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Abstract	Studying age-related changes in the physiology, biochemistry, and molecular biology of isolated skin cell populations in culture has greatly expanded the understanding of the fundamental aspects of skin aging. The three main cell types that have been studied extensively with respect to cellular aging in vitro are dermal fibroblasts, epidermal keratinocytes, and melanocytes. Serial subcultivation of normal diploid skin cells can be performed only a limited number of times, and the emerging senescent phenotype can be categorized into structural, physiological, biochemical, and molecular phenotypes, which can be used as biomarkers of cellular aging in vitro. The rate and phenotype of aging are different in different cell types. There are both common features and specific features of aging of skin fibroblasts, keratinocytes, melanocytes, and other cell types. A progressive accumulation of damage in all types of macromolecules is a universal feature of cellular aging in all cell types. A progressive failure of molecular maintenance and repair pathways is the ultimate cause of cellular aging in vitro and in vivo.	
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Aging and Senescence of Skin Cells in Culture

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Contents

Abstract

Studying age-related changes in the physiology, biochemistry, and molecular biology of isolated skin cell populations in culture has greatly expanded the understanding of the fundamental aspects of skin aging. The three main cell types that have been studied extensively with respect to cellular aging in vitro are dermal fibroblasts, epidermal keratinocytes, and melanocytes. Serial subcultivation of normal diploid skin cells can be performed only a limited number of times, and the emerging senescent phenotype can be categorized into structural, physiological, biochemical, and molecular phenotypes, which can be used as biomarkers of cellular aging in vitro. The rate and phenotype of aging are different in different cell types. There are both common features and specific features of aging of skin fibroblasts, keratinocytes, melanocytes, and other cell types. A progressive accumulation of damage in all types of macromolecules is a universal feature of cellular aging in all cell types. A progressive failure of molecular maintenance and repair pathways is the ultimate cause of cellular aging in vitro and in vivo.

Introduction

In modern biogerontology, the terms "cellular aging," "cell senescence," and "replicative senescence" most commonly imply the study of normal

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diploid cells in culture, which during serial subcultivation undergo a multitude of changes culminating in the permanent cessation of cell division. This process of cellular aging in vitro is generally known as the Hayflick phenomenon, and the limited division potential of normal cells is called the Hayflick limit, in recognition of the observations first reported by Leonard Hayflick in 1961 [1–4]. The study of age-related changes in the physiology, biochemistry, and molecular biology of isolated skin cell populations in culture has greatly expanded the understanding of the fundamental aspects of skin aging. The three main cell types that have been studied extensively with respect to cellular aging in vitro are dermal fibroblasts, epidermal keratinocytes, and melanocytes [5–11].

AU2 The aim of this article is to describe the experimental system of aging of skin cells in culture; to provide an overview of the age-related changes in the structural and functional aspects of cells including physiological, biochemical, and molecular changes; and to evaluate the use of such a system in testing and developing effective interventions for maintaining and/or re-achieving a healthy skin during aging.

Experimental Model System of Cellular Aging in Culture

Once the primary culture of normal cells is established in culture from the normal tissue (e.g., a skin biopsy), by using any of the standard methods such as the explant growth and enzymic dissociation of cells, the primary culture can then be subcultivated repeatedly each time it becomes confluent. This repeated subculturing of cells is also known as serial passaging [4]. In a description of the Hayflick phenomenon, Phase I is the period of the establishment of the primary culture from the normal tissue, Phase II is a relatively long period of serial passaging, growth, and cell proliferation at a constant rate, and Phase III is the final period of slowing-down of growth, which results in the cessation of cell division and end of replicative life span of cells. The whole duration of serial passaging is considered as the process of cellular aging, and the end-stage irreversible growth arrest in G_1 is termed as replicative senescence [1, 2].

After reaching a state of replicative senescence, some cells can still stay alive and be metabolically active at a minimal level for sometime and generally resist undergoing apoptosis [3, 4]. Although the exact culturing conditions, such as the type of the culture medium, the source of growth factors, the use of antibiotics, and the incubation temperature, humidity, and gaseous composition, may vary for different cell types, serial subcultivation of normal diploid cells can be performed only a limited number of times. This is in contrast to the high proliferative capacity of transformed, cancerous, and immortalized cells, whose cultures can be subcultivated and maintained indefinitely [3, 4].

The total number of cell divisions, measured as the cumulative population doublings (CPD), which can be achieved by a specific cell type in vitro, depends upon several biological factors. These include the maximum life span of the species, the developmental and adult age of the donor of the tissue biopsy, the site of the biopsy, and the health status of the donor [12]. For example, for human fibroblasts, the range of CPD for the cell strains originating from embryonic tissues is between 50 and 70, whereas for those originating from adult biopsies, it is generally less than 50 CPD. A similar range for CPD attained by human keratinocytes and melanocytes has been reported [5–10]. Additionally, gaseous composition, especially oxygen levels, and the quality of the nutritional serum and growth factors added to the culture medium can significantly affect the proliferative life span of cells in vitro. For example, culturing of fibroblasts in vitro in the air with about 20 % oxygen levels reduces their replicative life span which could be otherwise achieved at low-level (2 %) concentration akin to in vivo conditions [13, 14]. Furthermore, the site of the skin biopsy, for example, sun-exposed versus sun-protected area, has a significant effect on the CPD levels achieved by cells in culture [4, 15].

The Phenotype of Aging Skin Cells

Serial passaging of normal diploid skin cells is accompanied by a progressive and accumulative occurrence of a wide variety of changes before the final cessation of cell replication occurs. The emerging senescent phenotype of serially passaged normal diploid skin cells can be categorized into the structural, physiological, biochemical, and molecular phenotypes, which can be used as biomarkers of cellular aging in vitro, as summarized in Tables 1, 2, and 3. There are more than 200 such structural, physiological, biochemical, and molecular characteristics that have been studied during cellular aging, and a list of major characteristics that appear progressively in cell cultures and distinguish between young and senescent cells, generally before the end of proliferative life span and their irreversible arrest in the G_1 phase of the cell cycle, can be found in several publications [3, 4]. Here a summary of such phenotypic changes is given below.

(A) *Structural phenotype of aging skin cells*: Table 1 lists the major structural changes observed in aging skin cells in culture. Most commonly, a progressive increase in cell size and the loss of homogenous morphological pattern are the most dramatic and easily identifiable differences in early-passage young and late-passage old or senescent skin cells (Fig. 1).

Other structural changes during aging of skin cells include cytoskeletal and membrane rigidity, altered extracellular matrix, accumulation of intracellular debris, and incomplete cytokinesis leading to multi-nucleation.

In addition to the gross structural alterations listed in Table 1, there are several ultrastructural changes reported by using electron microscopic methods. These include the presence of distorted mitochondria, increased level of chromosomal aberrations, overcondensation of chromatin, increased nucleolar fragmentation, and the accumulation of lipid-protein conjugate lipofuscin in lysosomes [16–18].

Table 1 Structural phenotype of skin cells undergoing aging in vitro

Increased cell size
Change of shape from thin, long, and spindle-like to flattened and irregular
Loss of whorl-like arrangement in parallel arrays on the cell culture substrate
Rodlike polymerization of the cytoskeletal actin filaments and disorganized microtubules
Increased membrane rigidity
Increased multi-nucleation
Increased number of vacuoles and dense lysosomal autophagous bodies

Table 2 Physiological phenotype of skin cells undergoing aging in vitro

Altered calcium flux, pH, viscosity, and membrane potential
Reduced activity of ionic pumps
Reduced mobility
Reduced respiration and energy production
Reduced response to growth factors and other mitogens
Increased sensitivity to toxins, drugs, irradiation, and other stresses
Increased basal levels of autophagy
Increased basal levels of stress proteins

Table 3 Biochemical and molecular phenotype of skin cells undergoing aging in vitro

Permanent growth arrest in late G_1 phase of the cell cycle near the S phase boundary
Increased mRNA and protein levels of cell cycle inhibitors
Increased mRNA and protein levels of inhibitors of proteases
Decreased expression, levels, and activities of numerous housekeeping enzymes
Decreased expression, levels, and activities of macromolecular turnover pathways
Reduced levels of methylated cytosines in the DNA
Reduced length of telomeres
Increased levels of damage in nuclear and mitochondrial DNAs
Altered profiles of micro-RNAs
Increased levels of damaged and abnormal proteins
Increased levels of macromolecular cross-linking
Increased levels of reactive oxygen species
Altered profile of secreted proteins

Fig. 1 Figure shows Giemsa-stained light microscopic phase contrast pictures of serially passaged human skin fibroblasts at various points in their in vitro life span. Sparse and confluent cultures at three stages during replicative life span are compared: (1) early-passage young adult skin fibroblasts with less than 30 % life span completed, (2) middle-aged cells with 60–80 % replicative life span completed, and (3) late-passage senescent cells with more than 95 % life span completed

(B) *Physiological phenotype of aging skin cells*: Numerous studies have been performed elucidating changes in various functional and physiological parameters of skin cells undergoing aging. Table 2 lists some of the main such changes, which clearly indicate that almost all aspects of cellular function and physiology become impaired during aging. Collectively, these data show that aging skin cells progressively become less active, have reduced the ability to maintain various physiological functions, and become more prone to the negative effects of harmful substances.

Altered responsiveness of cells during aging is one of the most significant age-related changes, which can be a rate-limiting factor for the use of any potential modulators of aging. Several studies have been performed in order to understand the mechanisms for age-related alteration of responsiveness, and the pathways include unaltered receptor numbers and affinities, ineffective signal transduction, and interrupted networks [12]. Furthermore, increased levels of intracellular stress as manifested in increased basal levels of stress proteins and autophagy in serially passaged senescent skin cells are a hallmark of cellular aging [19].

(C) *Biochemical and molecular phenotype of aging skin cells*: At the biochemical and molecular levels, a large body of data is available which indicates that skin cells undergo a plethora of changes, which form the mechanistic bases of structural and physiological alterations. Table 3 gives a list of main categories of biochemical and molecular changes that have been reported in aging skin cells in culture.

Depending on the available technologies and the prevailing trends, changes in the amounts and activities of thousands of proteins and in the levels of thousands of mRNAs have been reported for aging skin cells. Recently, data are beginning to be collected for age-related changes in the so-called epigenome, micro-RNAs, metabolome, secretome, and proteome, including posttranslational modifications [15, 20]. All such data will further strengthen the descriptive understanding of the phenomenon of aging of skin cells.

Although every single piece of descriptive data for aging skin cells is yet to be collected, a generalized picture of the aging phenomenon has emerged, Therefore, based on the large amount of data collected so far, important inferences and generalizations can already be made, which have implications with respect to developing effective interventions for a healthy skin. These are as follows:

1. The rate and phenotype of aging are different in different cell types. There are both common features and specific features of aging of skin fibroblasts, keratinocytes, melanocytes, and other cell types.
2. A progressive accumulation of damage in all types of macromolecules is a universal feature of cellular aging in all cell types.
3. Replicative senescence of cells in culture is not due to the activation of any aging-specific genes but is an indirect consequence of occurrence and accumulation of molecular damage and molecular heterogeneity.
4. A progressive failure of molecular maintenance and repair pathways is the ultimate cause of cellular aging.

From Cellular Aging In Vitro to Understanding Aging In Vivo

The Hayflick system of aging of skin cells in culture has proved to be very useful in developing the cellular and molecular understanding of the overall process of aging. A loss of proliferative capacity of any of the cell types has a deteriorative impact on the functioning and survival of the entire organism. A loss or slowing-down of proliferation of osteoblasts, glial cells, myoblasts, epithelial cells, lymphocytes, and fibroblasts can lead to the onset of many age-related diseases and impairments including osteoporosis, arthritis, immune deficiency, altered drug clearance, delayed wound healing, and altered functioning of the brain. Furthermore, the occurrence of fully senescent or near-senescent heterogenous cells in vivo can promote dysfunctioning of the other tissues by producing harmful signals and can also promote and stimulate the growth of other precancerous and cancerous cells [21, 22].

However, the existence of the Hayflick-type senescent cells in vivo is not very well established so far. A commonly used biomarker of senescent cells is the so-called senescence-associated beta-galactosidase (SABG), which has been used to demonstrate the presence of senescent cells in the human skin and some other tissues [23]. However, there are several limitations regarding the use of SABG as a marker of cellular aging in vitro, since SABG can also be detected in immortal cells under various conditions [24]. More and multiple independent markers of senescent cells are needed for this purpose.

The correlation between cellular aging in vitro and in vivo is often based on the evidence gathered from studies on the effects of donor age, species life span, and premature aging syndromes on cellular proliferative capacity in culture. These studies indicate that the genetic and intrinsic Hayflick limit of diploid cell strains in culture is a true reflection of what is going on during aging of an organism. However, there are some recent critiques of this based on the replicative potential of stem cells, which in the case of the skin appear to be maintained throughout the life span [25, 26]. Similarly, some changes observed in the Hayflick system in vitro, such as increased basal levels of autophagy, may not be present in the aging skin tissues [19]. In contrast to this, there is evidence showing that the stem cell population in the skin also undergoes aging and the number of stem cells declines as a function of donor age and during aging of the skin equivalents in vitro [27].

Modulators of Aging Skin Cells

The Hayflick system of cellular aging in culture is primarily a model for the study of slow and progressive accumulation of damage resulting in the arrest of cells in a non-proliferative state [4]. This system has been proved to be very useful for testing various physical, chemical, and biological conditions for their harmful or beneficial effects and for understanding other aspects of cellular aging with implications in the origin of age-related diseases. For example, irradiation; severe oxidative stress by UV, hydrogen peroxide, or dicarbonyls; and gene transfection have been used to induce a sudden and rapid increase in molecular damage, resulting in premature appearance of the senescent phenotype [28]. On the other hand, insertion of catalytically active component of the telomerase gene can completely bypass the Hayflick limit in many cell types including skin cells, and such cells can proliferate indefinitely with or without becoming transformed [29]. Similarly, normal diploid cells can be transformed and immortalized by chemical carcinogens, irradiation, and viral genes. Such approaches are helpful for unraveling the molecular details of cell cycle regulation in normal cells and its dysregulation in cancer cells [29].

The Hayflick system of cellular aging in culture has also been very useful for testing various natural and synthetic molecules as potential antiaging and health-promoting compounds for the skin. Some of the well-tested examples are cytokinins kinetin and zeatin [30], a dipeptide carnosine [31], curcumin which is a component of the spice turmeric [32, 33], and extracts from medicinal plants and sea algae [34]. Several of

these tests have resulted in the successful development, production, and marketing of various products with pharmaceutical, cosmeceutical, and nutritional applications [34].

Another use of the model system of cellular aging in culture has been to test the principle of mild stress-induced beneficial and antiaging effects, which is the phenomenon of hormesis [35]. For example, human skin fibroblasts and keratinocytes exposed to repeated mild heat stress (41 °C, 1 h, twice a week) show several hormetic effects, such as improved protein degradation pathways, higher levels of chaperones, increased resistance to other stresses, improved differentiation, and increased proliferative life span [10]. Such studies can form the basis of testing novel hormetic agents, including potential hormetins of natural or synthetic origin, for improved skin care during aging [10, 35, 36].

Conclusion

It could be reemphasized that the present understanding of the cellular and molecular basis of aging of the skin owes a lot to the use of the Hayflick system of aging of skin cells in culture. Most importantly, studies performed by using this model system have demonstrated that aging of cells is characterized by the accumulation of damage in various molecules that results in the failure of maintenance and repair systems. Detailed genomic, proteomic, and metabolomic studies using this system can further identify the interacting networks of regulatory pathways, which will then be accessible to modulation for the maintenance of the structural and functional integrity of the skin.

References

1. Hayflick L, Moorhead PS. The serial cultivation of human diploid strains. Exp Cell Res. 1961;25:585–621.
2. Hayflick L. The limited in vitro lifetime of human diploid cell strains. Exp Cell Res. 1965;37:614–36.
3. Campisi J, d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol. 2007;8:729–40.
4. Rattan SIS (2008) Cellular senescence in vitro. In: Encyclopedia of life sciences, doi:10.1002/9780470015902.a0002567.pub2 AU4
5. Norsgaard H, et al. Distinction between differentiation and senescence and the absence of increased apoptosis in human keratinocytes undergoing cellular aging in vitro. Exp Gerontol. 1996;31:563–70.
6. Yaar M, Gilchrest BA. Ageing and photoageing of keratinocytes and melanocytes. Clin Exp Dermatol. 2001;26:583–91.
7. Lin JY, Fisher DE. Melanocyte biology and skin pigmentation. Nature. 2007;445:843–50.
8. Berge U, et al. Sugar-induced premature aging and altered differentiation in human epidermal keratinocytes. Ann NY Acad Sci. 2007;1100:524–9.
9. Berge U, et al. Kinetin-induced differentiation of normal human keratinocytes undergoing aging in vitro. Ann NY Acad Sci. 2006;1067:332–6.
10. Berge U, et al. Hormetic modulation of differentiation of normal human epidermal keratinocytes undergoing replicative senescence in vitro. Exp Gerontol. 2008;43:658–62.
11. Tran SL, et al. Absence of distinguishing senescence traits in human melanocytic nevi. J Invest Dermatol. 2012;132:2226–34.
12. Cristofalo VJ, et al. Replicative senescence: a critical review. Mech Ageing Dev. 2004;125:827–48.
13. Packer L, Fuehr K. Low oxygen concentration extends the lifespan of cultured human diploid cells. Nature. 1977;267:423–5.
14. Chen Q, et al. Oxidative DNA damage and senescence of human diploid fibroblast cells. Proc Natl Acad Sci U S A. 1995;92:4337–41.
15. Holly AC, et al. Comparison of senescence-associated miRNAs in primary skin and lung fibroblasts. Biogerontology. 2015;16(4):423–34.
16. Macieira-Coelho A. Ups and downs of aging studies in vitro: the crooked path of science. Gerontology. 2000;46:55–63.
17. Terman A, et al. Autophagy, organelles and ageing. J Pathol. 2007;211:134–43.
18. Swanson EC, et al. Higher-order unfolding of satellite heterochromatin is a consistent and early event in cell senescence. J Cell Biol. 2013;203:929–42.
19. Demirovic D, et al. Basal level of autophagy is increased in aging human skin fibroblasts in vitro, but not in old skin. PLoS One. 2015;10:e0126546.
20. Waldera Lupa DM, et al. Characterization of skin aging-associated secreted proteins (SAASP) produced by dermal fibroblasts isolated from intrinsically aged human skin. J Invest Dermatol. 2015;135:1954–68.
21. Campisi J. Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. Cell. 2005;120:513–22.
22. Blagosklonny MV, Campisi J. Cancer and aging: more puzzles, more promises? Cell Cycle. 2008;7:2615–8.

23. Dimri GP, et al. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci U S A. 1995;92:9363–7.
24. Yang NC, Hu ML. The limitations and validities of senescence associated-b-galactosidase activity as an aging marker for human foreskin fibroblast Hs68 cells. Exp Gerontol. 2005;40:813–9.
25. Rubin H. The disparity between human cell senescence in vitro and lifelong replication in vivo. Nat Biotechnol. 2002;20:675–81.
26. Giangreco A, et al. Epidermal stem cells are retained in vivo throughout skin aging. Aging Cell. 2008;7:250–9.
27. Youn SW, et al. Cellular senescence induced loss of stem cell proportion in the skin in vitro. J Dermatol Sci. 2005;35:113–23.
28. Sejersen H, Rattan SIS. Dicarbonyl-induced accelerated aging in vitro in human skin fibroblasts. Biogerontology. 2009;10:203–11.
29. Collado M, et al. Cellular senescence in cancer and aging. Cell. 2007;130:223–33.
30. Rattan SIS, Clark BFC. Kinetin delays the onset of ageing characteristics in human fibroblasts. Biochem Biophys Res Commun. 1994;201:665–72.
31. McFarland GA, Holliday R. Retardation of the senescence of cultured human diploid fibroblasts by carnosine. Exp Cell Res. 1994;212:167–75.
32. Lima CF, et al. Curcumin induces heme oxygenase-1 in normal human skin fibroblasts through redox signaling: relevance for anti-aging intervention. Mol Nutr Food Res. 2011;55:430–42.
33. Demirovic D, Rattan SIS. Curcumin induces stress response and hormetically modulates wound healing ability of human skin fibroblasts undergoing ageing in vitro. Biogerontology. 2011;12:437–44.
34. Rattan SIS, et al. Hormesis-based anti-aging products: a case study of a novel cosmetic. Dose Response. 2013;11:99–108.
35. Rattan SIS. Hormesis in aging. Ageing Res Rev. 2008;7:63–78.
36. Rattan SIS. Hormetic modulation of aging in human cells. In: Le Bourg E, Rattan SIS, editors. Mild stress and healthy aging: applying hormesis in aging research and interventions. Dordrecht: Springer; 2008. p. 81–96.

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