Kinetin-Induced Differentiation of Normal Human Keratinocytes Undergoing Aging *in Vitro*

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ABSTRACT: Kinetin (N^6-furfuryladenine) is a cytokinin growth factor having several anti-aging effects reported for human cells and fruit flies. We have observed that short-term culturing of human keratinocytes in the presence of 40 to 200 μM kinetin results in a significant inhibition of cell growth. Studies were undertaken to analyze the process of differentiation as a reason for growth inhibition. Keratinocytes at different passage levels were treated with fetal calf serum (FCS) and calcium as differentiation-inducing positive controls, with different concentrations of kinetin, and with a combination of kinetin and calcium. The induction and progression of differentiation was monitored by morphological observations and by using several differentiation markers, including keratins (K10 and K14), involucrin, epidermal transglutaminase, and some new keratinocyte-specific antibodies isolated by the phage display method. In young keratinocytes, two days of calcium treatment reduced the K14 level by 78%, and increased the levels of K10 and involucrin by 40% and 29%, respectively. In comparison, 40 μM kinetin had no effect on the K14 level, but increased the K10 level by 28% and that of involucrin by four-fold. The combination of calcium and 40 μM kinetin led to a decrease by 23% in the K14 level, to an increase in the level of K10 by 55%, and to a two-fold rise in the involucrin level. These results suggest that the rate, extent, and quality of differentiation depend on the inducing agent, and that kinetin may be useful in promoting the differentiation of human keratinocytes, especially in the presence of calcium.

KEYWORDS: cytokinins; skin; aging; anti-aging; epidermal cells; differentiation

Rattan and Clark published the first report of anti-aging effects of N^6-furfuryladenine or kinetin (Kn) on human skin fibroblasts.[1] Later on Sharma *et al.* reported an increased median and maximum life span of the

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fruit fly *Zaprionuns paravittiger* fed with low levels of Kn.[2] Furthermore, this cytokinin has been found to inhibit oxidative and glycoxidative protein damage *in vitro*,[3] to protect the DNA against oxidative damage,[4] and to be an effective free-radical scavenger *in vitro.*[5] Since there are reports showing that Kn improves the appearance of the skin[6,7] and may induce differentiation in keratinocytes,[8] we investigated the effect of Kn in young human keratinocyte cultures with respect to growth, survival, and differentiation using a battery of biomarkers.

EXPERIMENTAL METHODS

Primary cultures of human epidermal keratinocytes were established from a mammary skin biopsy of a healthy woman (age: 28 years). For serial subculturing, the cells were thawed quickly and grown in proliferative mode-keeping, low-calcium EpiLife medium (Cascade Biologics, Mansfield, UK) with 5% CO_2/95% air and 95% humidity for 3–4 days until the medium had to be changed. When the cells reached 80% confluency, the culture was split using the trypsin/EDTA method (BioWhittacker™ Cambex Bio Science,Vervierts, Belgium). Kn (Olechemim Ltd., Czech Republic) was prepared by dissolving in 1 M HCl (30 mg/mL) followed by dilution in PBS. The effects of Kn on one-step growth, cell survival, and extent of apoptosis were studied by the standard methods described before.[1,9,10]

Differentiation Markers and ELISA

Since *in vitro* differentiation does not lead to fully differentiated corneocytes, four early, suprabasal markers were chosen: cytokeratins, K10 (ab9025, mouse monoclonal; Abcam, Cambridge, UK) and K14 (as single-chain variable fragments [scFv][11]), and involucrin (ab14504, mouse monoclonal; Abcam Cambridge, UK). In addition, the scFvs clone 10, which is specific for keratinocytes, but whose antigen is not known, was also tested.[12] The protocol for the expression and purification of scFv and the scFv-conjugagted phages was as described (*E. coli*: TG1 [supE hsd D5 (lac-pro AB)thi F'{tra D36 pro AB+ lacIq lacZ}]).[12–14] For the enzyme-linked immunosorbent assay (ELISA), 750,000 cells were seeded per T_{25} flask. After 6 h, the medium was changed according to the different treatments: control, 1% FCS, 1.2 mM $CaCl_2$, 40 μM Kn, 80 μM Kn, 200 μM Kn, and 1.2 mM $CaCl_2$ + 40 μM Kn. Cells were harvested after days 1, 2, and 3 with the trypsin/EDTA treatment and were washed twice subsequently with PBS (0.1 M NaCl, 50 mM $Na_xH_yPO_4$). The cell pellets were lysated (0.5 M TrisHCl, pH 8.0; 150 mM NaCl; 10 mM $MgCl_2$; 10% glycerol; 0.25‰ SDS) and stored at −20°C for later use.

Specific protein amounts (0.25 μg to 1 μg, depending on the antibody) in 50 μL lysis buffer were used to coat 96-well plates overnight at 4°C in

triplicate. After one or two washing steps with PBS, the wells were blocked with 2% milk in PBS (MPBS) and incubated for 1 h at room temperature (RT). After the incubation time, the blocking MPBS was changed for 100 μL of MPBS containing the antibody and incubated for 1 h at RT. The plates were washed six times with PBS, incubated with 100 μL of the MPBS containing the secondary antibody, and washed again six times. The protein levels were quantified applying OPD, 2 HCl (1,2-phenylenediamine dihydrochloride [OPD, 2 mg for ELISA]: DakoCytomation Denmark A/S, Glostrup, Denmark) as the detection system.

RESULTS AND DISCUSSION

The one-step growth analysis showed that Kn (40–200 μM) inhibited the growth of early-passage keratinocytes (30% life span completed; data not shown). However, there was no induction of apoptosis due to Kn treatment (control: 5–7%; Kn-treated: 3–7%). Studies on cell viability (MTT assay) and DNA-synthesis (5-Bromo-2′-deoxy-uridine [BrdU]) showed that Kn had no negative effects up to concentrations of 80 μM. Thus, Kn-inhibited cell growth in keratinocytes *in vitro* is not due to impaired DNA synthesis or cell viability or increased apoptosis.

In order to study the effects of Kn on differentiation, early-passage young (30% life span completed) keratinocytes were treated either with known inducers of differentiation (10% FCS, 1.2 mM $CaCl_2$), with Kn (40 μM, 80 μM, and 200 μM), or with a combination of Kn and calcium (1.2 mM $CaCl_2$ + 40 μM Kn) for 2 days. TABLE 1 shows that calcium and FCS treatment induced differentiation markers K10 by 41% and 30%, and involucrin by 29% and 89%, respectively. At the same time the levels of the basal cell marker K14 were reduced by 78% and 82%, respectively. The keratinocyte-specific protein recognized by scFv 10 was significantly induced by FCS, whereas its levels were reduced in the presence of calcium.

TABLE 1. Changes in the levels of keratinocyte differentiation markers after different treatments

	Treatment					
Differentiation marker	10% FCS	1.2 mM $CaCl_2$	40 μM Kn	80 μM Kn	200 μM Kn	1.2 mM $CaCl_2$ + 40 μM Kn
Keratin 10	1.30	1.41	1.28	1.12	1.06	1.56
Keratin 14	0.18	0.22	1.04	0.76	0.90	0.77
Involucrin	1.89	1.29	4.25	1.65	1.47	2.09
scFv 10 antibody	2.79	0.70	1.37	1.40	1.75	0.90

NOTE: Data are presented as the ratios of normalized ELISA values for days 2 and 1.

Kn treatment of keratinocytes also increased K10 levels, but to a lower extent than that with calcium and FCS (TABLE 1). The K14 level was unaltered when keratinocytes were treated with 40 μM Kn, but 80 μM Kn caused a decrease by 24% and 200 μM by 10%. Involucrin levels were induced most by 40 μM Kn (four-fold). For 80 μM and 200 μM Kn, the increase in involucrin level was 65% and 47%, respectively. The signals for the scFv 10 revealed increasing levels of this protein with rising concentrations of Kn, suggesting that Kn has some effect on the expression of this protein (TABLE 1).

A combination of Kn and calcium caused expression patterns similar to those of calcium, although in all ELISAs of young cells higher ratios could be observed. Therefore, the highest ratios for K10 were detected (+56%). K14 levels were similarly reduced as in 80 μM Kn (−23%). The involucrin level was elevated two-fold compared with both positive controls (TABLE 1).

Our studies indicate that Kn has a differentiation-modulating property. Although on its own, Kn does not appear to be a strong inducer of keratinocyte differentiation, in combination with calcium, a significant enhancement of differentiation could be observed. This could have implications for the use of Kn in the maintenance and promotion of keratinocyte differentiation during aging when both the calcium levels and the extent of differentiation are generally reduced. This may also explain the skin thickness–promoting effects of Kn in human clinical studies.[7,8]

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