Chapter 6
Hormetic Modulation of Aging in Human Cells

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Introduction

An experimental model system that has been used in testing and applying hormesis as a modulator of aging is the so-called Hayflick system of cellular aging *in vitro*. In modern biogerontology, the terms "cellular aging", "cell senescence" or "replicative senescence" imply the study of normal diploid cells in culture, which during serial subcultivation undergo a multitude of changes culminating in the irreversible cessation of cell division. This process of cellular aging or replicative senescence *in vitro* is commonly known as the Hayflick phenomenon, and the limited division potential of normal cells is called the Hayflick limit, in recognition of the observations first reported by Leonard Hayflick in 1961. In many organisms, several cell types retain the capacity to divide during most of the adult lifespan, and are required to divide repeatedly or infrequently in carrying out various functions of the body. These functions include the immune response, blood formation, bone formation, and repair and regeneration of various tissues. Epithelial cells, epidermal basal cells (keratinocytes), fibroblasts, osteoblasts, myoblasts, glial cells and lymphocytes constitute major differentiated and proliferating cell types of an organism, and are distinct from the pluripotent stem cells. It is not only their differentiated and specialized functions that are critical for the organism, their capacity to divide is an integral part of their role in organismic growth, development, maintenance and survival (for details on the aging *in vitro* of various cell types (see Kaul and Wadhwa 2003).

The study of age-related changes in the physiology, biochemistry and molecular biology of isolated cell populations has greatly expanded our understanding of the fundamental aspects of aging. In addition to the normal diploid fibroblasts which

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have been the most frequently used cells for studies on cellular ageing *in vitro*, a variety of other cell types including epithelial cells, endothelial cells, keratinocytes, glial cells, lymphocytes and osteoblasts have also been used. Although the exact culturing conditions (such as the type of the culture medium, the source of growth factors, the use of antibiotics, and the incubation temperature, humidity and gaseous composition) may vary for different cell types, serial subcultivation or serial passaging of normal diploid differentiated cells can be performed only a limited number of time. The cumulative number of cell proliferations, measured as the cumulative population doubling level achieved *in vitro*, depends upon several biological factors, such as the maximum lifespan of the species, the age of the donor of the tissue biopsy, and the site of the biopsy. This is in contrast to the high proliferative capacity of transformed, cancerous and immortalized cells whose cultures can be subcultivated and maintained indefinitely.

Serial subcultivation of normal cells is accompanied by a progressive accumulation of a wide variety of changes before the final cessation of cell replication occurs. The progressively emerging senescent phenotype of serially passaged normal diploid cells can be categorized into the structural, physiological, and biochemical and molecular phenotypes, which can be used as biomarkers of cellular aging *in vitro*. Table 1 gives a summary of the major changes occurring during serial passaging and replicative senescence. For specific details for different cell types (see Kaul and Wadhwa 2003).

What is clear is that the development and use of the Hayflick system has been instrumental in creating a strong foundation for understanding the cellular and molecular basis of aging (Rattan 2003). Based on these studies, aging can be characterized as: (i) a progressive accumulation of macromolecular damage and increased molecular heterogeneity; and (ii) progressive shrinkage of the homeostatic/homeodynamic space due to the failure of maintenance and repair systems leading to increased vulnerability, diseases and eventual death (Rattan 2006). Application of hormesis as a modulator of aging in human cells is based in the

Table 1 Main categories of phenotypic changes occurring during cellular aging *in vitro*

1. *Structural phenotype*: Increase in cell size; change of shape from thin, long and spindle-like to flattened and irregular; loss of fingerprint-like arrangement in parallel arrays; increased number of vacuoles and dense lysosomal residual bodies containing UV-fluorescent pigments; rod-like polymerization of the cytoskeletal actin filaments and disorganized microtubules; and increased level of chromosomal aberrations and multinucleation.
2. *Physiological phenotype*: Reduced response to growth factors and other mitogens; increased sensitivity to toxins, drugs, irradiation and other stress; altered calcium flux, pH, viscosity and membrane potential; reduced respiration and energy metabolism; and increased duration of G1 phase of the cell cycle.
3. *Biochemical and molecular phenotype*: Decreased activity, specificity and fidelity of various enzymes; accumulation of post-translationally modified and inactivated proteins; reduced rates of protein synthesis and degradation; increased levels of oxidative damage in nuclear and mitochondrial DNA; reduced levels of methylated cytosines; reduced length of telomeres; and altered (increased or decreased) expression of several genes, including cell cycle check point genes.

above understanding of biological aging as a progressive failure of homeodynamics (Rattan 2001, 2004, 2006).

Mild Heat Shock-Induced Hormesis in Human Cells

High temperature stress is a widely used hormetic agent, not only because it is relatively easy to implement, but also because heat stress mainly acts through an evolutionarily highly conserved stress response pathway known as the heat shock (HS) response. HS response is one of the primordial intracellular defence mechanisms against stressful conditions in which extracellular stress, and intracellular stress from denatured proteins, initiates a series of events starting with signal transduction, activation and nuclear translocation of heat shock factors (HSF), DNA binding of HSF, preferential initiation of HS gene transcription, and preferential translation of heat shock proteins (HSP), which then perform various biological functions (Verbeke et al. 2001; Park et al. 2005). (*HS-induced hormesis in other aging organisms is discussed in other chapters in this book*).

In our labs, we have been testing the hormesis hypothesis of the beneficial effects of mild HS on the Hayflick system of cellular aging of normal human cells in culture. The mild HS conditions were selected from a series of pilot studies performed on testing the effects of 1 h HS at different temperatures, ranging from 37 °C to 45 °C, on the synthesis of HSP70 in the following 3 h period. Maximum HSP70 synthesis (more than eightfold synthesis as compared with that at 37 °C) was observed at 43 °C. However, at 41 °C, HS response was about one third of the maximum response, and so this temperature was selected for long-term studies in which the cells were exposed repeatedly to HS. Temperatures higher than 43 °C could not be used for repeated exposures.

Using a mild stress regimen of exposing serially passaged human adult skin fibroblasts to 41 °C for 1 h twice a week throughout their replicative lifespan *in vitro*, we have reported a wide variety of biological effects. A summary of our observations on the hormetic effects of repeated mild HS on human skin fibroblasts undergoing aging *in vitro* is given in Table 2.

We have also undertaken studies on the hormetic effects of repeated mild HS on normal human epidermal keratinocytes (NHEK), and the results obtained are very much similar to those for dermal fibroblasts (Rattan and Ali 2007). As previously observed for human skin fibroblasts, NHEK also showed a variety of cellular and biochemical hormetic anti-aging effects on repeated exposure to mild HS at 41 °C. These effects included maintenance of youthful cellular morphology, enhanced replicative lifespan, enhanced proteasomal activity, and increased levels of HSP (Rattan and Ali 2007). Additionally, we have also studied the effects of HS on Na, K-ATPase or the sodium pump. Mild HS significantly increased the content and activity of the pump in NHEK. However, the molecular mechanisms and interactions which bring about the mild HS-induced increase in the amounts and activity of Na, K-ATPase, and its consequences on other biochemical pathways, in NHEK

Table 2 Hormetic effects of repeated mild heat shock on human skin fibroblasts undergoing aging *in vitro*

Characteristic	Hormetic effect	Reference
Cell size	Reduced enlargement	Rattan 1998
Cellular morphology	Reduced irregularisation	Rattan 1998
Replicative lifespan	10–20% increase	Nielsen et al. 2006
Glycation, furasine level	50–80% reduction	Verbeke et al. 2001
Glycoxidation level	10–30% reduction	Verbeke et al. 2001
CML-rich protein level	20–85% reduction	Verbeke et al. 2001
Lipofuscin pigment level	6–29% reduction	Verbeke et al. 2001
Protein carbonyl levels	5–40% reduction	Verbeke et al. 2001
Reduced glutathione level	Threefold increase	Verbeke et al. 2001
Oxidised glutathione level	Twofold reduction	Verbeke et al. 2001
Induction of sugar-induced protein damage	Tenfold reduction	Verbeke et al. 2002
H_2O_2 decomposing ability	50–140% increase	Fonager et al. 2002
Survival after H_2O_2 exposure	10–18% increase	Fonager et al. 2002
Survival after ethanol exposure	10–40% increase	Fonager et al. 2002
Survival after UVA exposure	5–17% increase	Fonager et al. 2002
Hsp27 level	20–40% increase	Fonager et al. 2002
Hsc70 level	20% increase	Fonager et al. 2002
Hsp70 level	7–20-fold increase	Fonager et al. 2002
Hsp90 level	50–80% reduction	Fonager et al. 2002
Proteasome activities	40–90% increase	Beedholm et al. 2004
20S proteasome content	No change	Beedholm et al. 2004
19S activator content	No change	Beedholm et al. 2004
11S activator content	Increase	Beedholm et al. 2004
11S activator binding	Increase	Beedholm et al. 2004
Proteasomal oscillation	Enhanced stability	Kraft et al. 2006

during aging are yet to be elucidated. Notably, comparable hormetic effects could not be seen in NHEK repeatedly exposed to 43 °C, which underlines the differences between the beneficial effects of mild stress and the harmful effects of severe stress. Other hormetic effects of mild HS on NHEK include increased differentiation of keratinocytes in the presence of calcium, and reduced cytotoxic effects of glucose and glyoxal (Berge et al. 2007).

Other cell types in which we have initiated studies on the hormetic effects of mild HS are telomerase-immortalised human bone marrow stem cells, and human microvascular endothelial cells. In a pilot study we have reported that vitamin-D-induced differentiation of telomerase-immortalised bone marrow stem cells into osteoblasts could be enhanced by pre-exposure to mild HS (Nørgaard et al. 2006). Our more recent studies indicate that mild HS also promotes angiogenesis (measured by the tube formation assays) in human microvascular endothelial cells, and may stimulate the migration of human skin fibroblasts in a wound healing assay *in vitro* (*unpublished observations*). Further studies are in progress to unravel the molecular details of enhanced angiogenesis by endothelial cells and enhanced wound healing by fibroblasts exposed to single or multiple rounds of mild HS.

Possible Mechanisms of Hormetic Effects of Heat Stress

The possible pathways for the hormetic effects of repeated mild HS in human cells include an increase in the activities of the proteasome, increased levels of various HSP, and increased antioxidative enzyme activities, (Fonager et al. 2002; Beedholm et al. 2004). Furthermore, we have also shown that repeated mild HS at 41 °C, but not the severe HS at 42 °C, increased the replicative lifespan, and elevated and maintained the basal levels of MAP kinases JNK1, JNK2 and p38 in human skin fibroblasts (Nielsen et al. 2006).

Although the general mechanisms of severe HS response are well understood (Feder and Hofmann 1999; Verbeke et al. 2001; Sun and MacRae 2005), it is not clear whether there are any significant differences between mild HS which has hormetic effects, and severe HS, repeated exposure to which has deleterious effects (Park et al. 2005). It is likely that the physiological cost of stress in terms of energy utilisation, molecular damage overload and metabolic shift determine the difference between the outcome of mild and severe stress (Salvioli et al. 2001). Also, it is yet to be understood how the transient appearance of HSP leads to biologically amplified hormetic effects at various other levels of cellular functioning, such as improved proteasome activity, enhanced resistance to other stresses, maintenance of the cytoskeletal integrity and others.

Optimal HS response in terms of HSP synthesis and activity is essential for cell survival. In contrast, inefficient and altered HS response has been implicated in abnormal growth and development, aging and apoptosis (Söti et al. 2005; Verbeke et al. 2001). When a cell encounters a "stressor", modifications of the cytoskeleton, cytoplasmic structures, cell surface morphology, cellular redox status, DNA synthesis, protein metabolism and protein stability occur. Heat stress generates molecular damage, especially abnormally folded proteins, which can aggregate and initiate a sequence of stress response. The cellular stress response can be viewed as an adaptive response for the defence and maintenance of its structural and functional integrity.

Signaling pathways in HS: Signaling pathways involved in HS response are still largely unknown. However, some kinases in the stress pathways, such as stress activated protein kinase (SAPK) c-Jun terminal kinase (JNK or SAPK1) and p38 (SAPK2), are suggested to play an important role. HS activates within minutes the major signaling pathways involving mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) and SAPK (Gabai et al. 1998; Dorion and Landry 2002). These kinases are involved in both survival and death pathways in response to other stresses and may, therefore, contribute significantly to the HS response (Gabai and Sherman 2002). Activation of p38 occurs very early during stress and leads to the phosphorylation of HSP27. It is triggered by a highly specific HS sensing pathway and requires the activation of upstream kinases such as the MAPKK MKK3/6 and the MAPKKK apoptosis signal-regulating kinase-1 (ASK1) (Meriin et al. 1999). HS is also thought to activate (and thus phosphorylate) the epidermal growth factor (EGF) receptor in an agonist-independent way (Dorion

and Landry 2002). HS has also been shown to phosphorylate constitutive nitric oxide synthase at tyrosine residues and increase fas/CD95 expression on the cell membrane (Kiang et al. 2003). HS also increases the levels of calcium, sodium, cAMP, and inositol 1,4,5-trisphosphate (Kiang and Tsokos 1998).

It is suggested that JNK is preferentially associated with the protective effects of HS against severe stress (Park and Liu 2001). A major mechanism for HS-induced JNK appears to be the direct inhibition of the JNK phosphatase that normally inactivates JNK. In the absence of this phosphatase, the basal activity of MAPKK4 (MKK4) is sufficient to activate JNK (Meriin et al. 1999). An early and transient activation of the JNK and p38 pathways is usually associated with survival and differentiation, whereas a late and sustained activation might point to apoptosis (Dorion and Landry 2002). Therefore a balance between the JNK and p38 pathways (apoptotic) and ERK pathways (survival), and their interplay, determine whether a cell exposed to HS will die or survive and become stress tolerant (Gabai and Sherman 2002). We have observed a rapid activation of MAP-kinases in terms of phosphorylation after 41 °C or 42 °C HS, and cells exposed to multiple rounds of 41 °C or 42 °C HS seem to have an increased amount of total JNK and p38 as compared with unstressed cells (Nielsen et al. 2006).

Activation of heat shock factors: The induction of the HS response is facilitated through the HSF working as molecular links between environmental stresses and the stress response (Kiang and Tsokos 1998; Verbeke et al. 2001). The four vertebrate HSF are expressed constitutively and cooperate functionally. HSF1 is a long-lived protein, it is an inactive monomer considered to be a general stress responsive factor which is expressed ubiquitously and is activated by mild HS as well as multiple environmental or physiological stresses. HSF2 is a short-lived protein present as an inactive dimer refractory to typical stress stimuli except proteasome inhibitors and is considered to be important during embryogenesis and spermatogenesis. HSF3 is also an inactive dimer and an important co-regulator of HSF1, activated by severe HS and chemical stress. HSF3 may exhibit complex interactions with other transcription factors governing development, growth and apoptosis, such as c-Myc and p53. HSF4 constitutively binds DNA even in non-stressed cells and is preferentially expressed in muscle, brain and pancreas (Verbeke et al. 2001).

In unstressed cells, HSF1 is both located in the cytoplasm and in the nucleus. It is maintained as a non DNA-binding inactive complex both by internal coiled-coil interactions and by stoichiometric binding with HSP90, HSP70 and other chaperones. The synergistic interaction between these chaperones modulates HSF1 activity by feedback repression (Shamovsky and Gershon 2004). During and after stress, the cellular proteins undergo denaturation and/or polyubiquitination and sequester the chaperones capping HSF1. The inactive HSF1 becomes free and translocates into the nucleus. HSF have a nuclear localization sequence that is both necessary for the transition of HSF from inactive to active state and for nuclear import. HSF1 is activated by trimerization and subsequent phosphorylation (Kiang and Tsokos 1998). Using electrophoretic mobility shift assay, we have demonstrated that RMHS at 41 °C activates HSF1 and facilitates its nuclear translocation and DNA binding in human skin fibroblasts, thus initiating the HS response. No studies have

yet been performed on other HSF, and also it is not known whether mild stress activates HSF to the same extent as a severe stress at higher temperatures (Shamovsky and Gershon 2004).

Heat shock proteins (*HSP*): Genes encoding HSP are highly conserved. Many of their products can be assigned to families on the basis of sequence homology and molecular weight. In mammals, many HSP families comprise multiple members that differ in inducibility, intracellular localization and function (Verbeke et al. 2001; Sørensen et al. 2003; Park et al. 2005). HSP are known to play diverse roles as chaperones and/or proteases. In unstressed cells, HSP act in successful folding, assembly, intracellular localization, secretion, regulation and degradation of other proteins. Under conditions in which protein folding is perturbed or proteins begin to unfold and denature, HSP have been shown to assist in protein refolding, to protect cellular systems against protein damages, to dissolve protein aggregates to some extent, to sequester overloaded and damaged proteins into larger aggregates, to target damaged proteins to degradation, and to interfere with the apoptotic programme. Chaperones and proteases can recognise the same protein substrates and the abundance of both types of proteins suggests that HSP are able to distinguish between those proteins that can be refolded and those fated to enter the proteolytic pathway (Söti and Csermely 2000; Söti et al. 2003; Kiang and McClain 2003).

Some HSP are known to be chaperones and are involved in the renaturation of unfolded proteins. Chaperones recognize and bind to other proteins when they are in non-native conformations and are exposing hydrophobic sequences. Their role is to minimize the aggregation of non-native proteins formed during stress. Typically, chaperones function as oligomers, if not as a complex of several different chaperones, co-chaperones and/or nucleotide exchange factors (Feder and Hofmann 1999). In response to heat and oxidative stresses, different small HSP (sHSP) either become phosphorylated or dephosphorylated. Depending on their phosphorylation status, sHSPs form large (300–800 kDa) and active oligomers having an ATP-independent chaperone activity. sHSPs and HSP90 families capture unfolded proteins and create a reservoir of folding intermediates preventing further aggregation. Subsequently, HSP70 and HSP60 families, helped by cochaperones, bind to the stabilized unfolded proteins in the cytosol, mitochondria and endoplasmic reticulum and attempt to restore the structure of proteins in a cycle driven by ATP-hydrolysis. If the target protein is damaged by post-translational modifications, it could be repaired by specific cellular systems before refolding, but such systems exist for only few kinds of damages (Verbeke et al. 2001).

Acting as molecular chaperones, HSP protect many different systems involved in maintenance of cellular functions. sHSP induce an increase of the cellular GSH level leading to the protection of the mitochondrial membrane potential during stress (Préville et al. 1999). HSP70 contains a novel nuclear localisation signal in its C-terminal domain implying a role for HSP70 in the regulation of nuclear proteins and transcription factors such as HSF. Members of HSP70 and HSP90 families are associated with the centrosome, suggesting an involvement in microtubule nucleation or in centrosome assembly. The protection of protein synthesis during stress, called translational thermotolerance, is due to the association of

HSP72 with ribosomal subunits in polysomes of thermotolerant cells. Some chaperones such as the sHSP α_2-crystallin and HSP90 could stabilize a more active conformation of the proteasome (Verbeke et al. 2001).

Members of the HSP90 family constitute 1–2% of cytosolic proteins and have stress-related as well as housekeeping functions. HSP90 stabilize damaged proteins during and after stress. HSP90 interact and either modulate the assembly, the stability and/or the activity of particular cellular proteins such as protein kinases, calcineurin, calmodulin, nitric oxide synthase, telomerase, steroid receptors, oncogenes and transcription factors (Verbeke et al. 2001). HSP90 is presented as a suppressor of cryptic genetic variations by assisting mutant proteins to maintain a wild type structure and function (Rutherford and Lindquist 1998). HSP90 and p23 play also a direct role in the regulation of the HS response by modulating the HSF1 activation/deactivation process. Since HSP90 exists in homeostasis with intracellular hormone receptor and HSF1, it could be hypothesized that steroid hormones activate the HSF by altering this homeostasis. HSP90, HSP70, HSP60 and p23 make heterocomplex with a variety of transcription factors and protein kinases involved in mitogenic signal transduction. The major function of this complex may be to fold the client protein and to keep it inactive until it reaches its ultimate location. There is also a potential involvement of HSP70 and HSP90 in DNA replication since members of these families interact with components of the eukaryotic cell cycle. HSP70, HSP90, HSP27 and TCP-1 are known to bind and stabilize actin, tubulin and the microtubules/microfilament network playing a role in cellular morphology and signal transduction pathways. The HSP60/HSP10 chaperonin system is localized primarily in the matrix space of mitochondria where it assists in folding, refolding and/or elimination of mitochondrial proteins (Kiang and Tsokos 1998; Verbeke et al. 2001).

Our studies show that the basal levels of both the constitutive HSC70 and stress-inducible HSP70 and HSP27 proteins increase during cellular aging of human skin fibroblasts even without any HS (Fonager et al. 2002). A similar increase in the basal level of HSP22 in aged *Drosophila* (King and Tower 1999), and HSP70 in rat kidneys (Maiello et al. 1997) has been reported previously and is taken as the cells' adaptive response to increased intracellular stress during aging. Therefore, it appears that increased levels of HSP27, HSC70 and HSP70 in senescent cells are indicative of their failed attempt to maintain structural and functional ability and to survive for as long as possible. In comparison, exposing these cells to repeated bouts of mild stress stimulates the synthesis of these HSP, maintains their levels high and helps to improve the functional ability and survival of cells without interfering with their replicative lifespan (Fonager et al. 2002). Further analysis of the activities and different modes of action of these HSP and the molecular significance of their increased levels during cellular aging and RMHS treatment is yet to be performed.

In contrast to the increase in the basal level of some HSP discussed above, the basal levels of HSP90 decreased significantly during cellular aging with and without RMHS treatment (Fonager et al. 2002). Although the exact mechanism for the disappearance of HSP90 is not fully understood, it has been proposed that HSP90 during stress binds to partially unfolded proteins and is degraded together with

them in a manner similar to what can be observed for HSP70 after HS (Buchner 1999). Furthermore, HSP90 is a powerful modulator of the HS transcription factor HSF1 activation, and the deletion of HSP90 has been shown to promote yeast cells' ability to launch a stress response (Harris et al. 2001). Therefore, it is possible that a decrease in the level of HSP90 during cellular aging and after repeated mild HS treatment is also an adaptive response resulting in the activation of HSF1, which then stimulates the transcription and translation of other HSP.

Some HSP are known to be proteases or to make up the components of a protease system involved in the degradation of the damaged proteins. The irreparable state of a protein could be signalled to the HSP by the extent of irreparable modifications, such as carbonylation (Dukan et al. 2000). HSP70 and its cofactors as well as HSC70, HSP90 are involved in the recognition and the degradation of unnecessary and damaged proteins by the proteasome pathway (*discussed below*). Decreased association of certain proteins with HSP90 and increased association with HSP60/HSP70 lead to their 20S proteasome-mediated degradation. HSP70 has been shown to promote the poly-ubiquitination of damaged proteins. Ubiquitination seems also to be involved in the degradation of unfolded polypeptide by the lysosome. One major mechanism of the lysosomal degradation of proteins is dependent on HSC73 and is responsible for the degradation of a significant amount of the cytosolic protein (Cuervo and Dice 2000).

Protein degradation: One of the main effects of repeated mild HS on human cells is the reduction in the extent of accumulation of oxidatively and glycoxidatively damaged proteins (Verbeke et al. 2000, 2001). Although this may be due to an increase in cellular resistance of RMHS-treated cells to glucose and other protein damaging agents (Verbeke et al. 2002), another possibility is the enhanced removal of abnormal proteins by increased turnover. The bulk of proteolysis is carried out by the ubiquitin-proteasome system in eukaryotes. The proteasome is a multisubunit, multicatalytic proteinase complex, also known as multicatalytic proteinase (MCP). Oxidised proteins are preferentially degraded by the 20S proteasome in an ATP-independent manner, whereas the proteins marked by covalently attached ubiquitin are degraded in an ATP-dependent way by the 26S proteasome, which is ubiquitous among eukaryota, archaebacteria, eubacteria, and prokaryota (Grune 2000; Rivett et al. 2002; Shringaarpure and Davies 2002; Brégégére et al. 2006). The eukaryotic proteasome is present both in the nucleus and in the cytoplasm and constitutes approximately 1% of the total content of cytosolic protein. Polypeptides to be degraded are covalently attached to ubiquitin, which is itself an extremely conserved and heat-inducible HSP. The substrates for the proteasome can be categorised as either misfolded, denatured and otherwise damaged proteins, or perfectly healthy proteins, which have to be removed for normal functioning of the cell, such as cell cycle control, protein quality control, apoptosis and antigen presentation. During aging, there is a decline in the activities of the proteasome, including a decreased activity of the proteasome towards artificial peptide substrates as well as the ability to preferentially degrade oxidized proteins (Brégégére et al. 2006).

We have found that human skin fibroblast cells exposed to repeated mild HS had 20–100% increased proteasome activities, without any accompanied increase in the

20S proteasomal content. However, these hormetic effects of proteasome stimulation by mild heat stress can be dependent on the cell cycle status of the cells. Furthermore, we have observed that this increase in proteasomal activities was related to a significant increase in the amount of the proteasome activator 11S, which is an adaptor between the 20S proteasome and some of the chaperones in the cytosol. The increase of the 20S may be due to an increase in its transcription and translation of 11S activator, an increase in its binding to the 20S proteasome, and a higher level of HSPs in RMHS-treated cells. Although we have not yet determined the extent of transcription, it has been observed that the amount of 11S activartor bound to the 20S proteasome was significantly higher in RMHS-treated cells (Beedholm et al. 2004). Such an increased binding makes it possible for the RMHS-treated cells to activate the proteasome faster than the unstressed cells.

Lysosome is the other major cellular proteolytic system affected by aging. The HSC73-specific lysosomal-proteolytic-pathway is inhibited in senescent fibroblasts (Cuervo and Dice 1996; Cuervo and Dice 2000; Hallén 2002). Accumulation of lipofuscin, which is an aggregate of oxidized proteins and lipids, affects the lysosomal activities (Terman et al. 1999; Terman and Brunk 1998). Other typical cellular inclusions in senescent cells contain over-aggregated proteins as well as chaperones and proteasome components as if both chaperones and proteases have capitulated in face of various insults. A decline in HSF and HSP activity, if not always a decline in their expression, and decrease in the activities of antioxidant enzymes are thought to underlie human neurodegenerative diseases. This is because imbalances of the cellular redox status and lack of chaperone activity promote protein aggregation and favour the development of aging-linked pathologies including cataract, polyglutamine-related disorders or other neurodegenerative diseases as well as cancer (Verbeke et al. 2001; Söti and Csermely 2000; Söti et al. 2003). Severe stress may also promote some of these pathologies more directly by a transcription pathway. Accumulation of oxidized and aggregated proteins could be responsible for the increase in the constitutive expression of some HSPs such as HSP22, HSC70 and HSP70 observed in aged animals, especially in tissues formed by post-mitotic cells exposed to stress for a long period of time (King and Tower 1999). However, no studies have yet been done on the effects of repeated mild HS on lysosome-mediated protein degradation.

Thus, anti-aging hormetic effects of mild HS in aging human cells appear to be facilitated by reducing protein damage and protein aggregation by activating internal antioxidant, repair and degradation processes. HSP are involved in preventing the accumulation of highly damaged proteins during aging since they govern both the repair of weakly damaged proteins and the catabolism of highly damaged proteins. Hormetic pathways are suggested to activate several key proteins involved in the stress response. Indeed, hormesis leads to the maintenance of the HS response during aging and the concomitant transitory and moderate over-expression of HSP in cells and organisms is greatly beneficial. However, the extent of the beneficial effects of HS are also affected by the genotype, as shown by the observed differences in HS response of human lymphocytes in the context of polymorphism in HSP70 genes (Singh et al. 2004; Singh et al. 2006; Singh et al. 2006; Singh et al. 2007).

Cold shock: Almost all the studies described above have used HS at temperatures higher than the normal body temperature. There are no systematic and long-term studies performed on the effects of single or multiple exposures of normal human cells to lower temperatures. In Chinese hamster ovary cells, hypothermia-induced cold stress is reported to increase their resistance to hydrogen peroxide-induced apoptosis by enhancing the expression of bcl-2 gene (Slikker et al. 2001). Similarly, in the case of isolated mesenchymal stem cells from young and old rats, exposure to hypothermia at 32 °C increased the levels of some stress proteins, and reduced the levels of reactive oxygen species (ROS) and carbonylated abnormal proteins (Stolzing et al. 2006). It will be interesting to elucidate the effects of mild and severe cold shock on age-related changes in human cells, which will be useful in making a distinction between general stress response and temperature-specific stress response, and its applicability as hormetic agents.

Other Hormetic Stress Treatments in Aging Human Cells

Irradiation, mechanical stress, cortisols, proxidants and some natural and synthetic molecules are some of the other potential hormetic agents tested for their beneficial and anti-aging effects on human cells. For example, a very low dose rate of chronic ionizing radiation increased the division potential of human embryonic lung fibroblasts WI-38 (Icard et al. 1979; Croute et al. 1986). Similarly, the adaptive response of human embryonic cells to low dose gamma-radiation has been shown to increase the replicative lifespan by up to 160% compared to non-irradiated cells (Watanabe et al. 1992). Furthermore, human embryonic lung fibroblasts MRC-5 sequentially irradiated with 1 Gy gamma rays had their replicative lifespan increased to some extent (Holliday 1991). Hormetic effects of low dose X-irradiation on the proliferative ability, genomic stability and activation of mitogen-activated protein kinase pathways have been reported for other human diploid cells including embryonic fibroblasts (Tsutsui et al. 1997; Suzuki et al. 1998; Suzuki et al. 2001; Suzuki et al. 1998; Yang et al. 1998). Similarly, repetitive low-dose UVA irradiation of human skin fibroblasts enhanced their antioxidative ability and increased resistance to phototoxicity under selenium deficient conditions (Meewes et al. 2001). Recent studies on the exposure of human skin fibroblasts and keratinocytes to 900 MHz (GSM-900) radiofrequency radiation for 48 h showed an induction of HSP70, which can have some protective effects in terms of its chaperoning activity (Sanchez et al. 2006).

Other mild stress treatments which have been shown to have some beneficial hormetic effects in human cells include glucocorticoids in skin fibroblasts from Cushing's syndrome patients (Pratsinis et al. 2002; Zervolea et al. 2005), hydrogen peroxide in umbilical vein endothelial cells (HUVEC) and keratinocytes (Haendeler et al. 2004; Yokoo et al. 2004), and nitric oxide (NO) in keratinocytes (Krischel et al. 1998). Shear stressing of HUVEC in parallel plate flow chamber induced changes in gene expression including enhanced NO production and cytochrome

P450 expression (McCormick et al. 2001). However, no long-term studies on determining the effects of shear stress on age-related changes in human cells have yet been performed. With respect to nutritional stress, our preliminary studies have shown that partial food restriction by serum starvation (2% serum instead of normal 10% serum) of human skin fibroblasts for 24 h once a week increases their lysosomal autophagic activity, reduces the accumulation of intracellular debris, and enhances the replicative lifespan (*unpublished observations*).

Some natural and synthetic molecules which appear to have anti-aging effects on human cells through stress-induced maintenance and repair are also being tested, and these are collectively termed as hormetins (Ali and Rattan 2006; Rattan and Ali 2007). Such potential hormetins include thymidine dimers which enhance DNA repair in human keratinocytes (Eller et al. 1997) and enhance melanin production in melanocytes (Lin and Fisher 2007), celastrols from certain Chinese herbs which induce HS response (Westerheide et al. 2004), and curcumin which stimulates proteasome activity, sodium pump activity and HS response in human keratinocytes (Ali and Rattan 2006; Rattan and Ali 2007). It will be useful to test other natural and synteitc compounds which may be potential hormetins for human cells in culture and for other experimental model systems of aging.

Conclusions

The Hayflick system of cellular aging *in vitro* has facilitated testing the hormesis hypothesis of anti-aging modulation in human cells. That repeated mild stress, followed by a period of recovery and adaptation, has a wide range of biological effects which are generally beneficial is now well documented in this experimental system. At the cellular and molecular level it has been shown that the major pathways of hormetic response include heat shock proteins, other chaperones, antioxidant defenses, proteasomal and lysosomal activities and DNA repair systems. Studies performed on human cells in culture have provided the proof of the principle that hormetic modulation of the process of aging is a real possibility. The results obtained from cell culture studies can also be the basis for elucidating the mechanisms of hormetic effects of other stresses such as exercise, calorie restriction, nutritional components, and psychosocial factors.

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