Stress-Mediated Hormetic Modulation of Aging, Wound Healing, and Angiogenesis in Human Cells

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ABSTRACT: Aging is amenable to intervention and prevention by mild stress-induced hormesis. Previously, we have reported that repeated mild heat stress has antiaging and other beneficial effects on growth and a range of cellular and biochemical characteristics of normal human skin fibroblasts and keratinocytes undergoing aging *in vitro*. We have also established a model system of sugar-induced premature senescence in human cells, which can be useful for monitoring the protective and hormetic effects of other treatments. We have now initiated studies on testing the hormetic effects of glyoxal and heat shock on the wound-healing capacity of skin fibroblasts and on the angiogenic ability of endothelial cells. The effects of glyoxal on the extent of wound closure *in vitro* showed a typical biphasic hormetic curve with 20-40% stimulation at lower doses (up to 0.125 mmol) and more than 50% inhibition at concentrations above 0.5 mmol. In the case of angiogenesis by endothelial cells, measured by the standard tube formation assay on Matrigel, a prior exposure to mild heat shock at 41°C for 1 h increased the total tube length and total number of junctions by 30–60% and 10–14%, respectively. In contrast, a severe heat shock at 42.5°C had slightly inhibitory effects on total tube length and the number of junctions. These data add to the ever-growing body of evidence in support of the view that mild stress-induced hormesis can be a useful approach for the modulation, intervention, and prevention of aging and age-related impairments.

KEYWORDS: antiaging; hormesis; hormetin; stress; blood vessels

INTRODUCTION

The phenomenon in which adaptive or homeodynamic responses of cells to low levels of stress result in the improvement of their biological performance,

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in increased stress resistance, and in enhanced survival and longevity is known as hormesis.[1,2] Application of hormesis as an aging–modulatory approach for the prevention and/or slowing down of age-related alterations is gaining wide recognition and acceptance.[3–10] Some of the main hormetic agents used in such studies are exercise, calorie restriction, irradiation, heat stress, hypergravity, ethanol, and pro-oxidants.[10] Furthermore, many different types of experimental systems, such as fruit flies, nematodes, rodents, and cultured cells, are being used for testing the possibilities of hormesis applications in aging research and interventions. For this purpose, we have used the so-called Hayflick system of cellular aging *in vitro* in which serially passaged normal diploid human cells undergo a plethora of cellular and biochemical changes before entering an irreversible state of replicative senescence.[11] In this article, we present a brief review of our previous work and some novel results from the ongoing work on elucidating various hormetic effects and their mechanisms in different types of human cells undergoing aging *in vitro.*

MILD HEAT STRESS HORMESIS IN HUMAN SKIN CELLS

Since 1998, we have published a series of papers reporting the hormetic effects of mild heat shock (HS; 41°C, 1 h, two times per week) on cultured human skin fibroblasts, keratinocytes, and telomerase-immortalized bone marrow mesenchymal stem cells. Briefly, these effects include: (*a*) a reduction in age-related changes in cell morphology,[12,13] (*b*) an increase in cellular replicative life span,[13] (*c*) a reduction in the accumulation of damaged proteins,[14–16] (*d*) an increase in intracellular antioxidative abilities, and (*e*) an increase in resistance to ethanol, hydrogen peroxide, and UV-A irradiation.[17] The main mechanisms involved in bringing about the above beneficial effects of mild HS in fibroblasts require increased levels of various heat shock proteins (HSP),[17] increased proteasomal activities,[18] and efficient stress kinase activation.[13] Similar cellular and biochemical hormetic antiaging effects of repeated exposure to mild HS were observed in normal human epidermal keratinocytes. These effects included maintenance of a relatively youthful cellular morphology, enhanced replicative life span, enhanced proteasomal activity, increased levels of HSP, increased content and Na,K-ATPase activity of the sodium pump, and improved cellular differentiation.[19,20] Additionally, we have also reported that vitamin D-induced differentiation of bone marrow stem cells into osteoblasts could be enhanced by pre-exposure to 1 h HS at 41°C and 42.5°C.[21] We have now extended these studies to test the hormetic effects of HS and sugars on additional parameters of biological activities, such as wound healing by skin fibroblasts and angiogenesis by vascular endothelial cells.

SUGAR-MEDIATED MODULATION OF AGING AND WOUND HEALING

Recently, we have established a novel experimental model system of inducing premature senescence in human skin fibroblasts by treatment with glyoxal, which is a highly reactive intracellular metabolite of glucose.[22] Whereas the long-term aim of setting up this model system of sugar-induced premature aging is to test the antiaging and other modulatory effects of mild and severe stress conditions, certain hormetic effects of glyoxal on wound healing *in vitro* have also been observed.

Briefly, human skin fibroblasts ASF-2 were seeded at a density of about 100,000 cells per well in a six-well plate (growth area per well = 10 cm^2), and were allowed to attach, grow, and make a near-confluent layer in about 24 h.[22] A wound was made by scratching the center of the confluent layer. Scratched cells in the wells were removed by replacing the culture medium with fresh medium containing various concentrations of glyoxal.[22] Phase-contrast photos of live cells in a selected part of the scratched area were taken by using an inverted microscope equipped with a digital camera (Zeiss-Axiovert25; Carl Zeiss, Inc., Jena, Germany). Cells were then incubated at normal culturing conditions of 37°C, 5% CO_2, and 95% humidity. After 24 h, photos were again taken from exactly the same area as before and a composite image composed of at least six overlapping pictures was generated; this represented the wound area (about 3 mm^2). FIGURE 1 shows an example of before and after pictures of wound healing in ASF-2 cells with or without the presence of glyoxal. The lines drawn on the pictures mark the areas which are not occupied by migrating fibroblasts, and these demarcations are used to quantify the extent of wound healing by using an image analysis program known as ImageJ (available from http://rsb.info.nih.gov/ij/). Semiquantitative results are presented in FIGURE 2 as the extent of wound closure in glyoxal-treated cells compared with that in control cells.

ASF-2 cells at population doubling 17 (PD17), which is indicative of about 35% replicative life span completed, show a biphasic hormetic dose response for the effects of glyoxal on wound healing (FIG. 2). Although at concentrations above 0.25 mmol and up to 1 mmol, the wound-healing inhibitory effects of glyoxal were similar to its previously reported growth-inhibitory effects,[22] at lower doses (0.0625 mmol and 0.125 mmol) glyoxal treatment enhanced the wound healing by about 40% and 20%, respectively.

At this stage, it is not clear how low levels of glyoxal enhance wound healing *in vitro* and what are its implications *in vivo*. According to the general hormetic principle, glyoxal-induced macromolecular damage could stimulate counteractive maintenance and repair mechanisms, such as enhanced activity of the protein degradation machinery the proteasome, and an increase in the lysosomal autophagy. There is some evidence that glyoxal treatment stimulates nuclear proteasome activity in human keratinocytes[23] and increases lysosomal

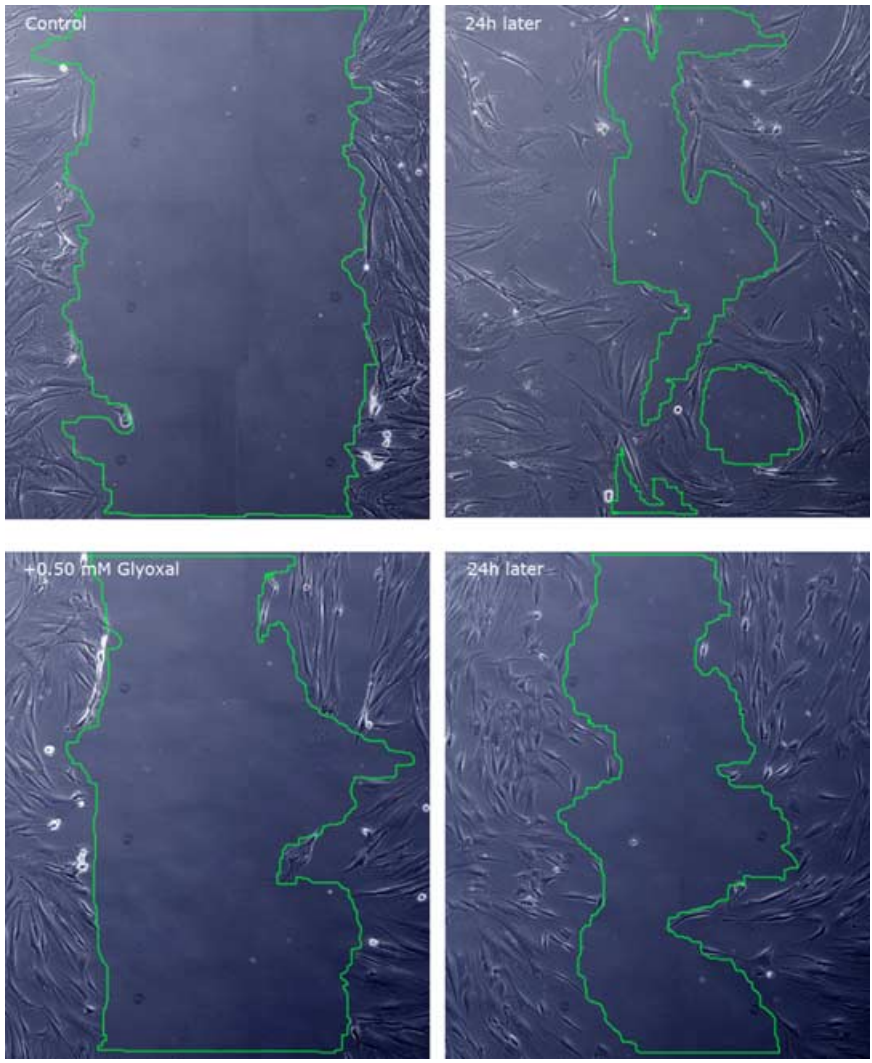

FIGURE 1. Phase-contrast microscopic pictures of early passage human skin fibroblasts during wound healing in the absence or presence of 0.5 mmol glyoxal. Marked areas on pictures indicate the region used to calculate the extent of wound closure after 24 h compared with the empty area immediately after wounding the confluent layer of cells.

activity in human bone marrow stem cells (I. Lundgaard and S. Rattan, personal observations). Our studies to elucidate the hormetic effects of glyoxal on the wound-healing capacity of human skin fibroblasts during aging are in progress. Some preliminary data indicate that late passage ASF-2 cells at PD34, which is about 70% life span completed, show a relatively lesser hormetic response

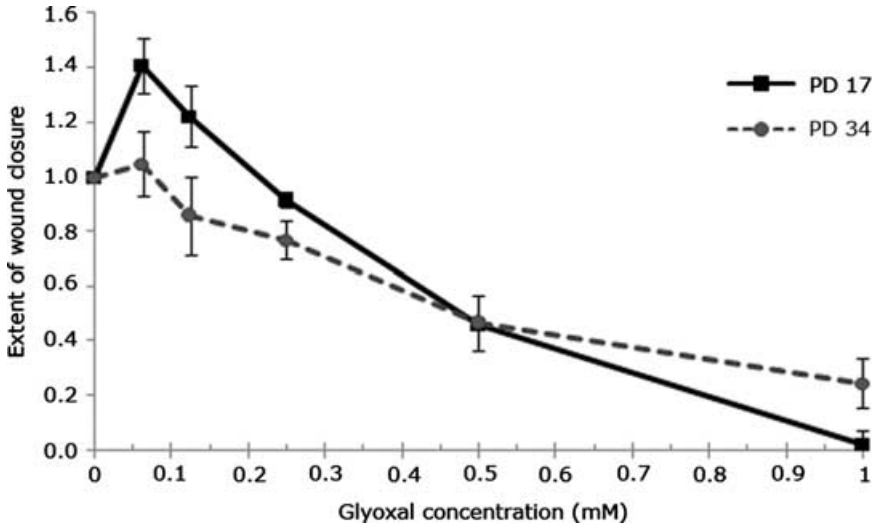

FIGURE 2. Hormetic effects of glyoxal on the extent of wound healing in early passage (PD17) and late passage (PD34) human skin fibroblasts. Data are presented with respect to the extent of wound healing in untreated control cells.

than early passage cells (FIG. 2). These results have implications with respect to the selection of correct timing and age at which cells and organisms could be exposed to a stress in order to achieve beneficial hormetic effects.

HORMETIC MODULATION OF ANGIOGENESIS BY HEAT SHOCK

Angiogenesis is a physiological process involving the growth of new blood vessels from pre-existing vessels, by the active participation of vascular and microvascular endothelial cells. Since there is an age-related decrease in angiogenesis,[24] we have initiated studies to determine the effects of mild and severe stress on angiogenesis *in vitro*, using normal human umbilical vein endothelial cells (HUVEC) undergoing aging *in vitro* and an immortal SV40-transformed human dermal microvascular cell line, HMEC-1. A standardized tube formation assay involving the growth of cells on commercially available basement membrane extract (Matrigel matrix, MM; BD Biosciences, San Jose, CA) was employed to analyze the kinetics and extent of new blood-vessel formation. Briefly, about 40,000 cells per well were seeded in a 24-well plate in which the wells were precoated with 0.2 mL MM forming a uniform layer. Cells were allowed to attach and form tubes by incubating them at 37°C, 5% CO_2, and 95% humidity with 0.5 mL endothelial cell growth medium per well

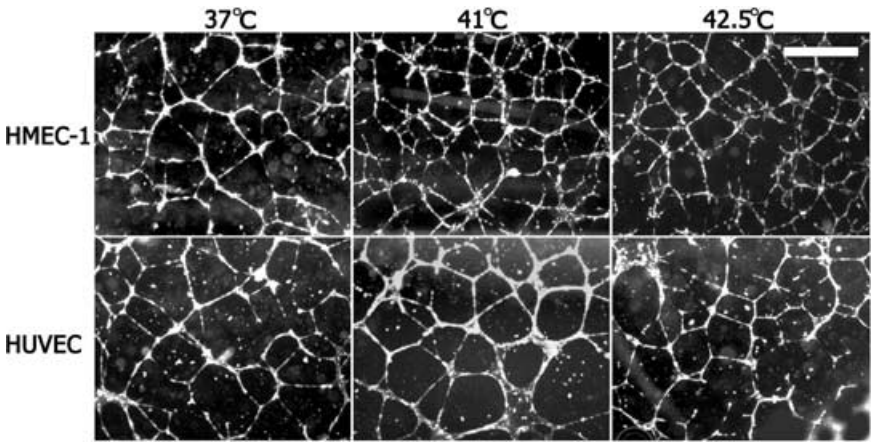

FIGURE 3. Artificially created dark-field illumination microscopic pictures showing the extent of tube formation in 24 h by immortal HMEC-1 and mortal HUVEC (PD10) pre-exposed to mild (41°C) or severe (42.5°C) HS 24 h earlier. (Scale bar: 1 mm.)

(Clonetics, Cambrex Biological Science, Verviers, Belgium). Formation of vascular tubes was monitored at various time points using an inverted microscope, and digital pictures were taken under dark field illumination. Three independent pictures from each well were analyzed for various parameters, such as tube length, tube size, and number of junctions, using the Angio-Quant image analysis program (http://www.cs.tut.fi/sgn/csb/angioquant).[25] For checking the effects of a pre-exposure to mild and severe HS, endothelial cells grown in T75 flasks were given a 1 h HS either at 41°C or at 42.5°C, followed by trypsinization and seeding on MM 3, 6, and 24 h after HS.

Both HMEC-1 and HUVEC, when seeded on MM containing appropriate nutrients and growth factors for angiogenesis, produce tube-like structures starting within 2 h after seeding and continuing for up to 72 h. FIGURE 3 shows representative pictures depicting the extent of tube formation in 24 h by immortal HMEC-1 and mortal HUVEC (PD10, which is about 20% life span completed) with and without a pre-exposure to 1 h HS at either 41°C or 42.5°C, 24 h before seeding. Since after about 36 h of tube formation, there was some debris accumulation in the medium followed by some cell shrinkage, all comparisons and analyses were performed at the 24-h time point.

Pre-exposure of HMEC and HUVEC to 1 h HS at 41°C or 42.5°C, followed by different periods of recovery at 37°C, appears to have hormetic effects with respect to angiogenesis. FIGURE 3 shows that the general extent and quality of the tubes formed by cells pre-exposed to 41°C was better than that in the controls, but a pre-exposure at 42.5°C resulted in a relative worsening of tube structures.

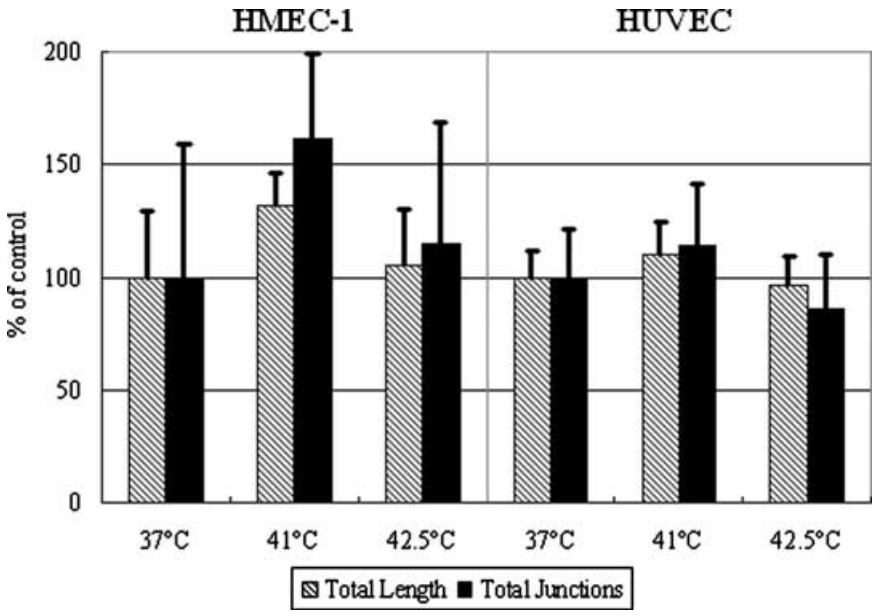

FIGURE 4. AngioQuant-based quantitative comparison of the total tube length and the total number of junctions in HMEC-1 and HUVEC with or without a pre-exposure to mild or severe HS.

FIGURE 4 presents preliminary results of a semiquantitative analysis with respect to total tube length and the total number of junctions made on 8–11 different pictures, using the AngioQuant program. Although there is a large scatter in the results obtained, these data indicate that a pre-exposure of HMEC-1 and HUVEC to mild HS at 41°C improves their tube formation ability *in vitro.* For example, there was a 31.8%–61.4% and 10.5%–13.9% increase in total tube length and the total number of junctions, respectively, in HMEC-1 and HUVEC pre-exposed to 1 h HS at 41°C compared with unstressed cells. In comparison, a pre-exposure to severe stress at 42.5°C did not have any stimulatory effect. Our studies are in progress to find out if the stimulatory effects of mild HS are variable in HUVEC at different PD levels during aging *in vitro*. Furthermore, we aim to elucidate whether the extent of hormetic effects of mild HS on angiogenesis are related to the levels of various HSP synthesized during this period, and what other pathways are involved in this. For example, there is some evidence that HSP90 stimulates tube formation by HUVEC via its role in enhancing the expression of the nitric oxide synthase gene and the production of nitric oxide.[26]

OTHER STRESSES AND FUTURE PERSPECTIVES

So far we have provided several lines of evidence in support of the view that hormesis can be applied successfully to aging research and intervention. This includes the effects of mild HS on various parameters of cellular aging and other functional characteristics, such as our new observations on wound healing and angiogenesis. At the mechanistic level, the induction of HSP as mediators of hormetic effects is only a partial explanation and cannot account for the wide-ranging and long-lasting biological effects.[27] The same may apply to other stresses, such as mechanical stress, irradiation, and intermittent fasting; and also hormesis-inducing molecules, termed *hormetins*, in nutritional components, spices, and natural and synthetic pro-oxidants. Therefore, it is important to determine how various components of the homeodynamic machinery[28] respond and interact during stress-induced hormesis, and how relatively small individual hormetic effects lead to a significant biological amplification that results in an overall improvement of the living system.

The main promise and potential of hormesis as a modulator of aging lies in its mode of action. Since hormetic effects occur by involving a series of molecular and physiological processes, the final target of hormesis is the overall homeodynamic machinery of the living systems. Although hormesis-inducing stress may be targeted at a single pathway, the cascade of biological effects and their amplification results in the modulation and strengthening of the total homeodynamic ability.[10]

The process of aging is primarily characterized by a progressive shrinking of homeodynamic space in terms of increased molecular heterogeneity, which leads to increased vulnerability, onset of diseases, and eventual death.[10] It is also important to realize that the dimensions of the homeodynamic space of an individual are determined by the interacting network of genes, milieu, and chance,[28] which are the basis of the uniqueness of the individual. Since the practical applications of mild stress-induced hormesis are critically dependent on individual variations in stress response, studies to establish the association between stress gene variants and stress response are highly important and informative.[29–31]

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