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REPEATED MILD HEAT SHOCK DELAYS AGEING IN CULTURED HUMAN SKIN FIBROBLASTS

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Summary: The effects of repetitive mild heat shock (30 min, 41° C) on growth and various cellular and biochemical characteristics of human skin fibroblasts undergoing ageing *in vitro* were analysed. Human skin cells not only tolerated more than 30 repeated heat shocks throughout their replicative lifespan, but also maintained several characteristics of young cells until late in life. Whereas the growth rates, population doubling rates, and cumulative population doubling levels achieved *in vitro* remained unaffected, age-related changes in cellular morphology, cell size, cytoskeletal organisation, autofluorescence and neutral β-galactosidase activity were significantly slowed down by repeated mild heat shock. These hormesis-like effects of stress-induced defence processes can be useful to elucidate the role of maintenance and repair mechanisms in ageing.

Key words: anti-ageing, homeostasis, hormesis, gerontogenes

INTRODUCTION

The ability of cells and organisms to respond to stress, the so-called heat shock (HS) response, is a well recognised homeostatic mechanism of cellular defence (1-3). Several studies have demonstrated that the extent of HS response decreases during ageing of cells and organisms (4-8). This altered responsiveness to HS is an important example of a failing homeostatic maintenance and repair process, which underlies the process of ageing (9, 10). Therefore, it has been hypothesised that if organisms are exposed to brief thermal treatment so that their stress response-induced gene expression is upregulated and this particular pathway of maintenance and repair is stimulated, it could delay the onset of various age-related changes. Indeed, anti-ageing and life-prolonging effects of heat shock have recently been reported for *Drosophila* (11, 12) and the nematodes (13, 14).

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In order to see whether the ageing process of human cells can also be modulated by HS, I have tested the effects of mild but repetitive heat shock on various cellular and biochemical characteristics of human skin fibroblasts undergoing ageing *in vitro*. The so-called Hayflick system of normal diploid cells in culture, which demonstrates a wide range of structural, physiological, biochemical and molecular changes during serial passaging, is a widely used experimental model system to study various aspects of cellular ageing *in vitro* (9, 10, 15). The results obtained in the present series of experiments show that repeated mild HS maintains several cellular, biochemical and growth characteristics and delays the onset of various age-related changes in human skin fibroblasts.

MATERIALS AND METHODS

Cell culture. All experiments were performed on a normal human adult female skin fibroblast line designated ASS, which has been used previously to test for the anti-ageing effects of the cytokinin hormone kinetin (16). At least three parallel cultures of control (series A1 to A3) and heat shocked cells (series H1 to H3) were serially passaged at 1:4 or 1:2 split ratio until the end of their proliferative lifespan, using normal culture conditions of medium (DMEM) containing antibiotics, 10% foetal calf serum, and incubation at 37° C with 95% humidity (16). The cells were considered to have reached the end of their proliferative lifespan when, in spite of weekly change of the culture medium, there was no further increase in cell number for more than 5 weeks.

Heat shock protocol. The H-series cells were given a 30 min heat shock at 41° C by immersing the culture flasks in a fine-regulated water bath. The cultures were kept at 37° C for 60 min before changing the medium. HS treatment was repeated twice a week with following restrictions: (a) cultures were not subcultivated within 24 h of HS; and (b) heat shock was not given to newly subcultivated cultures for at least 24 h. Growth rates, population doubling (PD) rates, cell yield and cumulative population doubling levels (CPDL) achieved *in vitro* were determined using standard procedures (16). In addition, morphological characteristics, actin filament organisation, and senescence-specific β-galactosidase staining pattern (17) of normal and heat shocked cells were compared. The extent of HS response in terms of heat shock proteins (HSP) synthesis was checked at various PD levels, by SDS-polyacrylamide gel electrophoresis (PAGE) after labelling the cells with [^{35}S]-methionine as described before (18).

RESULTS AND DISCUSSION

A series of pilot experiments were performed to determine suitable temperature conditions which fulfilled the following criteria: (i) the thermal treatment had no effects on immediate survival of the cells, as checked by the trypan blue exclusion test; (ii) the cells responded to the thermal treatment by inducing the synthesis of

major HSP, as detected by metabolic labelling of cells with radioactive amino acids followed by SDS-PAGE; and (iii) thermally treated cells could be subcultured normally without any effect on their attachment frequency.

The present series of experiments showed that repetitive and mild heat shock had several anti-ageing effects on human cells in culture. Human fibroblasts could be exposed to mild HS at 41° C repeatedly during their limited proliferative lifespan *in vitro* without any apparent negative effects on survival, attachment frequency, PD rates and CPDL potential. Although the temperatures higher than this (up to 43° C) could stimulate a more intense HS response in terms of HSP synthesis, cells could not survive more than 7 repeated thermal treatments. Continuous survival of human skin fibroblasts for 140 days during which time they underwent about 30 PDs and received 35 repeated HS at 41° C is a novel effect not observed before (Fig. 1).

Although there was no prolongation of the proliferative lifespan of human fibroblasts after repeated HS treatment, several other anti-ageing effects were observed. Most dramatically, age-related alteration in the morphology of cells, which is one of the most obvious changes during cellular ageing, was significantly slowed down in heat shocked cells. Fig. 2 (a-c) shows that the control cultures underwent the typical age-related increase in cell size, flattened appearance, increased morphological heterogeneity, loss of arrayed arrangement and increased number of lysosomal residual bodies. In addition, an increased number of highly polymerised actin filaments (19), higher levels of lipid peroxidation-related autofluorescent granules (20), and increased proportion of multinuclear cells was also observed during serial passaging. In comparison, the heat-shocked cultures showed a highly reduced rate of these age-related alterations and maintained a relatively young morphology even at the end of their proliferative lifespan. These cells did not undergo significant enlargement, maintained to a large extent their spindle-shape and arrayed arrangement, did not accumulate much residual bodies and had an almost complete absence of multinucleate cells (Fig. 2 d-f). Furthermore, heat shocked cells had low levels of autofluorescence and did not show many rod-like highly polymerised actin filaments *(pictures not shown)*.

A reduced rate of cell enlargement was also evident from the analysis of cell yield per cm^2 of cell culture flasks, which was reduced from about 4×10^4 cells in young cultures to 1×10^4 cells in senescent control cultures but was maintained at 2

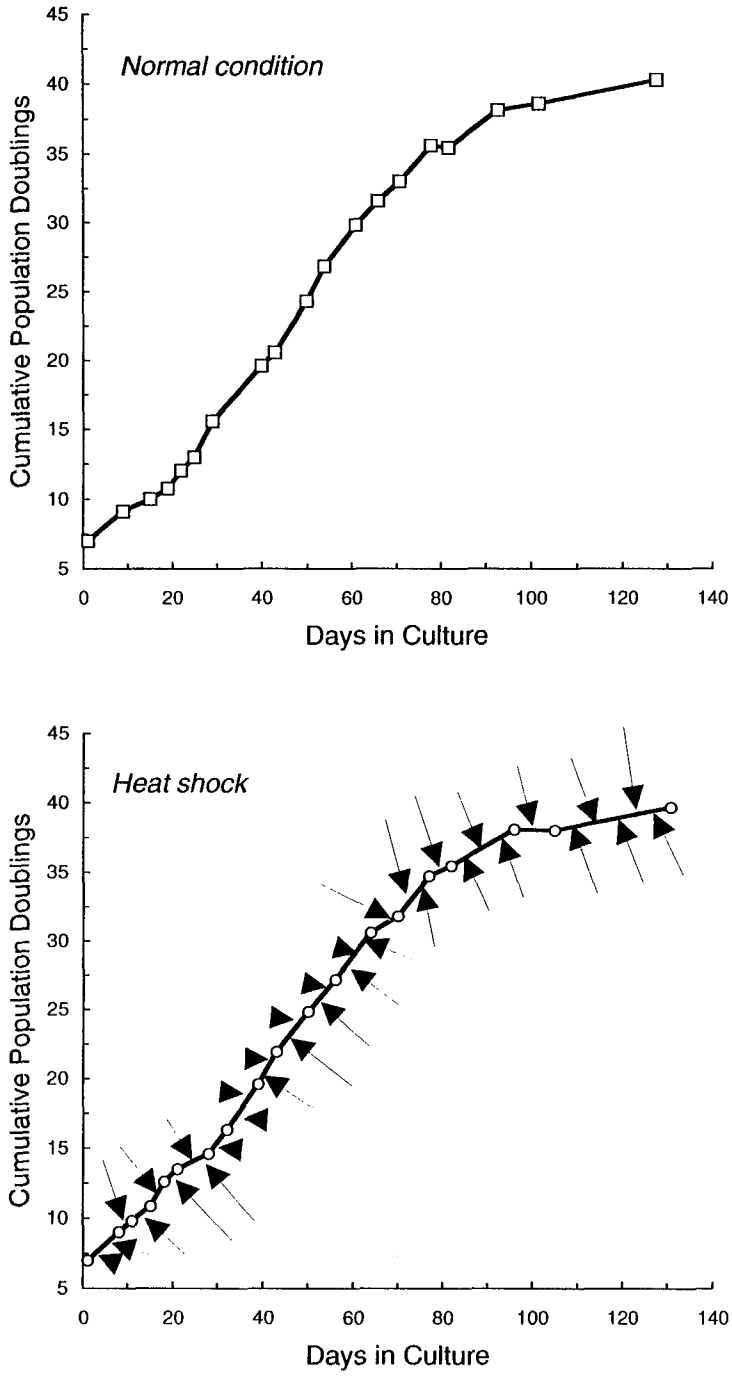

Fig. 1. Longevity curves showing the replicative lifespan in terms of cumulative population doublings for serially passaged human skin fibroblasts at normal culture conditions and after repeated mild heat shocks. Each thick arrow represents one heat shock at 41° C for 30 min followed by normal growth at 37° C.

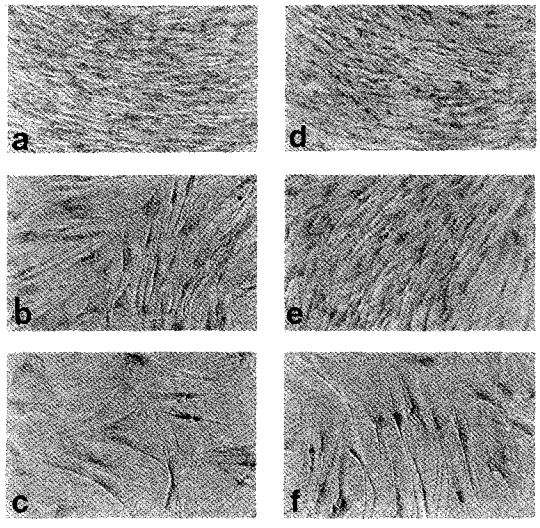

Fig. 2. The effects of repeated mild heat shock on the morphological alterations of human skin fibroblasts undergoing ageing in vitro. Control cells (a - c) at 37° C, and cells repeatedly heat shocked at 41° C (d - f), at various stages during their limited proliferative lifespan. (a, d) 25% lifespan completed; (b, e) 70% lifespan completed, and (c, f) 98% lifespan completed. (microscopic magnification: 50X)

to 3 times higher level (between 2.5 and 3 x 10^4 cells) in repeatedly heat shocked cultures. Maintenance of young morphology and reduced cell size is a strong indication of anti-ageing effects of heat shock, as also observed for other anti-ageing treatments such as carnosine (21) and kinetin (16).

Recently, it has been demonstrated that increased activity of neutral β-galactosidase is an indicator of senescent cells *in vitro* and *in vivo* (17). A comparison of the proportion of β-galactosidase positive cells in HS and control cultures indicates that repeated mild HS on human cells has anti-ageing effects. Whereas more than 95% of cells in late passage control cultures at the end of its proliferative lifespan were β-galactosidase positive, less than 5% of cells in heat shocked cultures were detectable by this marker *(colour pictures not shown)*.

Finally, these experiments show that by upregulating the longevity assurance processes of maintenance and repair, such as the heat shock response, the onset of various age-related changes can be delayed or minimised. Such a phenomenon in which stimulatory responses to low doses of otherwise toxic substances or stressful conditions improve health and enhance lifespan is known as *hormesis* (22, 23). Further

studies are in progress to investigate other cellular, physiological, biochemical and molecular effects of repeated HS in ageing cells. Since HS response is well known for its chaperoning activity through its ability to modulate the stability and turnover of many non-heat-shock proteins (1), it will be important to find out if repeated HS maintains a higher rate of protein degradation reducing thereby the accumulation of altered proteins and the consequent occurrence of other metabolic defects during ageing (9, 10).

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