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Hormetic modulation of differentiation of normal human epidermal keratinocytes undergoing replicative senescence *in vitro*

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ABSTRACT

Normal human epidermal keratinocytes (NHEK) show both the Hayflick phenomenon of replicative senescence and differentiation *in vitro*, depending upon the culture conditions. Using this experimental model system, we have studied age-related changes in the ability of serially passaged NHEK to enter into differentiation in the presence of calcium, as measured by the levels of differentiation markers involucrin, p38 and Hsp27. The results obtained in these studies show that calcium-induced differentiation of NHEK becomes progressively delayed during cellular aging *in vitro*, which can be modulated by treatments such as mild heat stress, kinetin and curcumin. Whereas all these treatments on their own were able to increase the levels of various differentiation markers to varying extents, their effects were synergistic and rapid in the presence of calcium. Furthermore, all three modulators tested in the present study bring about their effects by inducing stress response pathways in terms of an increase in the levels of stress proteins Hsp90, Hsp70 and heme-oxygenase-1 (HO-1), which is indicative of stress-induced hormesis bringing about the biologically beneficial effects.

1. Introduction

The skin, especially the epidermis as the borderline to the environment, is dependent on its highly controlled homeodynamic property critically balancing between proliferation, differentiation, desquamation and apoptosis. This balance is progressively impaired during aging. For example, intrinsically aged skin is thin, pale, wrinkled and less elastic, and has flattened dermal–epidermal junction (Yaar and Gilchrest, 2001a,b). One of the main reasons for this appears to be the decline both in the proliferative and differentiation potential of keratinocytes due to altered responsiveness to growth factors, hormones and cytokines (Norsgaard et al., 1996; Yaar and Gilchrest, 2001a,b). Since keratinocytes inhere the most important role in maintaining protection and functionality of the epidermis, *in vitro* cultures of keratinocytes can serve as a good experimental model mimicking cellular aging and differentiation.

It has previously been shown that normal human epidermal keratinocytes (NHEK), serially passaged in a proliferative mode in a low-calcium medium, undergo the typical Hayflick phenomenon of cellular aging and replicative senescence involving irreversible cell cycle arrest and other phenotypes of cellular aging (Norsgaard et al., 1996). Furthermore, although aging NHEK can be induced to undergo differentiation by the addition of calcium, the induction of differentiation becomes progressively reduced in NHEK undergoing cellular aging *in vitro* (Gandarillas, 2000; Kang et al., 2000; Norsgaard et al., 1996). In the present study, we have tested a variety of physical and chemical agents to modulate the process of differentiation in serially passaged NHEK and to find out what interventions may be effective for maintaining or for improving the functional abilities of such cells during aging. As modulators of differentiation, mild heat stress (HS), curcumin, and kinetin were chosen due to the following reasons.

Previously, we have demonstrated the beneficial and anti-aging effects of mild HS on human cells. Such effects of mild stress represent the phenomenon of hormesis, which is considered to be potentially a powerful approach in aging research and interventions (Rattan, 2004, 2008). The hormetic effects of mild HS on human skin fibroblasts included the maintenance of youthful morphology, reduced accumulation of damaged proteins, increased levels of various heat shock proteins (Hsp), increased proteasomal activities, increased antioxidative abilities, increased resistance to ethanol, hydrogen peroxide and UV-A irradiation, and maintenance of the stress kinase responses (Beedholm et al., 2004; Fonager et al., 2002; Nielsen et al., 2006; Rattan, 1998; Verbeke et al., 2000, 2001a, 2002). Several of the above anti-aging effects of repeated mild HS have also been observed for NHEK during serial passaging (Rattan and Ali, 2007). With respect to differentiation, we have reported that vitamin-D-induced differentiation of bone marrow stem cells into osteoblasts can be enhanced by pre-exposure to mild HS (Nørgaard et al., 2006).

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In the present study, we present novel data for the differentiation-modulatory effects of mild HS on NHEK undergoing aging *in vitro*. In addition, we have also tested the effects of a cytokinin, N^6-furfuryladenine or kinetin, which is known to have several anti-aging effects on human skin fibroblasts (Rattan and Clark, 1994), and promotes the differentiation of NHEK (Bolund et al., 1991). Furthermore, we have tested the effects of curcumin as a modulator of differentiation of NHEK, since we have observed the hormetic effects of curcumin as a stimulator of proteasomal activity in NHEK (Ali and Rattan, 2006).

2. Materials and methods

2.1. Serial passaging, proliferation and viability

Primary cultures of NHEK were established and maintained in a proliferative mode by using low-calcium EpiLife medium (Cascade Biologics, Mansfield, UK), as described previously (Ali and Rattan, 2006; Berge et al., 2006, 2007). Two frozen ampoules of NHEK, designated K5 cell line, were thawed at passages 5 and 6 and were separately serially passaged at a split ratio of 1:2 until they became replicatively senescent after 21 passages during a period of 160 days. A combined assay for cell proliferation and viability was performed as a modified BrdU-ELISA (BrdU Cell Proliferation ELISA (colorimetric), Roche Applied Science, Switzerland), with an integrated MTT reduction step. Protein content was determined by Lowry test (Dc Protein Assay Reagent A, S and B: Bio-Rad Laboratories, Hercules, Canada) with BSA as a standard. As an aging marker, the chymotrypsin activity of the proteasome was determined in cell extracts as described earlier (Ali and Rattan, 2006; Beedholm et al., 2004; Fonager et al., 2002).

2.2. Differentiation and other markers

Involucrin, Hsp27, and p38 were selected as the markers of differentiation induced by the addition of 1.2 mM calcium to the culture medium for NHEK (Duverger and Morange, 2005; Eckert et al., 2003, 2004). Other conditions used to test their effects on differentiation of NHEK with and without added calcium were: HS at 41 °C given for 1 h, 1 μM curcumin (Ali and Rattan, 2006), and 80 μM kinetin (Rattan and Clark, 1994). In the case of mild HS, calcium treatment was started 6 h after HS.

The quantification of differentiation markers was performed by ELISA, as described (Berge et al., 2006). Following antibodies obtained from Nordic Biosite, Sweden were used for various proteins: spa800 for Hsp27, spa810 for Hsp70, spa840 for Hsp90 and osa110 for heme-oxygenase-1 (HO-1). Other antibodies used were: ab14504 for involucrin (Abcam, Cambridge, UK); sc535 for p38 and sc99 for p53 (Santa Cruz Biotechnology, Santa Cruz, USA).

3. Results and discussion

3.1. Replicative senescence and calcium-induced differentiation

NHEK can be serially passaged for a limited number of times by maintaining the cells in a low-calcium containing medium, which ultimately results in their irreversible cell cycle arrest and replicative senescence (Norsgaard et al., 1996; Yaar and Gilchrest, 2001a). This process of cellular aging and replicative senescence *in vitro* is distinct from differentiation of NHEK, which can be induced at any stage by providing appropriate conditions such as high calcium (Gandarillas, 2000; Kang et al., 2000; Norsgaard et al., 1996).

In the present series of experiments, NHEK serially passaged at a split ratio of 1:2 achieved a cumulative population doubling (CPD) level of 21 in about 160 days. Further characterization of replicative senescence of NHEK was confirmed by using standard parameters of cellular aging *in vitro*, such as growth curves, longevity curve, morphology and the presence of more than 95% cells positive for senescence associated β-galactosidase staining. In addition, other parameters of cellular aging in terms of age-related decrease in the proteasomal activities (Brégégére et al., 2003, 2006; Petropoulos et al., 2000), and increased levels of oxidatively damaged glycated proteasomal subunit α7 (Gonzalez-Doasal et al., 2006) was tested and was found to be consistent with earlier studies on aging cells (data not shown).

In order to facilitate comparison of data among several independent experiments, the results are presented by adhering to the following nomenclature derived after one round of complete serial passaging: (a) MP: mid-passage cultures comprising samples from passages 11 to 15, which is 52–71% lifespan completed (LSC); (b) LP: late-passage cultures comprising passages 17 and 18 (80–86% LSC); and (c) SN: senescent cultures from the terminal passage 21 (100% LSC).

Fig. 1 presents novel data on the extent of calcium-induced differentiation in NHEK in three age groups, using involucrin, Hsp27, and p38 as reliable markers of differentiation (Eckert et al., 2003, 2004). All data are presented in comparison with the basal levels of these markers in undifferentiated cells, which were converted to 1 arbitrary unit (a.u.). Whereas MP-NHEK up to about 70% LSC rapidly entered differentiation in terms of the induction of involucrin (1.5-fold increase), p38 (2-fold increase), and Hsp27 (5.5-fold

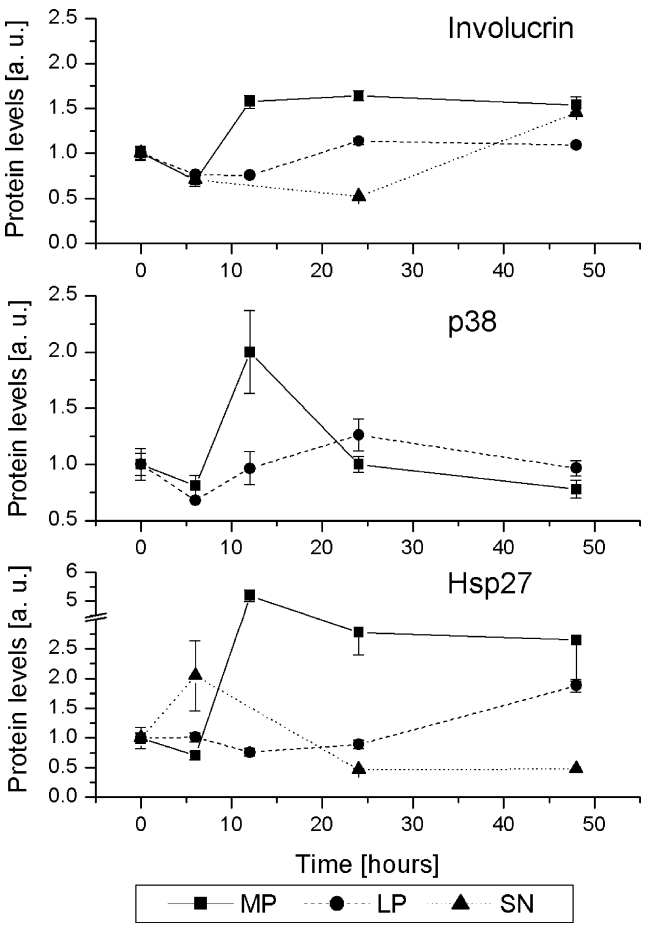

Fig. 1. Protein levels (±SD) of involucrin, p38 and Hsp32 during calcium-induced differentiation in normal human epidermal keratinocytes (NHEK). Data are presented as arbitrary units (a.u.) relative to the basal levels at the time of treatment. Each experiment was performed in triplicate samples prepared from serially passaged long-term cultures of NHEK.

increase) reaching peak values at about 12 h after the addition of 1.2 mM calcium in the culture medium, both late passage (LP) and senescent (SN) NHEK were much slower to enter differentiation. It is generally not possible to directly correlate the amount of a particular protein and the level of differentiation, and different proteins may be induced to different extents as shown in Fig. 1. However, for the purposes of data comparison, an increase of at least 25% and more in the level of a particular protein is considered as indicative of induction of differentiation.

Interestingly, SN cells were much slower in the induction of involucrin as compared with MP cells, but they could reach the same levels of induction in 48 h (Fig. 1). In comparison, the induction of p38 and Hsp27 was significantly slower and occurred to a lesser extent in LP and SN cells (Fig. 1). These results showed that the ability of NHEK to enter differentiation was significantly reduced during serial passaging and on becoming senescent. These data also showed that serial passaging of NHEK was not accompanied by spontaneous differentiation, since no differences in the basal levels of the three markers of differentiation were observed in the absence of high calcium. This provides further evidence in support of the view that serial passage-related cellular aging and calcium-induced differentiation of NHEK are two separate phenomena (Gandarillas, 2000; Kang et al., 2000; Norsgaard et al., 1996).

An unexpected observation in the present studies was the transient induction of three stress proteins, Hsp90, Hsp70 and heme-oxygenase-1 (HO-1), during calcium-induced differentiation of NHEK. Fig. 2 shows the changes in the levels of these proteins within 12–24 h. Whereas the levels of Hsp70 did not show much alteration during the period of NHEK differentiation, increased levels of Hsp90 and HO-1 indicate that differentiation process also induced a kind of intrinsic stress response in NHEK. Furthermore, this stress response at the level of Hsp90 was reduced in senescent cells as compared with mid-passage cells, which is in line with the general observations on the slowing down of differentiation and reduced stress response during cellular aging (Verbeke et al., 2001b; Yaar and Gilchrest, 2001a,b). However, in the case of HO-1, senescent cells showed a delayed response, whose biological relevance is not clear in the context of induction of differentiation.

In preliminary studies it was observed that calcium-induced differentiation in mid-passage NHEK was accompanied by a 30% increase in the chymotrypsin activity of the proteasome. However, this differentiation-related increase in proteasomal activity occurred to a much lower extent (less than 5%) in senescent cells which already had more than 80% less activity of the proteasome (data not shown). Although further studies are required to reach at any definitive conclusions, these preliminary results indicate that the age-related slowing down of differentiation of NHEK could be due to altered signaling and reduced responsiveness in terms of less induction of stress proteins Hsp70 and Hsp90, and lesser stimulation of proteasome during aging.

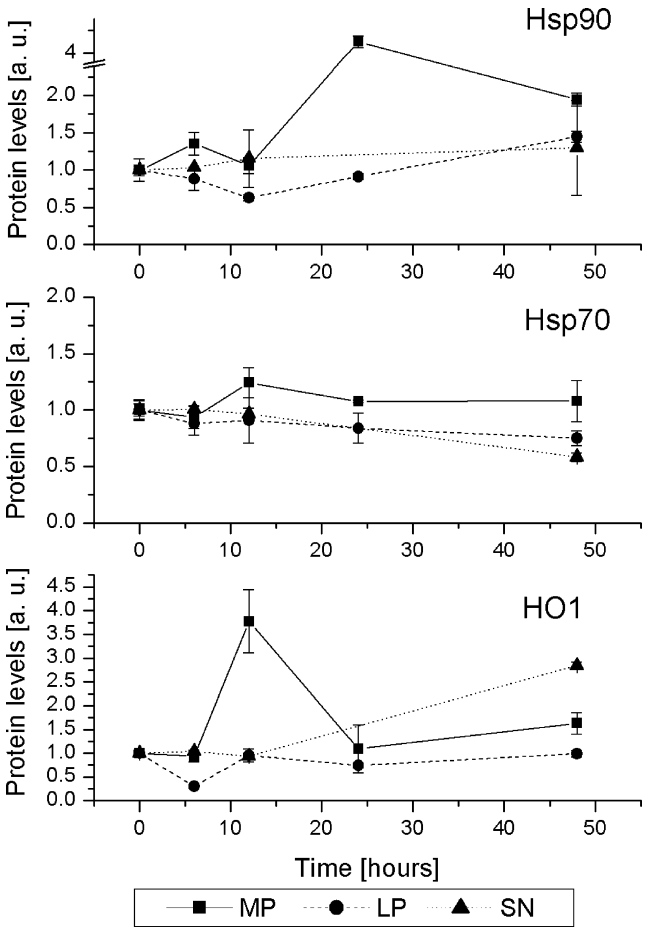

Fig. 2. Protein levels (±SD) of Hsp90, Hsp70 and HO-1 during calcium-induced differentiation in normal human epidermal keratinocytes (NHEK). Data are presented as arbitrary units (a.u.) relative to the basal levels at the time of treatment. Each experiment was performed in triplicate samples prepared from serially passaged long-term cultures of NHEK.

3.2. Modulation of NHEK differentiation

After establishing the criteria for calcium-induced differentiation in NHEK as described above, we tested various potential modulators of differentiation. First, we tested the effects of kinetin, which has been reported to have several anti-aging effects in skin fibroblasts (Rattan and Clark, 1994), antioxidative and longevity promoting effects in insects (Sharma et al., 1995, 1997), and differentiation-inducing effects in keratinocytes (Bolund et al., 1991). The results of those studies on kinetin-induced differentiation of NHEK undergoing aging were published previously (Berge et al., 2006), and had shown that although 40–80 μM kinetin on its own could induce some differentiation in NHEK, the effects of kinetin in the presence of calcium were additive, and may have beneficial applications *in vivo*.

Another condition that can be a potential modulator of NHEK differentiation is mild thermal stress in terms of single or repeated HS at 41 °C for 1 h. The reason for selecting this as a potential modulator of NHEK differentiation is our series of observations on the beneficial and anti-aging hormetic effects of mild HS on human skin fibroblasts and NHEK (see Section 1). In addition, we have used 1 μM curcumin, which has also been shown to have hormetic effects on stimulating proteasomal activities in NHEK (Ali and Rattan, 2006; Rattan and Ali, 2007). A summary of the results of studies on the effects of the treatment with various modulators of differentiation in MP-NHEK are given in Table 1. The data are presented as a generalized trend in terms of average change in percent, representing a ±range of less than 5–7%. However, no statistical analyses could be performed due to the limited number of independent experiments, and so these results should be considered as preliminary at this stage.

These data show that mild HS, curcumin and kinetin could induce differentiation in MP-NHEK, although the extent of induction was generally lesser than that by calcium. However, if different markers of differentiation are compared individually, then differentiation-related increase in involucrin levels was quite similar among various treatments, but the other two markers had a lesser induction. The physiological significance of these variable responses is not clear at present, and may reflect differences in the general quality of differentiation induced by different stimuli,

Table 1
Changes in the protein levels of differentiation markers in mid-passage normal human epidermal keratinocytes after treatment with potential modulators

Differentiation marker	Treatment			
	Calcium (%)	Mild heat stress (%)	Curcumin (%)	Kinetin (%)
Involucrin	+64.4	+75.7	+49.9	+69.2
p38	+100	−7.4	+8.6	+13.6
Hsp27	+418.4	+81.9	+98.9	+99.0

Table 2
Changes in the protein levels and in the kinetics of differentiation markers in late-passage normal human epidermal keratinocytes after treatment with potential modulators in the presence of calcium

Differentiation marker	Treatment					
	Mild heat stress		Curcumin		Kinetin	
	Level (%)	Time-shift (h)	Level (%)	Time-shift (h)	Level (%)	Time-shift (h)
Involucrin	+72.1	−6	+71.9	−18	+72.6	−18
p38	+3.7	+12	+31.8	+18	+76.4	+24
Hsp27	+441.3	−6	+133.3	−18	+387.3	−6

which requires further investigations. Furthermore, induction of differentiation by mild HS, curcumin and kinetin was significantly less pronounced in LP-NHEK and was almost absent in senescent NHEK, which is an indication of severely reduced responsiveness of NHEK during aging.

Since any potential modulators of keratinocytes to be used *in vivo* have to bring about their effects in the presence of calcium in the body, we have also tested the effects of those modulators in the presence of 1.2 mM calcium in the culture medium for NHEK. Table 2 summarizes the influence of those treatments in combination with calcium both in protein levels as well as in the time-shift of the response as compared with calcium alone in LP NHEK. The data in Table 2 are presented as a generalized trend in terms of average change in percent, representing a ±range of less than 5–7%, and the time of peak levels attained. However, no statistical analyses could be performed due to the limited number of independent experiments, and so these results should be considered as preliminary at this stage.

Treatment of LP-NHEK with either HS, curcumin or kinetin in the presence of calcium resulted in an increase in the levels of differentiation markers, and the extent of this increase was generally much higher than that by the treatment with modulators alone. Additionally, in combination with calcium, all treatments shifted the time of maximum induction of at least two of the differentiation markers by 6–18 h. However, for the third marker, p38, there was a delay in the differentiation-associated increase by 12–24 h. The reason for this difference in the time-shift for different markers requires further investigations. However, taken together, our data suggest that mild HS, curcumin and kinetin treatments can prevent and counteract to some extent the age-related slowing down of NHEK differentiation.

It may appear that our observations that curcumin-induced differentiation in NHEK is in contradiction to an earlier report where curcumin was shown to inhibit keratinocyte differentiation (Balasubramanian and Eckert, 2004). However, that study and other such studies on the inhibitory effects of curcumin are often performed by using relatively high concentrations of curcumin (above 10 μM), whereas we have previously shown that beneficial hormetic effects of curcumin are best observed at concentrations below 1 μM (Ali and Rattan, 2006). In addition to the differentiation modulatory effects of curcumin, we have some preliminary data which suggest that both kinetin and curcumin were also potent hormetins by inducing the synthesis of stress proteins Hsp27, Hsp70, and HO-1 (data not shown). Further investigations will resolve this issue completely.

In conclusion, we have demonstrated that calcium-induced differentiation of NHEK becomes progressively delayed during cellular aging *in vitro*, which can be modulated by treatments such as mild heat stress, kinetin and curcumin. Furthermore, our preliminary studies have shown that the proteasome may be an important entity during NHEK differentiation since its activity is enhanced during calcium-induced differentiation and after exposure of NHEK to differentiation-inducing hormetic treatments. This novel role of proteasome during NHEK differentiation deserves further detailed investigations. Finally, since the differentiation-inducing effects of the three modulators tested in the present study (thermal stress, kinetin and curcumin) are also accompanied by the induction of stress response pathways, these observations provide further evidence in support of the view that mild stress-induced hormesis can be a useful strategy in aging research and interventions.

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