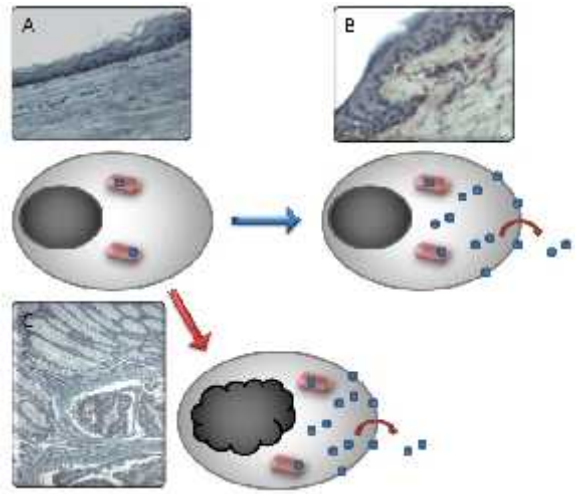

Figure 3. Hsp60 expression during senescence and carcinogenesis. Intracellular Hsp60 (small blue squares) levels can increase during cell senescence (blue arrow) as well as during carcinogenesis (red arrow), as demonstrated, for example, by immunostaining (performed by LSAB2 Kit, Dako, Carpintera, CA, USA, Cat. No. K677; human primary monoclonal anti-Hsp60 antibody: SIGMA, Milan, Italy, Cat. No. H4149, dilution: 1:400). Skin specimens of young human subjects (A) are commonly negative for Hsp60, while dermal fibroblasts of old subjects (B) show a diffuse positivity (red positivity) (unpublished data; see also Ref 69 for an *in vitro* demonstration). Hsp60 positivity (red) is commonly present in colon cancer cells (C, right-lower corner), while normal colonic mucosa is negative or low-positive (C; left-upper corner) (see Ref 83 for a review). Hsp60 overexpression may be associated to cell secretion (curved red arrow) as already demonstrated for tumor cells, see Ref 54). Mitochondria are shown as small pink capsules. Original pictures, magnification: 200X in A; 400X in B, and 100X in C.

7.1. Atherosclerosis and heart failure

Although atherosclerosis (ATS) has been proposed to be an "autoimmune disease due to an immune reaction against Hsp60" (67), the exact involvement of Hsp60 in the pathogenesis of ATS is still confused by contradictory results. Therefore, further studies are necessary to definitively clarify the roles of Hsp60 as etiologic factor and of the antibodies against it in sustaining the inflammation underlying the pathogenesis of ATS (84-87). Also, it is pertinent to consider the contribution of molecular mimicry between human and microbial Hsp60 in generating an autoimmune response that in turn causes endothelial damage and artery inflammation (88-94). A number of *in vivo* data strongly support the involvement of Hsp60 in the pathogenesis of coronary artery disease (59, 87, 95-98), but this view was not supported by data from others (99).

With regard to heart failure, Hsp60 is gaining ranks as a clinical marker for monitoring this condition (58, 80, 100). Hsp60 is overexpressed in the cytosol, localises in the cell membrane, and can also be secreted by stressed myocardiocytes (58, 80). Higher than normal anti-Hsp60 antibody levels were correlated with higher levels of brain natriuretic peptide, left ventricular end-diastolic dimension and with the extent of cardiac dysfunction (101). If confirmed, these data may become of clinical utility for heart failure management.

7.2. Neurodegenerative disorders

Several kinds of stresses, among which nitrosidative stress, may cause accumulation of aberrant proteins and neuronal cell damage or death (102). Thus, stress-induced proteins like some of the chaperones have been proposed as protective molecules for the nervous system cells. For example, overexpression of the *hsp60* gene was observed in experimental subarachnoidal haemorrhage in rats, possibly induced as a protective mechanism (103).

Hsp60 has been proposed as a good histological marker of the normality of unaffected cells in not damaged areas in pathological brains (104), although it is extremely difficult to value its levels *in vivo* in patients with Alzheimer's, Parkinson's, Huntington's, and prion diseases in clinical practice.

Hsp60 levels, as well as those of other stress proteins, were found elevated in lymphocytes from Alzheimer's disease patients when compared to controls (105). It could be of some utility to test levels of Hsp60 in patients with mild cognitive impairment (a clinical condition that precede Alzheimer's disease arise), for assessing the potential value of this protein as an early marker of the disease.

Lastly, since Hsp60 plays a role in the pathogenesis of ATS as discussed above, it can be inferred that by similar mechanisms this chaperonin is implicated in cerebrovascular disorders of the central nervous system, such as stroke, as also proposed by others (106).

7.3. Degenerative joint diseases

Hsp60 has been implicated in the pathogenesis of degenerative joint disease, both in young and elderly people (66, 107, 108). It has been postulated that a humoral response against bacterial Hsp60 (exogenous chaperonin) could elicit a cross-reaction against the infected-host's Hsp60 (endogenous chaperonin) and other antigens in the synovial tissue in rheumatoid arthritis, thus perpetuating the local inflammatory and destructive processes (108, 109).

7.4. Other pathologies

Hsp60 levels and the presence of anti-chaperonin auto-antibodies have been studied in relation to onset and progression of other aging-related diseases different from those already discussed above, such as atrial fibrillation (110-112), glaucoma (113, 114), periodontitis (115, 116), osteoporosis (117, 118), type II diabetes (119), and chronic obstructive pulmonary disease (120, 121). In these instances it is of the essence to determine to what extent Hsp60 contributes to pathogenesis when defective or to protection when normal, for elucidating the molecular mechanisms by which this chaperonin might trigger the

initiation, and/or modify the course, of these age-related disorders.

8. SUMMARY AND PERSPECTIVES

A positive correlation between longevity and capacity for mounting a strong heat-shock response has been reported (122, 123), implying that chaperones are key components of adaptive mechanisms for survival. Unfortunately, chaperone levels and/or functionality generally decrease with age. For example, Hsp60 could be overloaded by the increasing demand due to the accumulation of damaged proteins that occurs during senescence, which could result in a widespread chaperoning deficiency and lead to the onset of aging-related disorders (30, 124). A shift in the balance between misfolded proteins and available chaperonins, and other Hsps, in aging organisms, can bring about defects in signal transduction, protein transport, cellular organization, and immune functions (30, 32).

All the information available at the present time indicates that Hsp60 is a key factor for protein homeostasis and cell survival, and that this chaperonin is involved in various ways in the onset and progression of aging-related diseases. We have surveyed in this review some of the pathological conditions in which Hsp60 is implicated but there are many others which could also be listed here. In addition, one has to assume that other syndromes and diseases will in the near future be found to have a pathogenetic component involving Hsp60. In all these conditions, already described or to be found, it is necessary to investigate in depth the mechanism of Hsp60 participation and, thus, uncover clues useful for the development of diagnostic and therapeutic means, which ought to help considerably the management of aging-related disorders.

9. ACKNOWLEDGEMENTS

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Abbreviations: Hsps: heath shock proteins; ATS: atherosclerosis; COPD: chronic obstructive pulmonary disease

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