***In vitro* Senescence of Human Keratinocyte Cultures**

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ABSTRACT. Human keratinocytes have been serially cultivated in low (0.015 mM) and high (1.8 mM) calcium containing medium. The calcium concentration of the growth medium significantly influenced the cell growth period *in vitro*. Cells grown in low calcium medium underwent 35–40 population doublings over 16–17 passages, while cells grown in high calcium medium ceased to proliferate after 20 population doublings over 7 passages. Changing the keratinocytes from one *in vitro* environment to the other drastically altered the lifespan in culture of populations derived from the same primary tissue. The degree of DNA methylation of human keratinocytes was shown to decrease with age in both high and low calcium culture conditions but does not appear to be associated with differentiation.

Most of the studies on the ageing of cells *in vitro* have been performed on mammalian diploid fibroblasts. These cells, as is well known, have a finite capacity to multiply in culture and eventually undergo senescence. This limitation of the proliferative potential, which is determined by the number of cell divisions and is independent of the chronological age of the culture, has led to the use of these cells as models for cellular ageing (10). The mechanism by which this phenomenon takes place in cells is unknown and into this void have come several hypotheses to explain cellular senescence *in vitro* and to relate this phenomenon to *in vivo* ageing (1, 16, 17, 19, 25). The literature on fibroblast senescence *in vitro* and its possible causes has recently been reviewed (12, 13).

We have turned our attention to the human keratinocyte culture system which, although more complex, offers significant advantages over the fibroblast system. Human keratinocytes *in vitro* undergo terminal differentiation, a property which if not totally absent in the fibroblast, is at the very least, obscure. Moreover the differentiation of cultured keratinocytes can be experimentally manipulated by the extracellular calcium concentration in the growth medium (11). In high calcium (>0.1 mM) medium human keratinocytes differentiate whereas in low calcium (<0.1 mM) medium the cells are prevented from differentiating and are maintained in the stem cell configuration (23). The differentiation *in vitro* however is not identical to that which occurs *in vivo*. Fuchs and Green (8) have demonstrated differences in the keratin pattern of cells grown *in vitro* compared to *in vivo* skin whilst the keratin patterns of cells grown in either high or low calcium containing growth medium *in vitro* is the same (3). This culture system then offers the advantage of undergoing terminal differentiation which can be measured and experimentally manipulated and

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which approximates very closely the situation *in vivo*.

Several reports have indicated changes in cellular DNA with ageing of cells in culture (24, 29, 31, 32, 35, 36). More specifically a decrease in hybridisable ribosomal DNA has been demonstrated with age in human heart (31) and brain tissue (32). Shmookler Reis' laboratory has recently shown, both *in vitro* and *in vivo*, an increase in extrachromosomal inter-*alu* circular DNA molecules (29). Wilson and Jones (36) have reported decreases in the levels of DNA methylation occurring with time in culture of diploid mouse cells in contrast to the situation with immortal cell lines. Romanov and Vanyushin (23) have demonstrated decreases in the methylation of reiterated DNA sequences in cattle with ageing but not in unique sequences. These results would tend to indicate a degree of instability of the cell genome with time or cell division; however, Erlich *et al.* (6) have failed to detect any differences in the 5 methyl cytosine content of tissues from young and aged humans or in cultured human fibroblasts subdivided and allowed to grow over a period of 5 weeks compared to cultures maintained at confluence for the same period of time.

In this report we demonstrate that:

- i) the calcium concentration of the growth medium significantly influences the cell growth period *in vitro*. Human keratinocyte cultures under high calcium growth conditions cease proliferation sooner than cultures grown under low calcium growth conditions,
- ii) the fate of cell populations can be reversed by changing the growth conditions and,
- iii) the degree of DNA methylation of human keratinocytes decreases with age in culture but does not appear to be related to differentiation.

MATERIALS AND METHODS

Growth of cells. Human keratinocytes were derived from neonatal human foreskin and grown on irradiated fibroblast feeder layers according to Rheinwald and Green (22). During primary culture the cells were grown in Dulbecco's modified Eagle's medium (DME) containing penicillin (200 U/ml), streptomycin (100 µg/ml) and sodium bicarbonate (3.6 g/l) supplemented with 10% foetal calf serum, cholera toxin (10 ng/ml), hydrocortisone (10 µg/ml) and epidermal growth factor (10 ng/ml). After the first passage, cells were grown on irradiated fibroblast feeder layers in medium as above (high calcium) or in a 1 : 1 (v/v) mixture of calcium free DME and Ham's MCDB 151 (20) containing the additions indicated above except that foetal calf serum was replaced with chelex treated foetal calf serum (low calcium). The calcium concentration, determined by atomic absorption, for whole serum was 3.49 mM whilst for chelex treated serum, 0.01 mM. These sera were added at 10% to DME (1.82 mM Ca⁺⁺) and calcium free DME/MCDB151 (0.015 mM Ca⁺⁺) respectively.

At passage, cells were routinely washed with 0.02% EDTA in PBS to remove fibroblasts. The attached cells were harvested with 0.2% trypsin-0.02% EDTA in PBS, washed and resuspended in the appropriate growth medium, counted in a haemocytometer and an aliquot replated at the indicated densities in 25 cm² Corning T-flasks containing 1 × 10⁵ irradiated fibroblasts as feeder layers. Cumulative cell numbers were estimated as if all cells had been replated at every passage.

Cultures were maintained at 37°C in a humidified 7% CO₂ in air atmosphere. The medium was changed every 2 or 3 days and the cells passaged every week.

Extraction of DNA. Harvested cells were disrupted in 0.5% SDS-60 mM Tris-20 mM

EDTA (pH 8.4) by freezing, thawing and homogenisation. The lysate was incubated with proteinase K (100 $\mu\text{g/ml}$) for 3 h at 37°C and extracted with an equal volume of 1 : 1 (v/v) phenol : chloroform mixture. The interface was re-extracted with SDS/Tris/EDTA and the aqueous extracts combined and dialysed against 3 changes of 10 mM Tris—1 mM EDTA (pH 7.5). The dialysed extract was incubated with heat treated RNAase (50 $\mu\text{g/ml}$) for 1 h at 37°C. The mixture was re-extracted with phenol/chloroform and dialysed as above, ethanol-precipitated several times, washed with 70% ethanol, dried and redissolved in Tris/EDTA.

Estimation of DNA methylation. DNA samples were digested with endonucleases HpaII and MspI. To check that digestion was complete aliquot samples were incubated with PBR 322 DNA, submitted to electrophoresis on 7% agarose, containing ethidium bromide, gels and examined under UV light. After complete digestion, DNA was end labelled with ^{32}P -dCTP by the Klenhoff fragment of DNA polymerase. After labelling the DNA was precipitated several times, washed with 70% alcohol, dried and redissolved in Tris/EDTA. The samples were then submitted to electrophoresis on 5% agarose gels and the gels sliced into 2 mm sections, counted by Cerenkhov radiation and the radioactivity of each fraction plotted as a percentage of the total.

Chemicals. DME powdered formulation was from Gibco, calcium free DME from Flow Laboratories, MCDB 151, foetal calf serum and streptomycin from Commonwealth Serum Laboratories, Trypsin-Serva Biochemicals. PBR 322 DNA, HpaII and MspI endonucleases were from PL Biochemicals and Chelex 100 mesh size 100–200 was from Biorad Pharmaceuticals. Epidermal Growth Factor (EGF) was from Collaborative Research and cholera toxin and hydrocortisone from Sigma, Proteinase K from Merck, RNAase from Worthington Biochemicals, Klenhoff fragment of DNA polymerase was from Bohreinger-Manhoff and ^{32}P -dCTP 5 mCi/ml-1800 Ci/mmol was from Bresa.

RESULTS

Figure 1 shows the morphology of human keratinocytes grown in medium containing high (1.8 mM) (A) or low (0.015 mM) (B) calcium. Cells grown in low calcium medium (Fig. 1B) retain a very flattened morphology with a regular polygonal shape and relatively clear cytoplasm. The cells continue to grow as a monolayer never assuming the stratified differentiated appearance of cells grown in high calcium medium. Cells grown in high calcium medium (Fig. 1A) stratify, become densely packed and possess cytoplasmic granules.

Cells derived from the same primary culture demonstrate different replicative capacity depending upon which growth medium is employed (Fig. 2). Cells grown in the low calcium medium undergo 35–40 population doublings over 16–17 passages while cells grown in the high calcium medium ceased proliferation and die after 20 population doublings over 7 passages. Death is arbitrarily defined when there is insufficient cells after one week of growth to reseed at 3×10^5 cells and the experiment was terminated at this point.

More importantly however, if cells are switched from one growth condition to the other, the fate of the cell population is reversed. Figure 3 plots the cell number at passage against passage number and demonstrates that for cells grown in high calcium medium, limitation of proliferation begins after 3–4 passages and the population dies after 7 passages whereas cells derived from the same primary culture grown in low calcium medium, continue to grow and divide. When cells are trans-

Fig. 2. Population growth curves of human keratinocytes in (□—□) high calcium and (○—○) low calcium medium as described in Fig. 1. The cells were passaged by trypsinization after washing with EDTA every 7 days and 3×10^5 cells reseeded into T-25 Corning flasks.

ferred from low calcium conditions to high calcium conditions they immediately limit proliferation and die within a few passages. On the other hand, when a population of cells that was destined to cease proliferation in high calcium growth conditions was transferred to low calcium conditions it recovered and continued to grow and divide. These populations survived for a similar duration as those maintained solely in the low calcium medium (results not shown).

The level of DNA methylation in these cells was measured as a function of the passage number by digestion of isolated DNA with the endonucleases HpaII and MspI. Both of these enzymes cut DNA at the same site but HpaII is inhibited by methylation at the internal C residue (34, 30). This method does not give an absolute measure of DNA methylation but a decrease in methylation can be seen in HpaII digests by a decrease in the proportion of counts in the high molecular weight region of the gel with a concomitant rise in the proportion of counts in the lower molecular weight range.

DNA samples from high calcium cultures at passages 2, 4 and 5 were examined

Fig. 1. Photomicrographs (phase contrast $\times 300$) of human keratinocytes cultured in (A) DME, 10% FCS supplemented with cholera toxin, hydrocortisone and epidermal growth factor (high calcium) and (B) 1 : 1 (v/v) mixture of calcium free DME and MCDB 151 supplemented with 10% chelex treated FCS, cholera toxin, hydrocortisone and epidermal growth factor (low calcium).

Fig. 3. The fate of cell populations growing in (□—□) high calcium medium, (○—○) low calcium medium, (■—■) high calcium medium switched to low calcium medium at passage 6 and (●—●) low calcium medium switched to high calcium medium at passage 7. The cells were passaged by trypsinization after washing with EDTA every 7 days and 3×10^5 cells reseeded into T-25 Corning flasks.

Fig. 4. Molecular weight distribution from agarose electrophoresis gels of ³²P end labelled fragments of HApAI digests of DNA from (A) high calcium cells at passage (●—●) 2, (■—■) 4 and (□—□) 5 and (B) low calcium cells at passage (●—●) 2, (■—■) 4, (○—○) 8 and (△—△) 16.

(Fig. 4A). The results show a slight decrease in DNA methylation of cells from passage 5. In low calcium growth conditions DNA from cells at passages 2, 4, 8 and 16 was examined. The DNA from cells at passage 8 and 16 had a significantly lower degree of methylation than DNA from cells at the earlier passages.

DISCUSSION

Fibroblasts in culture have been shown to have a finite capacity for growth which is brought about by senescence of the population. In the keratinocyte system, under investigation here, there are two reasons for cessation of growth. In high calcium growth conditions, continued proliferation is limited by terminal differentiation (9). In low calcium medium the cells have an extended proliferative capacity however, eventually, they senesce and the culture has a finite growth period comparable to the "Hayflick limit". Thus we have demonstrated, (i) that the calcium concentration of the growth medium significantly affects the growth period *in vitro* of human keratinocyte cultures derived from the same primary culture and (ii) that keratinocytes grown in low calcium growth conditions are similar to fibroblasts and other differentiated somatic cells including glial cells, lymphocytes, endothelial and adrenal cells (12) in having a finite lifespan *in vitro*.

That the calcium ion concentration affects the differentiation and growth period of keratinocytes *in vitro* raises interesting speculation on the local control of both differentiation and ageing *in vivo*. It has been shown that the Ca^{++} content of skin varies significantly. The basal layer, which contains the proliferative cells, has an extremely low Ca^{++} content while the upper layer, which contains differentiating and differentiated cells, has a 20 fold increase in Ca^{++} content (7). It would be interesting to determine if the Ca^{++} level of these layers changed with age. Accumulation of cellular calcium has been discussed as a factor in ageing (33) and the susceptibility of cardiac myocytes to calcium induced death has been shown to be age dependent (4). Shapiro *et al.* have demonstrated premature senescence in cultured skin fibroblasts from subjects with cystic-fibrosis (27) although Holliday has reported that two out of four fibroblast cultures from cystic-fibrosis patients had longer lifespans than controls (personal communication). Increased sweat electrolytes is characteristic of the disease and has also been linked with ageing (27) whilst more specifically the intracellular Ca^{++} pool size is greater by about 30% in skin fibroblasts from cystic-fibrosis sufferers and carriers when compared to those obtained from normal donors (26).

When cultures were switched between the growth conditions the fate of the cell populations was reversed. These results are consistent with the observation that cells will differentiate in high Ca^{++} growth conditions and continue to proliferate until senescence in low Ca^{++} growth conditions. It appears from our results that a proportion of keratinocytes growing in low calcium growth conditions may be already committed to differentiate because of the rapid limitation in growth observed when cells were switched from low calcium growth conditions to high calcium growth conditions. Previous measurement of the proportion of committed cells, as estimated by the production of cornified envelopes after exposure to the Ca^{++} ionophore X537A, indicated a level of 30% for cells growing in low calcium culture conditions compared to 65% for cells in high calcium culture conditions (23). Figures as high as 55% have recently been reported for cornified envelope production of human

keratinocytes growing in low calcium growth conditions (2). These results with keratinocyte senescence in low calcium growth conditions may have some relevance to the commitment theory of cellular ageing (17) but it is questionable whether "commitment" to differentiate in the epithelial keratinocyte system is equivalent to "commitment" of a subpopulation of fibroblastic cells to a finite number of population doublings as envisaged in the Kirkwood-Holliday model.

Our results demonstrate that decreases in the level of DNA methylation occur with time in cultured human keratinocytes but they do not establish whether this loss of methyl groups is related to the number of cell divisions. There has been recent discussion on the role of methylation in gene expression with demethylation of DNA sequences being implicated in the transcription and expression of genes which would remain unexpressed in the methylated state (see reviews 5, 15). In regard to ageing, the level of DNA methylation in human diploid fibroblasts has been shown to decrease with age in culture in two out of three strains tested (28) and has also been demonstrated to decrease with age in culture in fibroblasts isolated from mice, hamsters and humans but not in "immortal" mouse cell lines (36). There is some evidence that DNA methylation declines with age *in vivo*. Romanov and Vanyushin have demonstrated decreases in the methylation of reiterated, but not unique, sequences in cattle with ageing (24) whilst Mays-Hoopers *et al.* have observed hypomethylation of specific moderately repeated sequences of the mouse liver genome with age (18). It is known that reiterated DNA sequences tend to have a higher proportion of methylated sites than other areas of the genome (21) and experiments are under way to look at keratinocyte reiterated DNA sequences in this regard.

It has recently been shown that a single treatment of early passage cells with the base analogue 5-azacytidine, which is known to reduce the level of 5-methyl cytosine in DNA, has a profound life shortening effect on populations of human fibroblasts (14). These results might suggest a direct role of methylation in ageing and Holliday, in reviewing these experiments, concluded "that the stochastic loss of epigenetic controls of gene expression may be an important component in the ageing process" (12).

In summary then we have demonstrated that the Ca^{++} concentration of the medium significantly affects the growth period *in vitro* of human keratinocyte cultures derived from the same primary culture and that the fate of the cell populations can be reversed by changing the growth conditions. Keratinocytes grown in low calcium conditions are similar to fibroblasts and other differentiated somatic cells in having a finite lifespan *in vitro*.

We have demonstrated a decrease in DNA methylation of human keratinocytes in culture which is associated with senescence but does not appear to be associated with differentiation. Whether or not this change can be traced to specific genes or reiterated DNA sequences controlling genes which may be related to ageing will have to await further study.

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