A novel culture medium ages keratinocytes without the need for pro-aging stimuli

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Background

A cell’s environment plays a dominant role in determining its behaviour, both in vivo and in vitro. The aging process in vivo is an excellent example of environmental changes altering cell function. Age-related loss of cell function is the result of a complex cascade of events (summarised in Fig. 1). The cascade begins with intrinsic and extrinsic aging stimuli, including telomere shortening, DNA damage, and metabolic stress, that over time are able to overwhelm the cell’s protective mechanisms. These stimuli cause a range of age-related cellular changes including mitochondrial disruption, altered signaling, DNA damage, apoptosis and senescence. These changes result in reduced cell function (in particular of stem/progenitor cells), that leads to a reduction in organ function and regenerative potential [1, 2].

The environment is equally critical in vitro, where the ability of specific factors to retain stem cells or drive differentiation is now well known. Culture media also contain a wide variety of highly protective and stimulatory factors to increase growth rate and longevity. As a result, culture media are strongly anti-aging. The anti-aging nature of standard media means that traditional methods to induce aging in vitro typically depend on either extended in vitro culture (repetitive aging), or exposure to highly concentrated pro-aging stimuli such as peroxide or methylglyoxal. Such methods can induce selected signs of cellular aging, but are expensive and time consuming, and are not always representative of in vivo aging stimuli.

The new VitroAge culture medium (CnT-AG2) uses an alternative approach in which keratinocytes are aged by a culture environment that lacks anti-aging and protective ingredients, but contains the addition of artificial pro-aging stimuli. Keratinocytes grown in VitroAge medium for 3 weeks were found to retain normal morphology, but demonstrated increased redox state and longevity, disrupted metabolism, and increased protein oxidation. Proteomic analysis revealed changes in key age-related protein groups: protein biosynthesis, metabolism, DNA/telomere repair, proteosome/progenitor cell markers, and redox/antioxidant mechanisms (Fig. 5). The MRM analysis of 90 significantly regulated age-associated proteins included the following:

- Mitochondrial dysfunction
- Altered signaling
- DNA damage, apoptosis and senescence
- Stress and inflammation
- Proteosome/oxidized-protein turnover
- Basal/stem cell markers

These results indicate that keratinocytes grown in CnT-AG2 undergo a variety of phenotypic changes known to occur in vivo in humans as peroxide or methylglyoxal-induced aging.

Reduced Proliferation and Longevity

Although epidermal stem cell number appears to remain fairly constant with age, it appears that transdifferentiating cells undergo a decrease in number, including increased cell death (up to 3x higher), and fewer divisions before differentiation [3, 4].

Primary keratinocytes growing in a Progenitor Cell Targeted (PCT) medium from CELInTEC display many features of transdifferentiating progenitor cells, including morphology and marker expression. In control medium (CnT-07), primary keratinocytes deliver consistent growth rates of 3-4 population doublings per week, typically for 30-40 population doublings. In contrast, after keratinocytes are transferred to VitroAge medium (CnT-AG2), the proliferation rate declines steadily over three weeks, and then nearly ceases in week 4 (Fig. 2). Normal keratinocyte morphology is maintained throughout the aging process (Fig. 3).

Increased Protein Oxidation

The accumulation of oxidised proteins (as measured by the protein carbonyl content) in aged tissues is a well-documented sign of aging. In many tissues, including skin, protein carbonyl content can double during the second half of an organism’s lifespan [5].

FACS analysis of primary keratinocytes grown for 3 weeks in CnT-AG2 (VitroAge) medium (Fig. 4) revealed that the level of protein carbonylation increased by 155% compared to young cells cultured in CnT-07 (standard medium) vs young cells aged with the CnT-420 Vitrage medium (“in-vitro”). Keratinocytes aged in VitroAge medium (CnT-AG2) for 3 weeks were found to demonstrate a range of changes in protein oxidation vs CnT-07 (standard medium) and CnT-AG2 (VitroAge) medium (Fig. 5).

Disrupted Metabolism and Membranes

It has recently emerged that the mitochondrial membrane is a key site of age-related changes [1, 2], where oxidative damage to the mitochondrial membrane is and its cytochromes results in increased production of ROS, additional DNA and membrane damage, plus the sustained p53 activation that is now thought to reduce stem cell function and increase apoptosis, senescence, and inflammation.

Keratinocytes grown for 3 weeks in CnT-420 medium were found to display a significantly reduced mitochondrial membrane potential (Fig. 6). Culture in CnT-420 with a protective active ingredient complex (AG2 + active ingredient) maintained increased membrane potential (+3% vs CnT-420). Integrity of the cell membrane has long been known to decrease with age, leading to increased permeability [6, 7]. Cell membrane permeability was evaluated by fluorescence in keratinocytes isolated from young and old donors (labeled “in-vitro”), for comparison with young cells cultured in CnT-420 (standard medium) vs cells aged with the CnT-420 Vitrage medium (“in-vitro”).

Age-Related Protein Expression

A novel Multiplex Reaction Monitoring (MRM) proteomics method developed by Biognoys AG now enables the quantitative determination of up to 100 proteins in a single sample. This method was used to identify changes in keratinocytes after 3 weeks of culture in either CnT-07 (standard) or CnT-AG2 (VitroAge) medium. Over 300 significantly up- or down-regulated proteins were identified (regression >0.9%, p-value <0.05), and grouped into functional clusters using the David Bioinformatics database. Keratinocytes aged in VitroAge medium demonstrated clusters of down regulated proteins associated with protein biosynthesis, metabolism, internal/external transport repair, Up regulated functional clusters included proteins involved in inflammation, and antioxidant mechanisms.

The MRM analysis of 83 significantly regulated age-associated proteins included the following:

- Mitochondria / Protein Synthesis
- p53
- Glutathione S-transferase
- Poly ADP ribose polymerase
- Mitochondrial membrane (CnT-AG2 + active ingredient)

Age-related changes in keratinocytes grown in VitroAge medium (CnT-AG2) were also assessed using an active ingredient complex (CnT-AG2 + active protective agent). Changes were found to be in percent, as control (Vitrage medium in CnT-420), and percentages are absolute values (denote up or down regulation).

Summary and Conclusion

Keratinocytes aged in VitroAge medium (CnT-AG2) for 3 weeks were found to demonstrate a range of changes in cell function and protein expression known to occur with age in vivo. Cells retained normal morphology, but demonstrated reduced cell growth rate and longevity, increased protein oxidation, and disrupted mitochondrial and cell membranes.


These results indicate that keratinocytes grown in CnT-AG2 undergo a variety of phenotypic changes known to associate with age, without the need for synthetic pro-aging treatments such as peroxide or methylglyoxal.

Changes in the model can be quantified using a multiplex MRM proteomics workflow, allowing detailed mechanistic insights into the cellular response to an aging environment, and into the ways in which active ingredients can ameliorate age-related changes.

3. Winter MC, Bickenbach JR. Aging epidermis is maintained by changes in transit-amplifying cell kinetics, not stem cell kinetics. JID 129, (2009)