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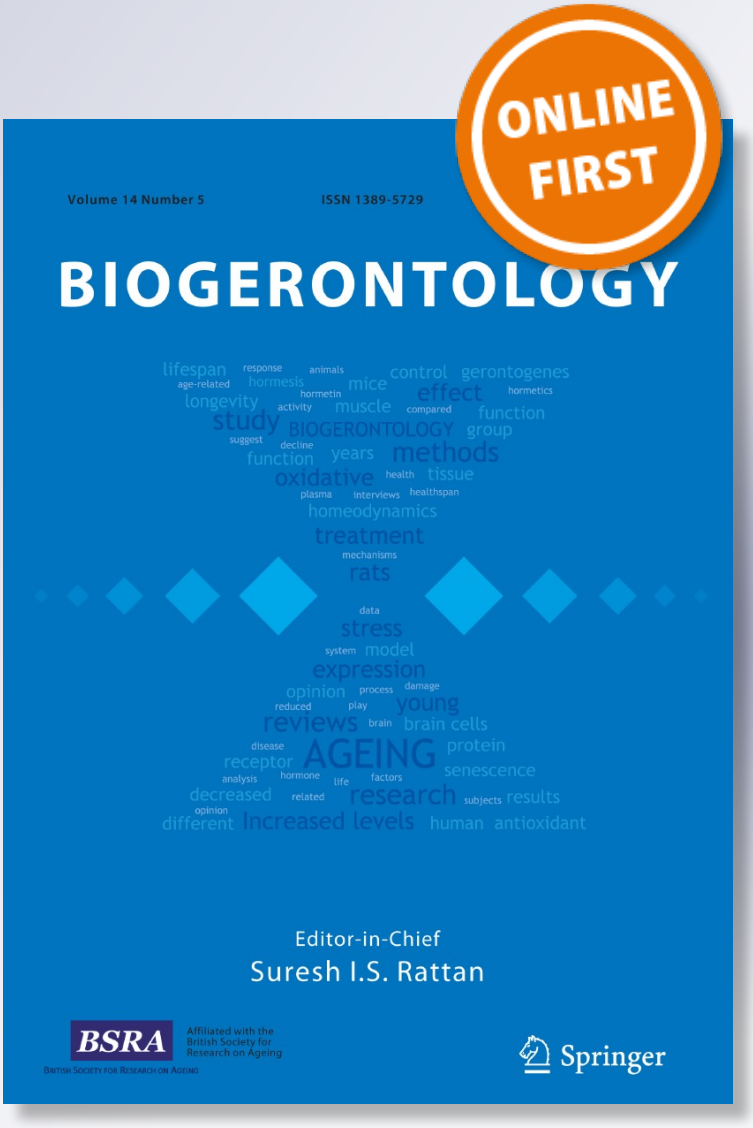

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PRELIMINARY REPORT

Lipid peroxidation-derived 4-hydroxynonenal-modified proteins accumulate in human facial skin fibroblasts during ageing in vitro

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Abstract The reactive aldehyde, 4-hydroxynonenal (HNE), is recognized as a product of lipid peroxidation, which binds to macromolecules, in particular proteins. HNE-modified proteins (HNE-MP) have been shown to accumulate during ageing, generally by using polyclonal antibodies, which increase the possibility of detecting false positives. Therefore, we have used a genuine monoclonal antibody specific for HNE-His adducts of proteins/peptides, which were revealed by immunoblotting method for whole-cell HNE-MP measurements in serially passaged human facial skin fibroblasts undergoing ageing in vitro. There was a significant increase in the levels of HNE-MP in serially passaged cells approaching a near senescent state at high passage level (P-61), as compared with low passage level (P-11) young and middle-aged (P-27) cells. However, if the cells were analyzed soon after re-initiation from the frozen samples with little further passaging, the amount of HNE-MP was low even in relatively high passage level (P-37) cells, which is an indication of selective elimination of cells with high molecular damage during the process of thawing and re-initiation in culture. This pilot study on normal human facial skin fibroblasts shows that HNE-MP detection by monoclonal antibody-based dot blot method can be used as a marker for age-related accumulation of lipid peroxidative molecular damage, and could be useful for testing and monitoring the effects of potential skin care products on ageing parameters.

Keywords Ageing · Intervention · Senescence · Lipid peroxidation · Molecular damage

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Introduction

Molecular damage in biological systems is caused by both internal and external sources, including reactive oxygen species (ROS), other free radicals, glucose metabolites and biochemical infidelity (Rattan 2006). One of the ROS-mediated products of lipid peroxidation is the reactive aldehyde 4-hydroxynonenal (HNE), which can further propagate oxidative damage (Zarkovic et al. 2013). HNE is generated by peroxidation of polyunsaturated fatty acids (PUFA), such as arachidonic acid and linoleic acid, and can covalently bind to and modify proteins through their cysteine,

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histidine, and lysine moieties (Siems and Grune 2003; Wildburger et al. 2009). The high lipophilicity of HNE means that it primarily reacts with proteins and phospholipids in the membrane (Guichardant 2002), but may also travel far away from the site of production, thereby making it potentially more dangerous than ROS. HNE is regarded as a marker of lipid peroxidation, and HNE-modified macromolecules are known to affect several cellular functions and interactions, including inhibition of DNA, RNA and protein synthesis, cell cycle arrest, mitochondrial dysfunction, protein aggregation, and even cell death (Negre-Salvayre et al. 2010; Zarkovic 2003; Zarkovic et al. 2013). Because HNE can cause such debilitating damage to cells and tissues, it is considered to play a major role in ageing and several age-related pathologies including neurodegenerative diseases, autoimmune diseases and bone diseases (Zarkovic et al. 2013; Dalleau et al. 2013).

Ageing of cells in vitro, also known as the Hayflick phenomenon, is a well-established and well-studied phenomenon, which has been instrumental in the elucidation of molecular basis of cellular ageing and its implications in health and diseases (Rattan 2012a). Accumulation of macromolecular damage is one of the hallmarks of ageing of cells, tissues, organs and organisms (Rattan 2008a; Nedić et al. 2013). HNE-modified proteins (HNE-MP) have been reported to accumulate during ageing in animals and humans (Levine and Stadtman 2001), as well as in serially passaged human keratinocytes and embryonic lung fibroblasts undergoing ageing in vitro (Ahmed et al. 2007; Petropoulos et al. 2000). However, the method of detection used in those reports generally employed using polyclonal antibodies to target HNE-MP, thus increasing the possibility of detecting false positives. Therefore, in this study we have used a monoclonal antibody and a relatively simple dot-blot method for whole-cell HNE-MP measurements, which has been successfully used before in detecting endogenous HNE in various biological samples (Spoljaric et al. 2011; Waeg et al. 1996; Weber et al. 2013). This method takes advantage of the fact that all modified proteins, whether membrane-bound or soluble, can be trapped on a nitrocellulose membrane allowing for whole-cell HNE-MP quantification.

Here we report the results of our pilot study showing increased levels of HNE-MP in human facial skin fibroblasts undergoing ageing in vitro, which have implications for using HNE-MP as a marker for testing novel skin care interventions.

Materials and methods

Cell culture

Normal diploid skin fibroblast cell strain, designated FSF-1 (provided by LVMH Research, St. Brays, France), was derived from the facial skin of a healthy middle-aged woman. FSF-1 cells were cultured and maintained by serial passaging throughout their proliferative lifespan of about 65 population doublings in vitro (Demirovic and Rattan, in preparation). Standard culturing conditions for FSF-1 consisted of complete culture medium comprising Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10 % foetal calf serum (FCS; Biological Industries, Beit Haemek, Israel), and 100 U/ml penicillin and streptomycin (Biowhittaker), and incubated at 37 °C, 5 % CO_2 and 95 % humidity. Cell samples stored in liquid nitrogen were thawed rapidly and seeded into a T75 plastic flask (COSTAR, Cambridge, MA, USA) along with 10 ml complete media and left undisturbed for about 12 h. The culture medium was then replaced with fresh complete media, and the cells allowed to proliferate until ready for splitting at near confluence. Cultures were serially passaged by trypsinizing each time they became 80–90 % confluent, splitting them at 1:2 or 1:4 ratio into new T75 cell culture flasks, adding 1 or 2 passage (P) number(s), respectively, to the in vitro age of the culture. All experiments were performed under sterile conditions in a laminar flow hood.

Dot-blot analysis of HNE-MP

Samples for the dot-blot analysis were prepared by scraping the cells from near confluent T75 flasks, in triplicate, at various P levels (P-11, 27, 37 and 61), in ice-cold PBS and stored at −80 °C. Cell lysates were prepared by a freeze/thaw cycle. The method for HNE determination was as described before (Weber et al. 2013). Briefly, prior to the dot-blot analysis, protein content was measured in each sample by the Bradford method (Bradford 1976) and protein concentration was adjusted to 0.25 mg/ml. For the application of proteins to nitrocellulose membrane, the appropriate

size of the membrane was first pre-wetted in distilled water for 5 min and assembled in the 96-well Bio-dot apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Then, the membrane was washed twice with PBS, followed by loading of 100 µl of each sample into the wells and second washing with PBS. After each step, the membrane was drained by vacuum-assisted microfiltration. Following the application, the apparatus was disassembled and the membrane was blocked in 2 % (w/v) non-fat dry milk in PBS for 1 h at room temperature (RT), and incubated overnight with mouse monoclonal antibody 1g4 directed against HNE-histidine epitope diluted 1:10 (Waeg et al. 1996). Next day, the membrane was incubated with 3 % H_2O_2 to block endogenous peroxidases, washed 4 × 5 min in PBS, followed by the incubation with the secondary antibody diluted 1:25 (EnVision; Dako North America, Carpinteria, USA) for 1 h at RT and with the second washing of 4 × 5 min in PBS. Immune complexes were visualized using 3,3′-diaminobenzidine staining (DAB; Dako North America, Carpinteria, USA), and scanned for signal quantification. Signals were analyzed in ImageJ, a Java-based image-processing program. The data were recalculated from the standard curve prepared with HNE-bovine serum albumin (BSA) standards (0.25 mg/ml) spiked with HNE-protein adducts ranging from 0 to 2,000 pmol/mg protein, and were expressed as pmol of HNE-MP/mg of protein.

Statistical analysis

Dot-blot analysis was performed in triplicates and repeated three times. Values were expressed as mean ± SD. Comparisons were assessed by a two-tailed Student's *t* test considering values of $p < 0.05$ as significant.

Results and discussion

FSF-1 cells used in this study were analyzed at 4 age-points during their limited proliferative lifespan in vitro of P-65, which is here considered as 100 % lifespan completed. Figure 1 shows the phase-contrast microscopic pictures of the cells at P-11, 27, 37 and

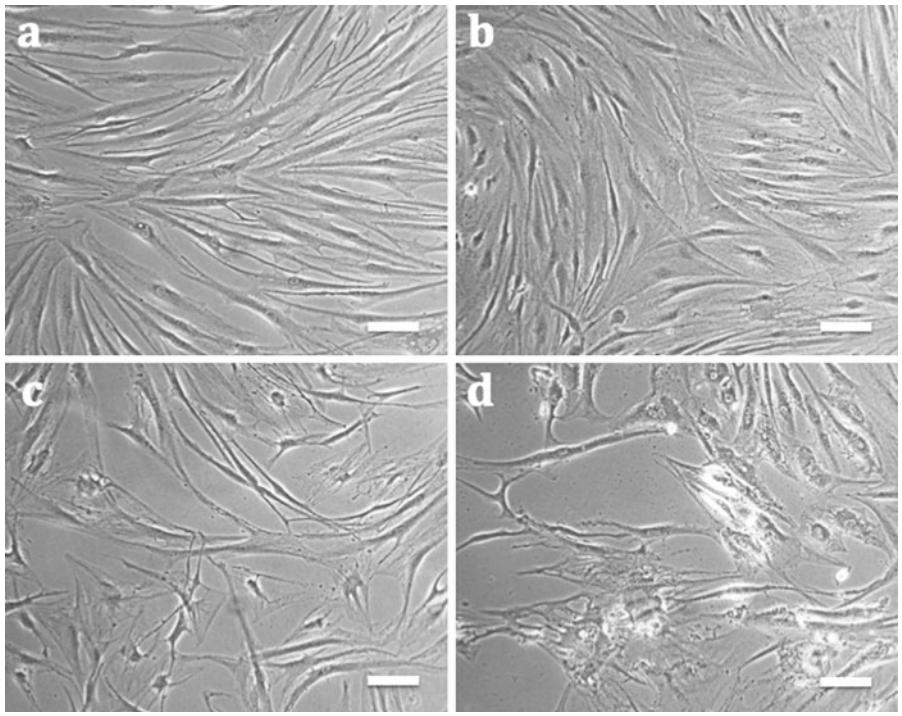

Fig. 1 Phase-contrast microscopic pictures of the serially passaged facial skin fibroblasts FSF-1 used in this study. **a** P-11 young (17 % lifespan completed), **b** P-27 early middle-aged (42 % lifespan completed), **c** P-37 late middle-aged (57 % lifespan completed), and **d** P-61 near senescent cells (94 % lifespan completed); *scale bar* = 100 µm

61, which were equivalent to 17, 42, 57 and 94 % lifespan completed, respectively. Since some of the cell cultures were re-initiated from the frozen samples, the exact numbers for their extent of serial passaging were 3, 19, 3 and 31, respectively. Therefore, for data presentation and analysis, cells with <30 % lifespan completed were considered as young cells, those between 30 and 60 % lifespan completed were considered as middle-aged cells, and those with >90 % lifespan completed were considered as late-passage near senescent cells.

Figure 1 shows that FSF-1 cells at different passage levels had the typical morphological features of serially passaged ageing cells, as described for the Hayflick system (see Rattan 2012a). The main morphological features of ageing cells include increase in cell size, change of shape from thin, long and spindle-like to flattened and irregular, loss of whorl-like spiral arrangement in parallel arrays on the cell culture substrate, increased number of vacuoles and dense lysosomal autophagous bodies containing ultraviolet (UV)-fluorescent age-pigments such as lipofuscin, polymerized actin filaments, and disorganized microtubules in the cytoskeleton. There are numerous other physiological, biochemical and molecular changes known to occur during serial passaging leading to the ultimate replicative senescence of normal diploid cells (Rattan 2012a).

The results of a dot-blot analysis of HNE-MP in FSF-1 cells undergoing ageing in vitro are shown in Fig. 2. The dot-blot traps the entire proteome in one dot for easy quantification, and because of its simplicity the method is quick and reliable (Spoljaric et al. 2011). Figure 2a (top row) shows the detection of HNE-MP in a serially diluted HNE-BSA standard sample with six concentrations (from 0 to 2,000 pmol HNE-BSA/mg protein), and the four cell samples (P-11, 27, 37 and 61) in triplicates coming from three independent flasks. There was a general increase in the amounts of HNE-MP in serially passaged FSF-1 cells. An interesting and important observation made in this study was that the accumulation of HNE-MP during ageing was best seen in serially passaged cells and not in re-initiated cultures from liquid nitrogen frozen samples (also see below). The possibility that the freezing and thawing process could influence the amounts of HNE-MP detected in the cells was apparent by including the P-37 cells in the experiment without any further serial passaging in vitro. Figure 2a shows that P-37 cells had a significantly lower amount (670 pmol/mg protein) of HNE-MP than P-11 cells (771 pmol/mg protein; $p < 0.05$). The most probable reason for this may be the selective elimination of high HNE-MP containing cells during thawing and re-initiation of the culture. This is well known that only about 50 % cells attach to the culture flask growth surface on thawing and reculturing, and these surviving cells may be the healthiest with lowest levels of molecular damage. Therefore, in our analysis of the age-related changes in the levels of HNE-MP, we have omitted the inclusion of data from P-37 cells, which had undergone only three passages after reculturing (Fig. 2b).

Figure 2b presents the quantitative data for the three age groups (young, middle-aged and senescent), showing that there was a significant increase of about

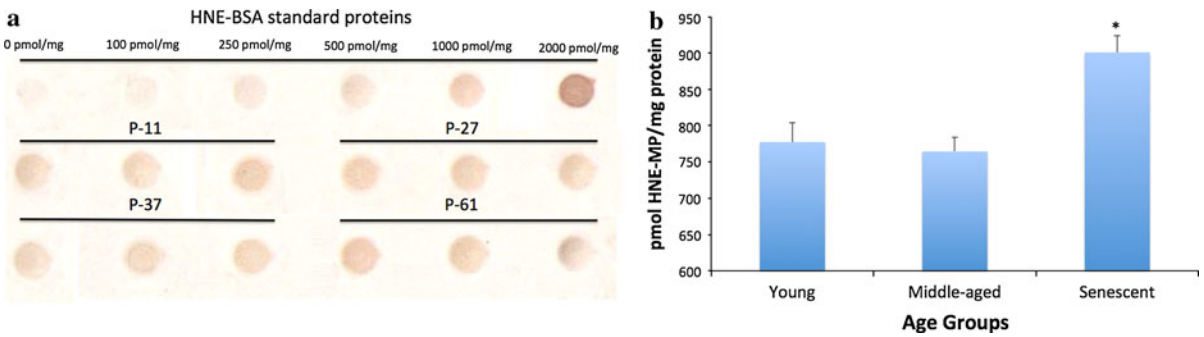

Fig. 2 A dot blot analysis of the differently passaged FSF-1 cells. **a** A scan of the nitrocellulose membrane dot blot analysis of FSF-1 cells in triplicates at P-11, 27, 37 and 61, with standard HNE-modified bovine serum albumin, concentration ranging from 0 to 2,000 pmol HNE-BSA/mg protein. The exact serial passages in vitro before collecting the samples were 3, 19, 3 and 31, respectively; **b** quantification of levels of HNE-modified protein (HNE-MP) in FSF-1 cells at three age groups, with significant difference between young and senescent cells (*$p < 0.01$)

17 % in the amount of HNE-MP between young/middle-aged (771 pmol/mg protein) and senescent cells (901 pmol/mg protein; $p < 0.01$). It should be noted that the detectable levels of HNE-MP were present even in early passage young and middle-aged cells, but the level increased in late passage cells approaching replicative senescence. These observations are in agreement with earlier reports on the accumulation of macromolecular damage, including HNE-MP, in cells undergoing ageing. Furthermore, the range of HNE-MP detected by our method, using a genuine monoclonal antibody, is comparable to a previous report (Petropoulos et al. 2000), underlining the validity of this method for the quantification of HNE-MP in ageing human cells.

Although the biological significance of increased levels of HNE-MP is yet to be fully understood, these adducts are known to inhibit proteasomal activity (Ahmed et al. 2007). This inadvertently results in the accumulation of HNE-MP leading to the elevation of oxidative stress. Furthermore, the ability of cells to defend themselves against additional HNE-induced oxidative stress is reduced, since the enzyme known to neutralize HNE-MP adducts can be deactivated after prolonged exposure to them (Riahi et al. 2010). Consequently, this could lead to the further build-up of HNE-MP, as seen in this study, which again, in a cyclic manner, reduces the cells' defense and, along with the accumulation of other forms of damage and stressors, can ultimately lead to cell senescence or death. Therefore lipid peroxidation and consequently accumulation of HNE-MP are known contributors in pathologies of certain diseases and ageing (Wildburger et al. 2009).

Our pilot study on determining the levels of HNE-MP in human cells by using a specific monoclonal antibody can be the basis for using this method as a marker for various oxidative damage-related diseases, and for screening and testing potential ageing interventions. For example, natural or synthetic compounds can be tested for their potential health beneficial effects in terms of their ability to reduce or prevent the accumulation of HNE-MP and/or enhance their removal during ageing. Similarly, this method can be used to distinguish between the beneficial and harmful effects of mild and severe stress, respectively, as proposed by the theory of hormesis (Rattan 2008b, 2012b). HNE-MP in human facial skin fibroblasts could be used as a marker for normal and sunlight-induced photo-ageing, and can be useful for testing and monitoring the effects of potential skin care products on molecular parameters of ageing (Rattan et al. 2013).

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