Altering basal medium components increases the *in vitro* lifespan of normal human keratinocytes.

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Abstract

We are interested in the regulation of cellular senescence. Typically, normal human epidermal keratinocytes (HEK) cultured without serum and fibroblast feeders can undergo ~20 population doublings after the primary culture prior to senescing. In contrast, HEK lifespan is ~ 50 population doublings when serum and fibroblast feeders are included in the culture environment, implicating hormones, growth factors, and/or extracellular matrix components in regulating cellular senescence. However, by manipulating only the composition of the basal medium, which contains no hormones or macromolecules, we can culture HEK for ~50 population doublings without serum or fibroblast feeders. Our medium requires only the same protein and growth factor supplements that are added to basal media that allow only 20 population doublings. Cells cultured in our formulation: (1) acquire senescent characteristics, such as the appearance of large non-dividing cells, at much higher population doublings than cells cultured in typical formulations, (2) maintain normal epithelial morphology until senescence, and (3) can be induced to express the differentiation markers K10 and involucrin under typical differentiation conditions (removal of EGF, insulin, etc.). We are currently investigating the role(s) of the medium components in the process of lifespan extension. This medium formulation should be very useful for understanding the underlying mechanisms of *in vitro* cellular aging.

Introduction

It has been nearly 25 years since replicating *in vitro* cultures of human epidermal keratinocytes were first established (see [1], for review). Richard Ham and his colleagues were the first to develop an in vitro system that did not require serum or feeder layers to support clonal growth, serial propagation, and differentiation of these cells [2, 3]. This system was the basis for the development of current commercial media that do not rely on the use of serum or feeder layers. These media permit the growth of keratinocytes to essentially confluence. However, the replicative lifespan of normal keratinocytes cultured in these media is limited, typically 15-25 population doublings after the primary culture stage [4, 5]. In contrast, keratinocyte lifespan is over 50 population doublings when serum and fibroblast feeders are included in the culture environment [6], implicating hormones, growth factors, and/or extracellular matrix components in regulating cellular senescence. Starting with formulations that have been shown to support keratinocyte growth, we optimized the components of the basal medium for the long-term growth of keratinocytes. The resulting optimized formula, EpiLife™, has increased the lifespan of cultured keratinocytes to >30 (HEK adult) or > 50 (HEK neonatal) population doublings without serum or fibroblast feeders. EpiLife™ requires only the same protein and growth factors that are added to standard basal media that allow only 20 population doublings. Proliferative keratinocytes cultured in standard medium express basal cell markers such as keratin 14 [7]. Basonuclin, a zinc finger protein, is also expressed in keratinocytes and has been shown to be associated with proliferative potential and absence of terminal differentiation in these cells [8]. When cultured under differentiation conditions, that is with growth factors removed and with the calcium concentration in the basal medium increased, keratinocytes can be induced to express keratin 10 and involucrin, terminal differentiation markers expressed in committed keratinocytes [7,9]. We examined the expression of these genes and the correlation of that expression with proliferation and differentiation conditions for keratinocytes cultured in EpiLife™ basal medium. Our results demonstrate that cells actively growing in EpiLife™ medium maintain the ability to express basal cell markers and can be induced to express suprabasal markers when switched to differentiation conditions, even after long term culture.

Methods and Materials

Primary Isolation of HEKn and HEKa. Neonatal foreskin or adult skin from reduction mammoplasty were obtained through the National Disease Research Interchange (NDRI). Epidermal cells were isolated as previously described [10], with modifications.

Preparation of media. Medium 154 and EpiLife™ medium were prepared from individual chemical components dissolved in 18 megOhm water. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Where indicated, media were supplemented with Human Keratinocyte Growth Supplement (HKGS) which contains EGF, Insulin, Hydrocortisone, Transferin and BPE. Medium KGM-2 was obtained from BioWhittaker, Inc. (Walkersville, MD).

Cell culture and calculation of population doublings. All determinations were made in triplicate. Cultures of HEK were established from cryopreserved cultures by plating 2,500 trypan blue-excluding cells/cm^2 in 25 cm^2 tissue culture flasks in the indicated media. The cultures were incubated at 37 degrees C in a humidified atmosphere of 95% air/5% CO_2. When cultures reached approximately 80% confluence, the cells were harvested using trypsin/EDTA and the total number of cells determined. Subsequent cultures were established by pooling cells from each set of triplicate cultures, centrifuging the cells, resuspending in fresh medium and seeding new cultures at a density of 2,500 trypan blue-excluding cells/cm^2 in 25 cm^2 tissue culture flasks. The population doublings (y) achieved during each culture interval (passage) was calculated as 2^y= the fold increase in cell number during each passage.

Northern Blots. Cells were grown in either Medium 154 (0.2 mM calcium) or EpiLife™ medium (0.06 mM calcium) supplemented with HKGS. For "rapidly growing" cultures, the RNA was isolated from cells that were 50-80% confluent. For "differentiation conditions", the cells were grown to confluence in the indicated medium supplemented with HKGS, the medium removed, cells washed with medium containing no supplement and the medium replaced with unsupplemented medium containing 0.2 mM calcium. After five days of additional incubation in differentiation conditions, poly-A+ enriched RNA was isolated as previously described [11].

Figure 1. (By manipulating only the composition of the basal medium, the new formulation, EpiLife™, supports extended lifespan of neonatal (1a) and adult (1b) keratinocytes.) Cryopreserved primary cultures of normal human epidermal keratinocytes (neonatal, HEKn, 1a; or adult, HEKa, 1b.) were thawed and plated at a density of 2,500 cells/cm^2 into EpiLife™ medium supplemented with Human Keratinocyte Growth Supplement (HKGS), or standard serum-free keratinocyte media: Medium 154 (M154) supplemented with HKGS (1a and 1b), and KGM-2, prepared as directed by the manufacturer (1a). When the cell density reached approximately 80% confluence, triplicate cultures in each condition were harvested, cell numbers determined and new subcultures established. The number of cumulative population doublings was calculated as described in the Methods and Materials.

Fig. 1a HEKn

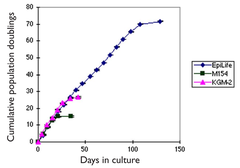


Fig. 1b HEKa

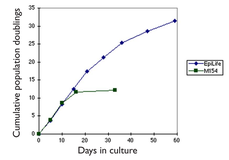


Figure 2. Keratinocytes cultured in EpiLife™ maintain normal epithelial morphology and acquire senescent characteristics at much higher population doublings. HEKn were cultured in the indicated conditions as described in Figure 1a. Phase contrast photomicrographs of cells growing in each condition were taken at indicated culture levels.

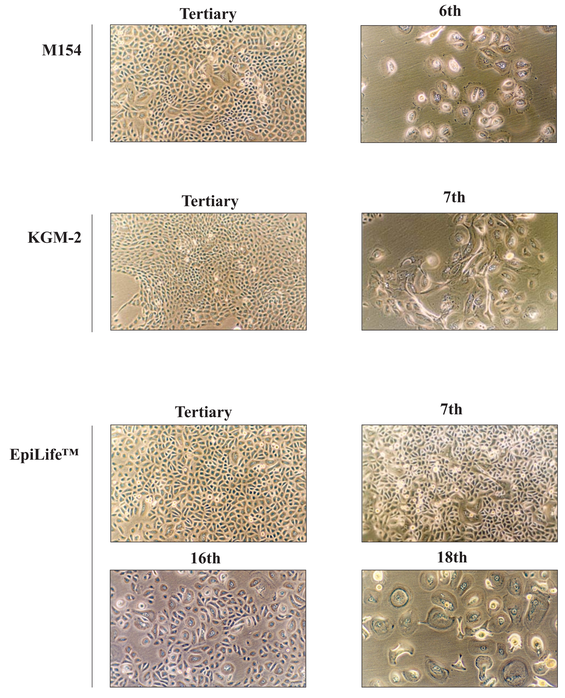


Fig. 3. "Rapidly growing" keratinocytes cultured in EpiLife™ express basal keratin (K14) and basonuclin mRNA. Under "differentiation conditions", the cells lose basonuclin expression and are induced to express K10 and involucrin mRNA. Keratinocytes were grown in Medium 154 or EpiLife™ Medium supplemented with HKGS to the tertary culture (M154 and EpiLife™) or the eighth culture (EpiLife™ only). For "rapidly growing" cultures, RNA was isolated from cells that were 50-80% confluent. For "differentiation conditions", the cells were grown to confluence in the indicated medium supplemented with HKGS, and then switched to unsupplemented medium containing 0.2 mM calcium. RNA was isolated after five days of incubation in differentiation conditions. Two identical northern blots were prepared (5 µg per lane) and hybridized with ^{32}P-labeled probes for either Keratin 10 mRNA (K10, 2.0 kb), Panel A, or Keratin 14 mRNA (K14, 1.6 kb), Panel B. The probed blots were exposed to film and the resulting exposures scanned for this presentation. The blots were subsequently hybridized with a 28S ribosomal RNA probe to insure that intact RNA was loaded into each lane. The blot shown in Panel B was allowed to decay and was subsequently hybridized with a ^{32}P-labeled probe for involucrin mRNA (2.1 kb). The results for involucrin are shown in Panel C. The blot in Panel A was allowed to decay and was subsequently hybridized with a ^{32}P-labeled probe for basonuclin mRNA (5.3 kb). The results for basonuclin are shown in Panel D.

Left to Right in Each Panel:
Lane 1. Tertiary culture, rapidly growing in Medium 154 supplemented with HKGS
Lane 2. Tertiary culture, confluent, 5 days in Medium 154, differentiation conditions
Lane 3. Tertiary culture, rapidly growing in EpiLife™ Medium supplemented with HKGS
Lane 4. Tertiary culture, confluent, 5 days in EpiLife™ Medium, differentiation conditions
Lane 5. Eighth culture, rapidly growing in EpiLife™ Medium supplemented with HKGS
Lane 6. Eighth culture, confluent, 5 days in EpiLife™ Medium, differentiation conditions

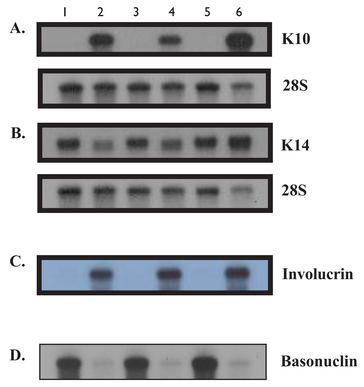
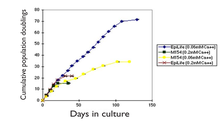


Table 1. Common Components of Basal Media for Human Keratinocytes

A. Major ions (7)		C. Vitamins (9)		E. Trace elements (10)	
•calcium	•potasium	•biotin	•pyridoxine	•copper	•selenium
•chloride	•sodium	•folate	•riboflavin	•iron	•silicon
•magnesium	•sulfate	•lipoate	•thiamine	•manganese	•tin
•phosphate		•niacinamide	•vitamin B12	•molybdenum	•vanadium
		•pantothenate		•nickel	•zinc
B. Amino Acids (20)		**D. Other organic compounds (11)**		**F. Buffers and indicators (4)**	
•alanine	•isoleucine	•acetate	•phospoethanolamine	•bicarbonate	
•arginine	•leucine	•adenine	•putrescine	•carbon dioxide	
•asparagine	•methionine	•choline	•pyruvate	•HEPES	
•aspartate	•phenylalanine	•ethanolamine	•thymidine	•phenol red	
•cysteine	•proline	•glucose			
•cystine	•serine	•i-inositol			
•glutamate	•threonine				
•glutamine	•tryptophan				
•glycine	•tyrosine				
•histidine	•valine				

Fig 4. A low concentration of calcium in the basal medium is required but not sufficient for the extended in vitro lifespan of keratinocytes. Cryopreserved primary cultures of normal human epidermal keratinocytes (neonatal, HEKn) were thawed and plated at a density of 2,500 cells/cm^2 into EpiLife™ medium or Medium 154, both prepared with calcium concentrations of either 0.06 mM or 0.2 mM, respectively. All media were supplemented with Human Keratinocyte Growth Supplement (HKGS). When the cell density reached approximately 80% confluence, triplicate cultures in each condition were harvested, cell numbers determined and new subcultures established. The number of cumulative population doublings was calculated as described in the Methods and Materials.



Conclusion

We have shown that manipulating only the composition of the basal medium, which contains no hormones or macromolecules (see Table 1), extends the lifespan of human epidermal keratinocytes cultured without serum or fibroblast feeder layers. Keratinocytes cultured in the optimized formula that we developed maintain a normal epithelial phenotype and acquire senescence at much higher population doublings. We believe that this medium will be useful for investigators studying cellular aging mechanisms. In addition, the new medium should be a useful tool for scientists requiring large banks of keratinocytes for research, diagnostic or therapeutic purposes.

References

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