HUMAN GENETICS '99: THE CARDIOVASCULAR SYSTEM
Stress-Response Proteins in Cardiovascular Disease

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Recent years have witnessed a quiet revolution in biology as seemingly disparate fields, as far apart as biophysics and medical specialties such as human genetics and cardiovascular diseases, have begun to merge. Studies of molecular chaperones have bridged the gaps between biophysics and medical genetics, and they provide a clear example of promise of the new synthetic approach. Biophysicists, using nuclear magnetic resonance spectroscopy and other techniques in cell-free systems, have elucidated some of the basic mechanisms of protein folding, and they have shown that chaperone proteins participate in multiple steps in the folding pathway of model substrates (Dobson and Ellis 1998; Richardson et al. 1998). Microbial geneticists and cell biologists have also found that similar chaperones also act in off-pathway events such as aggregation and protein degradation (Ciechanover 1998; Glover and Lindquist 1998). Meanwhile, medical scientists have become acutely aware that protein aggregates and protein damage are significant factors in the pathogenesis of heritable diseases such as cystic fibrosis and of acquired disorders such as ischemic strokes and heart attacks (for a review, see Thomas et al. 1995; Benjamin and McMillan 1998). In addition, the degree of aggregate formation in a cell can often be modified by the expression of specific chaperones, suggesting a possible avenue to alter the phenotype of many human pathological states. Because several chaperones are also heat shock proteins (HSPs), the well-established cytoprotective and regulatory properties of HSPs may be exploited as therapeutic targets for similar disorders.

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Stress Proteins in Endogenous Cellular Defense Mechanisms

HSPs are best known as cytoprotective agents, and several families of these proteins are conserved in all living cells, ranging from bacteria to human cells. Sudden heat stress (~5°C above normal growth temperature) up-regulates many classes of HSPs, a response often called the "heat shock response." Since diverse stresses (e.g., ischemia, heavy metals, amino acid analogues, and mutant or abnormal proteins) also induce the expression of HSP genes, the term "stress proteins" may be preferred. HSPs function as molecular chaperones, under both stressed and normal conditions, to assist polypeptide chain folding and assembly into its secondary and higher-order structures (Hartl 1996). In addition, HSP chaperones assist in the repair of denatured proteins or their degradation after heat shock.

The induction of HSP expression is crucial for the survival of cells and, ultimately, organisms under stressed conditions. Rapid induction is thus a central conserved feature of the HSPs (Morimoto 1998). This property helps explain the long-standing observation that a brief, sublethal pretreatment of cells and organs with heat stress elicits a powerful transient protection against a subsequent severe heat shock (thermotolerance) or different forms of stress (cross-tolerance). While studies (discussed below) in ischemic brain and cardiac protection have begun to establish direct causal relationships, the potential roles of stress proteins in protecting different cells and organs have now been extended to include a range of human pathologic conditions.

Regulation and Function of the Heat Shock Response

How diverse physiological stimuli are sensed in the cell and transmitted to evoke the heat shock response is the focus of active investigation with potential clinical significance. In vertebrates, stress-inducible up-regulation of *Hsp* genes is controlled by the family of DNA-binding proteins, or heat shock transcription factors (HSFs) (Wu 1995). Under unstressed conditions, eukaryotic HSFs are maintained in the latent, non–DNA-bind-

ing state by one or more negative regulators such as Hsp90 or Hsp70 (Morimoto 1998). Physiological stresses, such as heat and ischemia, result in the activation of HSF monomers to form homotrimers, translocate into the nucleus, and bind, with high affinity, to the conserved upstream heat shock elements (HSEs) contained in the promoters of all heat shock genes (for a review, see Wu 1995).

Prior exposure of cells to arachidonic acid, a potent mediator of the inflammatory response, lowers the temperature threshold for eliciting the heat shock response in cultured cells (Jurivich et al. 1994). One potential implication of these findings is that supraphysiologic heat shock is not necessary for the heat shock response in humans. In principle, multiple factors, such as cytokines and prostaglandins, could induce this response at temperatures present during febrile illness. Although recent studies suggest heat shock and oxidative stress can act directly on HSF activation in vitro, a growing body of knowledge supports the current concept that misfolded or damaged proteins themselves are the proximate signals in the activating of gene expression in specific intracellular compartments and organelles.

Recent genetic studies have identified discrete sensing that up-regulates molecular chaperones in various subcellular compartments. Basal levels of protein folding and misfolding are apparently regulated by the constitutive pool of chaperone networks to maintain cellular homeostasis. If this capacity is exceeded in the cytosol, because of either environmental or metabolic changes, the HSF1-mediated pathway is activated. However, an independent regulatory pathway has evolved to respond to excess levels of unfolded protein in the endoplasmic reticulum—termed the "unfolded protein response" (UPR). Activation of the UPR pathway requires the endoplasmic reticulum (ER)–resident transmembrane receptor, Ire1p, which senses the presence of unfolded proteins and transduces this signal by stabilizing the transcription factor, Hac1p (Cox et al. 1996). Hac1p uses a novel mechanism of mRNA splicing, which is extremely short-lived, to up-regulate the expression of ER-Hsp70 homolog, Grp78, thus enabling tight control of the unfolded response in this compartment (Sidrauski et al. 1996). Recent evidence also suggests this mechanism may be conserved in mammalian cells as well (Sidrauski et al. 1998).

Denatured and misfolded proteins that cannot be refolded by the molecular chaperones are efficiently targeted for removal by intracellular proteolysis. The ubiquitin-proteasome machinery is used in the turnover of some naturally short-lived cytoplasmic proteins that are targeted for ubiquitin-proteasome–mediated protein degradation. Ubiquitin, a 76–amino acid polypeptide, is covalently linked to intracellular proteins, which are subsequently degraded by the multisubunit 26S proteasome (for a review, see Ciechanover 1998). Available evidence suggests that the ubiquitin-proteasome machinery plays a pivotal role in disease pathogenesis through effects for increased protein degradation of mutant or misfolded proteins in many inherited and acquired pathological states (Ciechanover 1998).

Protein aggregation is a hallmark of many human disorders, such as Huntington disease, Alzheimer disease, amyloidosis, cystic fibrosis, and hypertrophic cardiomyopathy. Several classes of molecular chaperones have been shown to mitigate protein aggregation, either by preventing protein aggregation or by solubilizing existing aggregates (Glover and Lindquist 1998). Thus, Hsp104, a yeast cytosolic molecular chaperone, appears to be in the latter category and is required for recovery of cell growth after heat shock. Many of the other classes of chaperones, including the major Hsp70 and small–molecular weight Hsps (heme oxygenase [Hsp32], Hsp25/27, and αB-crystallin), facilitate protein folding and prevent protein aggregation. In the endoplasmic reticulum, similar functions are thought to be mediated by the Ca^{2+}-binding glucose-regulated class of HSPs, Grp170, Grp94, Grp78/BiP, Grp58, and Hsp47. Grp78/BiP, one of the best characterized of the ER molecular chaperones (immunoglobulin-binding protein), interacts with many secretary and membrane proteins in the ER during their maturation (for a review, see Gething and Sambrook 1992).

Chaperones that prevent aggregate formation most likely act by binding transiently to exposed hydrophobic surfaces on partially folded proteins, thereby reducing the concentration of free intermediates of protein folding. Recent studies of the common ΔF508 mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) by Johnston and colleagues (1998) have shown that multiubiquinated, mutant CFTR aggregates as a pericentriolar structure, termed the "aggresome." Aggresome formation is accompanied by microtubule redistribution, which forms a cage around the aggresome.

Biochemical studies have shown that the cytosolic Hsc70 chaperone increases the productive folding of the common ΔF508 mutation of the CFTR and decreases its aggregation (Strickland et al. 1997). Treating the cells with lowered temperature or osmolytes, such as glycerol, stabilizes protein folding by favoring the hydration of molecules in the native state. Interestingly, the chemical chaperones—glycerol, dimethylsulfoxide, and trimethylamine N-oxide—have been shown to improve the folding and processing of ΔF508 CFTR significantly in cultured cells. Studies of chemical chaperones provide a rationale basis for potential therapeutic strategies for correcting other protein-folding defects.

Protein Oxidative Damage Occurs during Ischemic Stroke and Myocardial Disease

Timely restoration of coronary blood flow after stroke or infarction is required to salvage ischemic organs such as the brain and heart. The amount of tissue damage after a catastrophic event such as a heart attack or stroke is the primary determinant of an individual's morbidity and survival. Although cell death does not occur immediately after such stress, subsequent tissue deterioration is due primarily to the limited capacity of postmitotic cells (e.g., cardiomyocytes and neurons) to proliferate after lethal injury. Because thrombolytic therapy is contraindicated in many patients with acute ischemic syndromes, treatments that speed the recovery of injured cells may be the most promising strategy for minimizing long-term damage in the heart and brain.

Protein unfolding and aggregation remain a hazard even after blood flow is restored to ischemic tissue. Highly reactive oxygen-free radicals may be generated from the mitochondrial electron-transport system, a potential source of protein damage during reperfusion. Reduced efficacy of antioxidant networks and the increased production of reactive oxygen species could, in theory, increase oxidative stress/damage, the main pathogenic mechanism in oxidative protein damage and lipid peroxidation associated with age-related syndromes. Such concepts have inspired the hope that molecular chaperones could mitigate specific deleterious events in the aging process.

Stress Proteins Afford Protection in Intact Organs

A substantial amount of literature has described the protective effect of HSPs, especially Hsp70, against myocardial ischemia–reperfusion injury, (for a review, see Benjamin and McMillan 1998). Early observations indicated that increased expression of Hsp70, induced by hyperthermal stress or sublethal ischemia, could reduce infarct size and arrhythmias and improve postischemic functional recovery. Forced expression of Hsp70 provided a cytoprotective effect in the in vitro cell culture subjected to simulated ischemia. More recently, Suzuki et al. (1997) reported that intracoronary infusion of the human *hsp70* gene carried by virus liposome improved postischemic recovery (Suzuki et al. 1997). Studies in transgenic mice show that stress protein–mediated cytoprotection after transient global or regional ischemia extends even to the intact heart and brain (Marber et al. 1995; Plumier et al. 1995, 1997; Radford et al. 1996). In addition to Hsp70, the small HSPs αB-crystallin and Hsp27, when overexpressed in isolated adult or neonatal cardiomyocytes, protected against simulated ischemia (Martin et al. 1997). The effect of αB-crystallin in this assay is particularly striking in light of recent evidence that the endogenous αB-crystallin gene (*CRYAB*) is required for normal cardiac function.

Missense Mutation of αB-crystallin Chaperone Causes Cardiac and Skeletal Myopathy

Since the early 1980s, several reports have described neuromuscular disorders with cardiac and skeletal muscle involvement in which the muscle-specific intermediate protein desmin is overexpressed and deposited in disordered form in cardiac and other muscle cells (Osborn and Goebel 1983). This desmin-related myopathy (DRM) is characterized by variable, but delayed, onset of proximal and distal muscle weakness and all three major forms of cardiomyopathy (i.e., dilated, hypertrophic, and restrictive). Patients with this condition typically exhibit first-degree atrioventricular (AV) block and other AV conduction abnormalities, progressive deterioration of ventricular function, congestive heart failure, and death from either intractable pump failure or lethal arrhythmias.

The histologic hallmarks of DRM in endomyocardial or skeletal muscle biopsy samples are accumulations of dense deposits and aggregate formation of intermediate filaments (IFs). These comprise 8–10-nm filaments that are intermediate between actin filaments (5–7 nm) and myosin filaments (14 nm) (Fuchs and Weber 1994). Besides desmin, other proteins found in the aggregates include the αB-crystallin chaperone and ubiquitin. Excess amounts of IFs and associated proteins, desmin and αB-crystallin chaperone, have been confirmed in the affected tissues by western blot analysis. On the basis of ultrastructural analysis, Goebel (1997) has recently proposed two major classifications of desmin myopathy—namely, granulomatous (type I) and cytoplasmic inclusion (type II). Both familial and sporadic cases have been described in the literature worldwide. The autosomal dominant inheritance pattern is typically the granulomatous type, which is not found in other forms of dilated cardiomyopathy (Goebel 1997).

A significant breakthrough into the etiology of desmin myopathy came recently when a missense mutation was mapped to the muscle-specific IF desmin in affected individuals (Goldfarb et al. 1998). Soon thereafter, Vicart and colleagues (1998) excluded mutations of the desmin gene in a French pedigree but found a missense mutation of αB-crystallin (Arg120Gly), which mapped to the locus of DRM on chromosome 11q21–23 and cosegregated with the disease phenotype. These are the first findings to implicate an HSP with chaperone properties in a human cardiovascular disorder. Moreover, these aggregates in αB-crystallin^{R120G} myopathy, found in cardiac or skeletal biopsy samples, are strikingly reminiscent of the

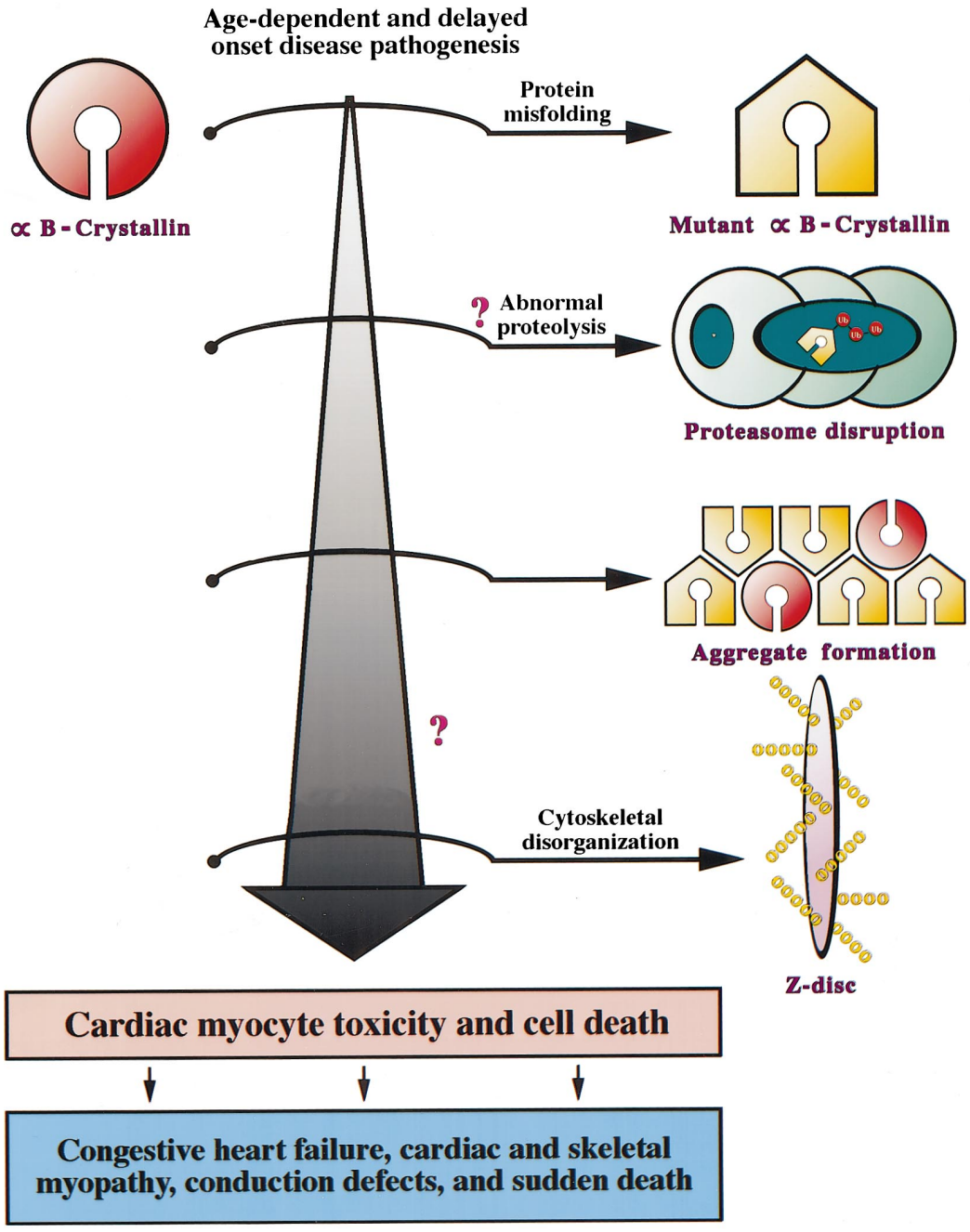

Figure 1 A hypothetical model of mutant αB-crystallin chaperone in cardiomyopathy. Besides being expressed in the vertebrate lens, the αB-crystallin chaperone is an abundant cytosolic protein of striated muscle, such as the heart and skeletal muscle. This chaperone shows a tendency to transiently interact with components of the cytoskeleton, such as Z-discs, a major site of force generation, and IFs (e.g., vimentin and desmin). A missense mutation in the αB-crystallin gene (*CRYAB*) is proposed to cause misfolding of mutant protein whose major fate in the cell is determined by the ubiquitin-proteasome degradation. Potential factors for triggering aggregate formation may include the overproduction of mutant protein, perhaps the result of the stress-response HSF1 regulatory pathway or alteration in the capacity of proteasome degradation, or both. Besides ubiquitinated mutant proteins, a common pathogenic mechanism has been proposed because similar aggregates, termed "aggresomes," have been shown recently to contain IFs (Johnston et al. 1998). It is conceivable that the myopathic effects of aggresome formation are causal factors in disease pathogenesis through effects that disorganize the cytoskeleton and impair contractile performance, resulting in congestive heart failure and lethal arrhythmias.

CFTR mutant aggresomes with the IF protein vimentin (Johnston et al. 1998), suggesting a common pathogenic mechanism in these inherited diseases.

Perspectives

Many lines of evidence now indicate that molecular chaperones are key mediators of protein-folding pathways and that misfolded or abnormal proteins arising from acquired disorders or genetic errors play primary etiologic roles in the pathogenesis of cardiac diseases. The effects of the αB-crystallin^{R120G} mutation in cardiomyopathy remain uncertain, but several models appear to be readily testable. The hypothesis that this mutation compromises αB-crystallin activity as a chaperone has yet to be shown biochemically. This mutation is presumed to act as a dominant negative allele that interferes with the integrity and function of the IF protein in muscle cells. Indeed, Bennardini et al. (1992) report that desmin serves as a substrate of the αB-crystallin chaperone in vitro. Conceivably, the αB-crystallin chaperone may play complementary roles along with hsp27 in cross-bridging actin filaments in the Z-disc of the sarcomere. The absence of dynamic remodeling and the abnormal architecture of the actin cytoskeleton would be predicted to interfere with force generation during muscle contraction. The physiological milieu inside the cell may also be adversely affected by accumulating abnormal peptides that escape from proteolysis and ubiquitin-proteasome degradative pathways. It is conceivable that pathogenic mechanisms that promote aggregate formation may also activate protective mechanisms, and, indeed, it is well established that increased contractile activity up-regulates αB-crystallin expression (Neufer et al. 1996). Onset of symptoms may then occur only when such protective mechanisms prove inadequate, tipping the balance toward cumulative myocyte loss, compensatory hypertrophy, and secondary left ventricular dilatation (fig. 1).

A major goal of future research is to determine whether the tendency for aggregate formation is a critical factor in the pathogenesis of human diseases. Small-animal models of human genetic disorders provide a powerful experimental approach to determine the effects of physiological perturbations in the intact organism, such as misfolded protein expression that mimics the condition of cardiomyopathy in humans. Results of such studies can help in establishing a cause-and-effect relationship and in explicating potential mechanisms by the alterations in the functions of a muscle-specific chaperone as related to a cardiomyopathy in mammals. *Hsf1*-null mice, now under development (McMillan et al. 1998), will permit the testing of other crucial questions. These animals should lack the endogenous heat shock response, although it might be restored, under controlled conditions, to test the physiological adaptation to mutant protein expression. This system might be advantageous in studying the effects of specific chaperone alleles, such as the αB-crystallin^{R120G}, in an intact animal. Such studies have major implications for cardiac diseases with similar clinical features (Geisterfer-Lowrance et al. 1996); furthermore, unambiguous answers could challenge the belief that aggregate formation is a secondary, and not a primary, cause of the pathogenesis of certain human diseases.

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