Vol. 201, No. 2, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 15, 1994 Pages 665-672

KINETIN DELAYS THE ONSET OF AGEING CHARACTERISTICS IN HUMAN FIBROBLASTS

Suresh I. S. Rattan and Brian F. C. Clark

Laboratory of Cellular Ageing, Department of Chemistry,
Aarhus University, DK-8000 Aarhus - C, Denmark

Received April 15, 1994

Kinetin (Kn) is a synthetic cytokinin plant growth hormone having some senescence-retarding effects in plants. Its effects on animal cells have been, until now, best considered as growth inhibitory and anti-tumorigenic. However, we have observed that an addition of 40–200 μM Kn in the culture medium of human diploid fibroblasts can both delay the onset and decrease the extent of many of the ageing characteristics that appear during serial passaging of normal cells in culture. Age-related changes that are affected by Kn include morphological alterations, growth rates, cell size, cytoskeletal organisation, macromolecular synthesis and the intensity of autofluorescence due to the oxidative damage product lipofuscin. These anti-ageing effects of Kn are achieved without any increase in the cell culture lifespan in terms of maximum proliferative capacity *in vitro*.

Like several other N^6-substituted purine cytokinins, such as zeatin, dihydrozeatin and benzyladenine, N^6-furfuryladenine or kinetin (Kn) retards senescence in plants [1, 2]. However, cytokinins have been reported to have growth inhibitory, anti-tumour, and anti-transformation effects in human lymphocytes [3-5], Ehrlich ascites tumour cells, leukemic myoblasts [6], fibroblasts, mouse sarcoma cells and 3T3 cells [7]. Although some earlier studies had claimed short-term growth promoting effects of Kn on the outgrowth of epithelial cells and fibroblasts from human skin [8, 9], no long-term studies have been performed in this respect. Therefore, we undertook studies in order to see the long-term effects of Kn on various cellular and biochemical characteristics of serially passaged normal human fibroblasts in culture.

Normal diploid cells in culture have a finite proliferative capacity, known as the Hayflick limit [10]. Innumerable studies using fibroblasts, epithelial cells, keratinocytes, chondrocytes, lymphocytes, glial cells and bone cells have shown that normal cells undergo a large number of cellular,

0006-291X/94 $5.00

physiological, biochemical and molecular changes, which are progressive and accumulative, and lead to an irreversible cessation of cell proliferation followed by cell death. These changes have been considered as indicative of cellular ageing *in vitro* [11-13].

We have observed that an addition of 40–200 μM Kn in the culture medium delays the onset of many age-related characteristics in normal human skin fibroblasts without disturbing their Hayflick limit of cell proliferation. To our knowledge, this is the first report of the anti-ageing effects of cytokinin category of plant growth hormones on human cells without any potential harmful effects, such as increased cell proliferation, immortalization and transformation leading to carcinogenesis.

MATERIALS AND METHODS

Cell culture Primary cultures of dermal fibroblasts were established from mammary skin biopsies obtained from normal healthy adult female volunteers of different ages (biopsies were taken by Dr. T. Lidet, Kommune Hospital, Aarhus). Skin fibroblast cultures, designated ASS, were maintained at 37°C, using DMEM supplemented with 10% foetal calf serum and antibiotics, by following routine cell culture methods described previously [14]. For long-term lifespan studies, confluent cultures were serially passaged at 1:2 split ratio until the end of their proliferative capacity *in vitro*, by following well-established methods in the field of cellular ageing for serial passaging and for determining the rate of population doubling (PD) and the cumulative population doubling level (CPDL). Cells were considered to have reached the end of their lifespan when the cultures failed to grow further and to form a confluent layer even after 8 weekly changes of medium.

Kinetin treatment At least 4 parallel cultures at each dose were maintained with or without the presence of 40, 80 and 200 μM Kn (N^6-furfuryladenine, purchased from Serva, Heidelberg, Germany) in the culture medium. 8 mM stock solution of Kn was prepared in Hank's buffer containing HCl at a final concentration of 5.7%. The concentrations of Kn selected for long-term studies were based on pilot testing of a wide concentration range from less than 1 μM to 1 mM. These studies showed that whereas no effects could be detected at concentrations below 40 μM, Kn was slightly growth inhibitory above 200 μM and was toxic above 500 μM. In comparison, the nucleoside derivative kinetin-riboside was growth inhibitory and toxic for human cells even at concentrations below 40 μM. Furthermore, pilot testing also ruled out any effects of various concentrations of HCl on growth, morphology and other characteristics of human cells.

Macromolecular synthesis and fluorescence microscopy At various CPDLs during the entire lifespan of Kn-treated and untreated cultures, the extent of DNA, RNA and protein synthesis was determined by measuring the incorporation of radioactive [^{3}H]thymidine, [^{3}H]uridine and [^{35}S]methionine, respectively, into TCA insoluble material, as described previously [15, 16]. All experiments were performed at least five times. The proportion of S-phase positive cells was determined at various passages, by autoradiographic methods [14]. The cytoskeletal organization of actin microfilaments and tubulin

microtubules was analysed by phalloidin staining and immunofluorescence staining, respectively, as described [17]. Intracellular autofluorescence due to the accumulation of the "age pigment", lipofuscin, was observed by UV-mediated fluorescence microscopy [18]. The protein content of cells was determined in NaOH lysates of cells by Coomassie Blue method, using bovine serum albumin as the standard.

RESULTS

Changes in the morphology of normal diploid cells during serial passaging is one of the most dramatic alterations associated with ageing of cells in culture [11-13]. For example, normal adult skin fibroblasts of about 20% lifespan completed *in vitro* are long, thin, spindle shaped, and are arranged in regular arrays in a confluent layer. After completing about 70% lifespan, these cultures progressively become heterogeneous with the appearance of large and flattened cells until the end of the culture lifespan when almost all cells are extremely large, full of lysosomal residual bodies, and many of them contain more than one nucleus. We have observed that cells grown in the presence of Kn do not undergo such morphological changes to the same extent as control cultures, even when they have reached the end of their proliferative lifespan (Fig. 1).

In the example shown here, adult human mammary skin fibroblast cultures, designated ASS-1 (donor age 52 yr), grown with or without the presence of 40 μM Kn throughout their lifespan *in vitro* are compared for age-related alterations in their morphology. Fig. 1 shows the phase-contrast pictures of Giemsa-stained cells at 50x microscopic magnification: (a) untreated control cultures of young cells of less than 20% lifespan completed; (b) untreated ageing cells of 90% lifespan completed; (c) moribund, non-dividing and senescent control cells two months after growth cessation; (d) Kn-treated young cultures of less than 20% lifespan completed; (e) Kn-treated ageing cells of 90% lifespan completed but still maintaining young characteristics and a lack of significant cell enlargement; and (f) moribund and non-dividing cells of Kn-treated cultures two months after the cessation of growth. Note a lack of significant cell enlargement, absence of multinucleate cells and reduced levels of accumulated cellular debris in Kn-treated cells.

In this series of experiments, Kn-treated and untreated cultures reached a final CPDL (100% lifespan completed) ranging from 29 to 31 at all doses of Kn. The representative longevity curves shown in Fig. 2 are of adult human skin fibroblasts, ASS-1, which were treated with 40 μM Kn from CPDL 8 until the end of their lifespan at CPDL 31. There is no difference in growth characteristics or proliferative lifespan of the cultures grown in the presence or absence of Kn. Similarly, Kn was not seen to affect the maximum lifespan or proliferative capacity of other cell strains including embryonic lung fibroblasts MRC-5, and

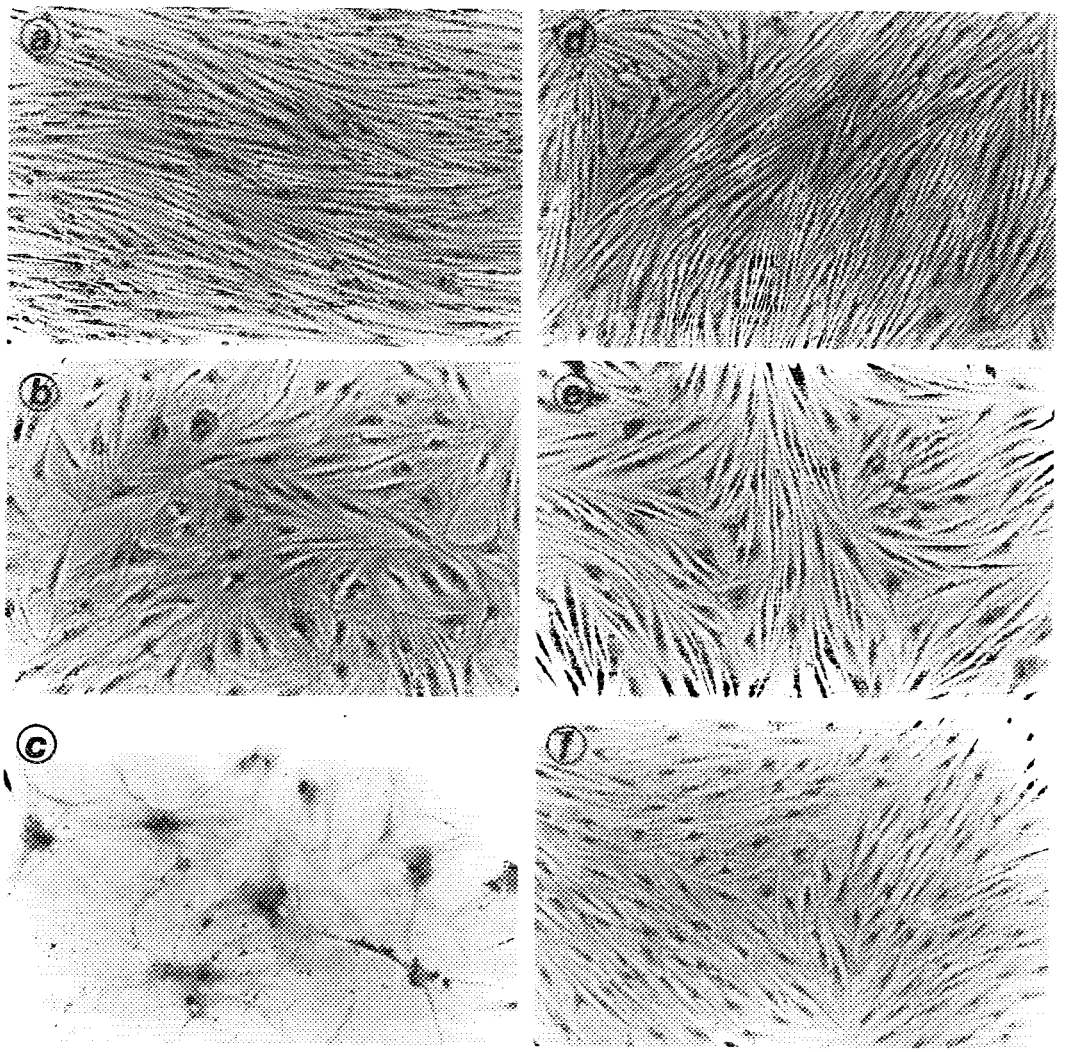

FIG. 1. Effect of long-term Kn treatment on the age-related morphological characteristics of human adult mammary skin fibroblasts *(see text for details).*

adult mammary skin fibroblasts ASS-2 (donor age 20 yr) and ASS-3 (donor age 54 yr) *(results not shown).*

Several other cellular and biochemical parameters of ageing were studied in Kn-treated and untreated cells. Table 1 compares various characteristics of early passage (less than 20% lifespan completed) and late passage (more than 90% lifespan completed) of ASS-1 fibroblasts in the presence or absence of 40 μM Kn throughout their proliferative lifespan. It is clear that whereas there were no significant effects of Kn on the lifespan or growth characteristics, including population doubling (PD) time, DNA, RNA and protein synthesis and the percentage of cycling cells, some other cellular and biochemical characteristics

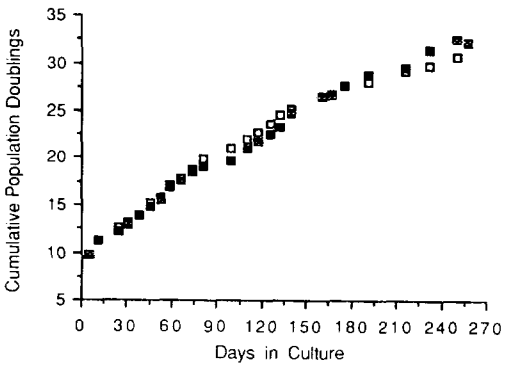

FIG. 2. Longevity curves of human adult mammary skin fibroblasts grown in the presence(■)or absence(□)of 40μM Kn.

associated with ageing were affected significantly. For example, the decrease in cell yield, that is, the number of cells per unit area in a confluent layer, which is inversely proportional to cell size, during ageing, is delayed in Kn-treated cultures. Whereas the cell yield in late passage untreated cultures decreased from an early passage average of 4 x 10^4 to 1 x 10^4 cells per cm^2, Kn-treated cultures maintained a high cell yield until more than 95% lifespan was completed (Table 1).

Table 1. Cellular and biochemical characteristics of ageing human skin fibroblast cultures maintained in the presence or absence of kinetin

Characteristic	Untreated cultures		Kn-treated cultures	
	early passage	late passage	early passage	late passage
PD time (days)	8 ± 1	20 ± 2	8 ± 1	15 ± 3
Cell yield (10^4 cells per cm^2)	4 ± 0.5	1 ± 0.2	4 ± 0.5	3 ± 0.3
DNA synthesis (10^4cpm/10^6 cells)	35 ± 4	5 ± 0.5	39 ± 5	5 ± 0.4
S-phase positive cells (%)	92 ± 4	5 ± 1	93 ± 3	8 ± 3
RNA synthesis (10^3cpm/10^6 cells)	5 ± 0.4	3 ± 0.5	5 ± 0.5	3 ± 0.4
Protein synthesis (10^6cpm/10^6 cells)	4 ± 2	1 ± 0.6	4 ± 2	3 ± 2
Protein content (μg/10^6 cells)	325 ± 50	985 ± 70	320 ± 60	535 ± 45
Autofluorescence	low	high	low	low
Actin filaments	diffused	highly polymerized	diffused	less polymerized
Microtubules	orderly	disorganized	orderly	orderly

Other characteristics of young cells that remained either unaltered or changed to a smaller extent during serial passaging in the presence of Kn include lower levels of autofluorescence due to accumulation of lipofuscin, a lack of highly polymerised and rod-like actin microfilaments, and a lack of multiple and disorganised microtubular network, as observed by fluorescence microscopic methods (pictures not shown). Although there was an age-related decline in DNA, RNA and protein synthesis of ageing human fibroblasts, no significant differences between Kn-treated and untreated cells was observed. However, the total protein content of cells, which increased several fold during ageing of untreated cells, did not increase to the same extent in Kn-treated cells (Table 1). Furthermore, the proportion of multinucleate cells in Kn-treated high CPDL cultures was less than 0.5% as compared with more than 5% multinucleate cells present in untreated senescent cultures.

DISCUSSION

Our results show that the cytokinin Kn delays the onset of several cellular and biochemical characteristics associated with cellular ageing *in vitro*. This delay was most pronounced in cultures in which Kn was present continuously in the culture medium. On removal of Kn from the culture medium, some ageing characteristics began to reappear within few population doublings. Similarly, although most of the ageing characteristics could be reversed in late passage cultures the extent of reversal was less than the extent of maintenance of young characteristics in long-term treated cultures *(results not included)*.

The above effects of Kn on human cells in culture were observed apparently without any effect on their maximum proliferative capacity, which is genetically determined. There were no differences in cell attachment frequencies, growth characteristics or the CPDL of Kn-treated and untreated cultures. These observations were confirmed by autoradiographic studies to determine the number of cells entering the S phase of the cell cycle. Although Kn helps to complete cytokinesis (as indicated by an almost complete absence of mutinucleate cells in Kn-treated cultures), it does not promote cell proliferation by inducing cells to enter S phase.

The lack of stimulation of cell proliferation by Kn is an extremely interesting characteristic of cytokinins, which distinguishes them from other compounds which affect proliferative capacity of human cells in culture. Such compounds include EGF, hydrocortisone and dexamethasone [19], acidic and basic FGF [20], neurotropin [21], retinoic acid [22], hyaluronidase [23], and carnosine [24]. This "forced" cell proliferation or dysregulation and escape from the Hayflick limit is considered as one of the early events associated with cellular immortalization, transformation and carcinogenesis [25-27]. Therefore,

it is of great interest that Kn can delay the onset of many ageing characteristics without forcing the cells to undergo additional proliferation.

At this stage, we are unable to explain the biochemical and molecular mechanisms of Kn action in human cells. Even in plants, the mode of action of cytokinins is not well understood [28]. Various hypotheses have been put forward to explain the mode of action of cytokinins. These include direct interaction of cytokinins with rRNAs and tRNAs for increased protein synthesis [29], stimulation of protein elongation factors [30], interaction with ribosomal proteins [31], and indirect action through receptor-mediated pathways of signal transduction including cAMP- and cGMP-mediated pathways [28, 32] and through the action of specific cytokinin-binding proteins [33]. Furthermore, a superoxide-dismutase-like antioxidant activity has been observed for Kn and 6-benzylaminopurine complexed with copper [34]. However, all these hypotheses remain to be tested.

We have shown that Kn is incorporated into prokaryotic and eukaryotic tRNAs in an exchange reaction catalysed by a putative tRNA-kinetin transglycosylase present in cell-free extracts from *E. coli* and human cells, respectively [35]. We have also observed receptor-mediated uptake and cytoplasmic and nuclear accumulation of a radioactive derivative of Kn by human fibroblasts *(Rattan and Clark, in preparation)*. These results suggest that Kn may act through receptor-mediated action on the components of protein synthetic machinery, thereby improving the efficiency of various maintenance and repair pathways, including fidelity of protein synthesis, scavenging free radicals and removing abnormal and damaged macromolecules. Therefore, it appears that Kn and possibly other cytokinins, by a series of as yet unidentified processes, may modulate the action of genes influencing ageing, gerontogenes, and thereby prevent some of the age-related detrimental alterations in human cells.

Most importantly, our observations on the effects of Kn in human cells lead to a range of lines for further investigation, including a search for cytokinin receptors, intracellular uptake and metabolism, their modes of action at the levels of gene expression and the maintenance of cellular organization and function. Furthermore, it will be interesting to test various other synthetic and natural cytokinins and their derivatives on human fibroblasts and other cell types both *in vivo* and *in vitro* in order to elucidate the action and applications of plant hormones in animal cells.

Acknowledgments: This work was supported by a grant from Senetek PLC. We thank Dr. N. J. Coppard for critically reading the manuscript.

REFERENCES

1. Skoog, F., and Armstrong, D.J. (1970) *Annu. Rev. Plant Physiol.* 21, 359-384.
2. Van Staden, J., Cook, E.L., and Noodén, L.D. (1988) In *Senescence and Aging in Plants.* (L.D. Noodén and A.C. Leopold, Eds.), pp. 281-328, Acad. Press, N. York.
3. Gallo, R.C., Whang-Peng, J., and Perry, S. (1969) *Science* 165, 400-402.
4. Saito, T., Shibata, Y., Kurokawa, T., and Ishida, N. (1979) *Experientia* 35, 685-686.
5. Mookerjee, B.K., Ballard, J., Bentzel, C.J., Allen, J.C., and Jung, C.Y. (1979) *J. Reticul. Soc.* 25, 299-314.
6. Adair, W.L., and Brennan, S.L. (1986) *Biochem. Biophys. Res. Commun.* 137, 208-214.
7. Johnson, G.S., D'Armiento, M., and Carchman, R.A. (1974) *Exp. Cell Res.* 85, 47-56.
8. Orr, M.F., and McSwain, B. (1957) *Cancer* 10, 617-624.
9. Orr, M.F., and McSwain, B. (1960) *Canc. Res.* 20, 1362-1364.
10. Rattan, S.I.S. (1991) *Mutat. Res.* 256, 67.
11. Hayflick, L. (1991) *Mutat. Res.* 256, 69-80.
12. Macieira-Coelho, A. (1988) Biology of Normal Proliferating Cells in vitro. Relevance for in vivo Aging. Basel: Karger.
13. Stanulis-Praeger, B.M. (1987) *Mech. Ageing* Dev. 38, 1-48.
14. Cavallius, J., Rattan, S.I.S., and Clark, B.F.C. (1986) *Exp. Gerontol.* 21, 149-157.
15. Rattan, S.I.S., Cavallius, J., and Clark, B.F.C. (1988) *Biochem. Biophys. Res. Commun.* 152, 169-176.
16. Derventzi, A., Rattan, S.I.S., and Clark, B.F.C. (1992) *Biochem. Int.* 27, 903-911.
17. Derventzi, A., Rattan, S.I.S., and Clark, B.F.C. (1992) *Biochem. Biophys. Res. Commun.* 182, 1423-1428.
18. Rattan, S.I.S., Keeler, K.D., Buchanan, J.H., and Holliday, R. (1982) *Biosci. Rep.* 2, 561-567.
19. Finlay, C.A., Rosner, B.A., Miller, J.E., Wallace, J.M., and Cristofalo, V.J. (1985) *Exp. Cell Res.* 156, 462-470.
20. Froger-Gaillard, B., Charrier, A.M., Thenet, S., Ronot, X., and Adolphe, M. (1989) *Exp. Cell Res.* 183, 388-398.
21. Okada, Y., Kimura, H., and Aoyama, T. (1986) *Mech. Ageing Dev.* 35, 133-143.
22. Varani, J., Mitra, R., Gibbs, D., Phan, S.H., Dixit, V.M., Mitra, R., Wang, T., Siebert, K.J., Nickoloff, B.J., and Voorhees, J.J. (1990) *J. Invest. Dermatol.* 94, 717-723.
23. Moczar, M., and Robert, L. (1993) *Exp. Gerontol.* 28, 59-68.
24. McFarland, G.A., and Holliday, R. (1994) *Exp. Cell Res.* 212, in press.
25. Macieira-Coelho, A. (1990) In *Cancer and Aging.* (A. Macieira-Coelho and B. Nordenskjöld, Eds.), pp. 11-37, CRC Press, Boca Raton, Florida.
26. Cohen, S.M., and Ellwein, L.B. (1991) *Canc. Res.* 51, 6493-6505.
27. Martin, G.M. (1992) In *Oxford Textbook of Geriatric Medicine.* (J.G. Evans and T.F. Williams, Eds.), pp. 41-48, Oxford Univ. Press, Oxford.
28. Kamínek, M. (1992) *Trends Biotech.* 10, 159-164.
29. Romanov, G.A. (1990) *Plant Cell Env.* 13, 751-754.
30. Spirin, A.S., and Ajtkhozhin, M.A. (1985) *Trends Biochem. Sci.* 10, 162-165.
31. Yakovleva, L.A., and Kulaeva, O.N. (1987) *Biochem. Phys. Pflanzen* 182, 359-365.
32. Vesely, D.L., Hudson, J.L., Pipkin, J.L., Pack, L.D., and Meiners, S.E. (1985) *Endocrinol.* 116, 1887-1892.
33. Kulaeva, O.N., Karavaiko, N.N., Moshkov, I.E., Selivankina, S.Y., and Novikova, G.V. (1990) *FEBS Lett.* 261, 410-412.
34. Goldstein, S., and Czapski, G. (1991) *Free Rad. Res. Comms.* 12/13, 173-177.
35. Barciszewski, J., Otzen, D., Rattan, S.I.S., and Clark, B.F.C. (1992) *Biochem. Int.* 28, 805-811.