How might replicative senescence contribute to human ageing?

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Summary

Cell senescence is the limited ability of primary human cells to divide when cultured in vitro. This eventual cessation of division is accompanied by a specific set of changes in cell physiology, morphology, and gene expression. Such changes in phenotype have the potential to contribute to human ageing and age-related diseases. Until now, senescence has largely been studied as an in vitro phenomenon, but recent data have for the first time directly demonstrated the presence of senescent cells in aged human tissues. Although a direct causal link between the ageing of whole organisms and the senescence of cells in culture remains elusive, a large body of data is consistent with cell senescence contributing to a variety of pathological changes seen in the aged. This review considers the in vitro phenotype of cellular senescence and speculates on the various possible routes whereby the presence of senescent cells in old bodies may affect different tissue systems. *BioEssays* 20:985–991, 1998.

Introduction

With the proportion of the elderly within the population set to increase dramatically, ageing will become the major health-care challenge of the next century. It has been predicted that by the year 2015 approximately a fifth of the UK population will fall into the over 60 age category.(1) Human ageing is associated with an increased chance of death, mostly due to a subset of diseases largely restricted to later life. These diseases, which were first recognised as being age-related by the nineteenth century mathematician Gompertz,(2) include potentially fatal cardiovascular disorders, diabetes and neoplasms, and crippling conditions such as cataract, macular degeneration, auditory impairment, and neurodegeneration, all of which can greatly reduce the quality of later life. Although at first glance these diseases might appear more common nowadays, this is largely because most people in the past simply did not survive long enough to suffer from them. Cancer, heart disease, and cataract are by no means new diseases, having been reported throughout recorded history. For example, the Greek statesman Timoleon was forced to retire from public life in 336 BC due to age-related blindness, and the Byzantine empress Theodora died from throat cancer in 548 AD.(3) The diseases of later life have become the major causes of morbidity and death in the West today simply because there is practically nothing else left to routinely kill people.

Whilst living longer is vastly preferable to the alternative, the downside is a population which contains more people with distressing or fatal age-related conditions. The prospect of an increasing fraction of the population with such chronic health-care needs is not reassuring, whether viewed from a social or a financial perspective. Since these diseases are age-related, understanding the normal process of human ageing may give important insights into the means by which they develop.

Why do we age? Evolutionary considerations argue that organisms do not age "for" any particular reason but rather as a side effect of the optimisation of evolutionary fitness in animal populations. This concept of antagonistic pleiotropy

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stems from the idea that the force of natural selection declines with age and that late-life deleterious effects are thus not effectively selected against. Postreproductive years are essentially removed from the force of natural selection since the phenotype of the organism in this period can no longer affect its ability to contribute to the next generation. Accordingly, any mutation which favours early reproductive success but produces detrimental effects later in life will be selected for; the result are a series of late-life emergent phenotypic changes which we call ageing.

These themes are explored in some detail in the disposable soma theory of ageing.[4] This argues that in nature a trade-off occurs between reproduction and somatic maintenance. For example, a short-lived animal, such as a vole, obtains resources in the form of food and distributes them between somatic repair and maintenance and reproduction. If more is used for reproduction, correspondingly less is available for somatic repair. In the wild, voles do not live for very long because of environmental hazards such as predation. Thus, there is no selectable advantage to a vole in having a body that could live for 50 years if it has a minimal chance of living for more than 2 before being eaten. A more sensible strategy to maximise offspring is thus to allocate the bulk of resources for a high rate of reproduction. Although this balance between somatic repair and reproduction was originally set in the natural environment, it can still be seen when animals are placed in artificial environments without major hazards. Immortality cannot and does not occur.

Cellular senescence itself is readily explainable in such evolutionary terms. It seems to have arisen because of selection for its tumour-suppressive function (see accompanying article by Reddel). The doubling potential of mammalian cells may well be set at a point sufficient to allow for normal growth, development, and cellular turnover during life but not so great as to allow the large-scale cell division seen during the establishment and progression of a potentially life-threatening cancer. For this to occur, replicative life span checkpoints must be bypassed, for example, by mutation in genes such as p53. However, the side effect of a mechanism (limited cellular life span) that successfully delays most human cancers until the postreproductive years is the occurrence of senescent cells and, thus, perhaps ageing.

Many molecular mechanisms may contribute to human ageing, but here we focus on the potential involvement of cellular senescence. The cell hypothesis of ageing was first advanced in its modern form by Hayflick[5] and proposes that senescence, the intrinsic upper limit to growth in normal cells, can also play a role in the ageing of tissues of which such cells form a part. It does not suggest that cellular senescence is the sole cause of ageing in humans, and indeed, it is singularly ill-suited to explain the ageing of tissues principally composed of nondividing cells. However, there are several regenerative tissues whose decline could have a significant impact on the ageing of an organism as a whole.

What is cellular senescence?

Normal human cells have a limited ability to proliferate in vitro before they enter a phase of viable cell cycle arrest called cellular senescence, a state associated with various changes in cell behaviour and gene expression.[6] ''Senescence'' in this context has a very specific meaning, as shown by the qualified term ''replicative senescence.'' Such a qualification avoids many potential confusions, such as the description of cells from nonregenerative tissues as ''senescent'' because they show impairment in aged individuals or growing cells from the elderly as ''senescent'' because they came from an old body. That neurones and other nondividing cell types show true ageing-related changes is unquestioned. However, these are preeminently progressive functional deficits, rather than specific changes resulting in viable permanent growth arrest. Replicative senescence occurs in cell populations with an intrinsic capacity for replacement. There is thus a basic distinction between regenerative adult tissue, which can show replicative senescence, and postmitotic cell populations, such as mature neurones, which cannot.

Replicative senescence of a culture of primary cells is its failure to grow under conditions which had previously allowed cell division. This finite life span, measured in population doublings (PDs), yields a final culture almost entirely composed of permanently growth-arrested senescent cells whose phenotype differs markedly from their dividing counterparts.[6] Senescence is not quiescence or terminal differentiation, nor is it cell death by either necrosis or apoptosis. Indeed, one of the earliest observations concerning the senescence of cell populations was that the phenomenon did not result from necrosis.[7] More recent observations have also demonstrated that, under normal conditions, senescent cells themselves are neither more apoptosis-prone nor apoptosis-resistant than their growing counterparts.[8] It has been suggested that senescence might represent a form of terminal differentiation.[9] However, merely because a cell is at the end of its developmental lineage (''terminally differentiated''), it is not necessarily nondividing.[10,11] A good example of this distinction is provided by epidermal keratinocytes, where a well-established differentiation pathway exists. A useful feature of keratinocyte cultures in vitro is the calcium dependence of the differentiation pathway, which is inhibited when the calcium in the medium is substituted with strontium or its concentration reduced below 0.1 mM. Keratinocyte cultures serially passaged at low calcium concentrations nonetheless enter senescence,[12] as do cells from the *NDK* (nondifferentiating keratinocyte) strain, in which the differentiation pathway is inoperative.[13] Furthermore, keratinocytes grown to senescence under low calcium conditions will still differentiate if the concentration is raised.[8] The evidence that senescence is

distinct from terminal differentiation is thus, in keratinocytes at least, rather strong.

Do senescent cells occur in vivo?

Cellular senescence as an in vitro phenomenon has been known for over 30 years, but only recently has direct proof of a similar in vivo process been obtained. Prior to this, evidence for the presence of senescent cells in ageing tissues was essentially circumstantial. For example, cell cultures initiated from embryonic fibroblasts achieve consistently higher PD levels than those grown from neonates.[5] Studies with very large numbers of subjects with chronological ages ranging from foetal to 100 years[14] demonstrate that the growth potential of these cultures declines at a rate of 0.2 PD per year of donor life. Similar data have been obtained for lens epithelial cells,[15] keratinocytes,[16] corneal endothelial cells,[17,18] and T cells.[10,19] These data can be interpreted as meaning either that all of the cells in the sample were closer to the end of their lifespan with none yet senescent or that significant numbers of senescent cells were present or both. For this reason, such data are at best merely consistent with the presence of senescent cells in old tissue and do not formally demonstrate their existence. However, it is worth stressing that the very fact that cells grow out of explanted tissue from elderly donors does in no way exclude the presence of significant numbers of senescent cells in that sample.

One operational, if somewhat indirect, criterion of senescence is a cell which fails to divide under appropriate mitogenic stimuli. Such growth-refractory cells can be visualised by continuous labelling of tissue and organ cultures (and even living animals) with bromodeoxyuridine (BrdUrd). Endothelial wound healing studies using organ culture of corneae from donors of different ages reveals an age-related increase in refractory cells.[20] Similar observations have been made in blood vessels, suggesting the presence of senescent vascular endothelial cells in regions of atherosclerotic stress.[21,22] However, the most direct evidence for senescent cells in vivo comes from a modified histochemical stain for β-galactosidase that is specific for senescent fibroblasts in vitro.[23] When applied to dermal tissue sections from donors with an age range of 20 to 90 years, a clear correlation between the number of senescent cells present and the age of the donor tissue is seen. To date, this study constitutes the best formal proof that senescent cells arise in aged tissues. It is this key observation that makes it timely to explore some of the potential ways whereby senescent cells might contribute to age-related decline in the elderly.

What causes cellular senescence?

Potential triggers and effectors of cellular senescence have been extensively reviewed.[6] There are no conclusive data regarding the nature of the hypothesised cell division counting ''clock.'' One widely discussed hypothesis is that the shortening of chromosomal telomeres in telomerase-negative human somatic cells eventually leads to ''critically short'' telomeres that signal cell cycle arrest. The recent observation that introduction of the catalytic subunit of human telomerase into mortal human cells prevents replicative senescence greatly strengthens the telomere-shortening hypothesis (see accompanying article by Reddel). However, the potential exists for senescence to be triggered by multiple pathways. For example, acute overexpression of activated H-*ras* (following infection with a retrovirus expressing ras^{V12}) leads rapidly to a phenotype similar to senescence, although this does cause highly elevated levels of p53, which is not the case for normal senescence.[24]

There are data to suggest that mitogenic signalling pathways can influence the decision to become senescent. For example, vascular endothelial growth factor delays the senescence of human dermal microvascular cells.[25] Antisense inhibition of the expression of the cytokine interleukin (IL)-1α significantly delays the onset of senescence in human umbilical vein endothelial cells (HUVECs).[26] As is usual with cellular signalling, it seems likely that senescence is triggered by complex mechanisms which can integrate numerous signals and are open to a variety of quantitative and qualitative modulations. This may result in marked tissue and species specificity; what is true in humans may not hold for mice.[27]

The link between cellular senescence and ageing

Cellular senescence and the division potential of explant cultures are dependent on donor age. Although this indicates that cellular senescence is a codependent variable, it gives no information regarding causality. Similarly, many of the changes in senescent cell behaviour are consistent with known changes in tissue and organ function with age, but again, this gives no insight into causal relationships. What then is the basis for suggesting that cellular senescence may contribute to human ageing? An important observation is that the growth potential of fibroblasts in culture correlates extremely well with the mean maximum life span of the species from which the tissue was derived.[28] Furthermore, cellular senescence and ageing co-vary in response to caloric restriction or mutation.

Currently, the only known way to retard the ageing process in animals (as measured by maximum life span, mean life span, or Gompertzian rate of increase in age-specific mortality) is to markedly reduce calorie intake below that seen in animals fed ad libitum. The numbers of division-competent cells in the lens epithelial tissue of young, old, and calorie-restricted mice were compared by continuous BrdUrd infusion in vivo (followed by histological examination) and by the analysis of colony size distribution in explant cultures of lens cells. Both assays showed that old mice had more senescent lens epithelial cells than their young counterparts and that

diet-restricted animals had lower levels of senescent cells than age-matched mice fed ad libitum.[29] Even though it falls short of a formal proof, the observation that calorie restriction not only extends life span and reduces a spectrum of age-related changes[30] but also reduces the rate of accumulation of senescent cells indicates a close relationship between replicative senescence and organismal ageing.

Cellular senescence can also be modulated by genetic mutations which alter the ageing process. Patients with Werner's syndrome (WS) display a range of in vivo changes which appear similar to those seen in normal ageing.[31] Fibroblast cultures from WS patients have a very limited proliferative ability owing to a greatly increased rate of entry into senescence.[32,33] There is some evidence for an increased rate of telomeric shortening[34] in such cells, perhaps related to the WS gene product being a DNA helicase,[35] mutation of which renders the cell prone to delete large regions of DNA.[36] WS patients show greatly elevated frequencies of age-related diseases such as atherosclerosis, arteriosclerosis, and cancer[31] together with impaired wound healing likened to that seen in elderly individuals.[31]

Potential impact of cellular senescence on human tissues

Despite what is known regarding the mechanisms whereby senescent cells occur, it should be stressed that this is not central to the cell hypothesis of ageing. This focuses on the occurrence of senescent cells, not the mechanism by which they become senescent. In the following section, we review some of the potential routes whereby the limited life span of somatic cells and the occurrence of senescent cells could impact on human ageing. These hypothetical routes are based on known patterns of the gene expression and growth dynamics of in vitro cultures and the assumption that if senescent cells are present in vivo, their phenotype will not be substantially different. Table 1 gives a series of representative changes in biochemistry and gene expression seem in senescent cells.

One route is via a reduction in the division potential of regenerative tissues. This could result from either the presence of significant numbers of fully senescent cells, an overall approach of cells towards (but not yet at) the end of their life span, or both. The prior cell division necessary to reach this state could reflect the tissue having a continuous high degree of cell turnover or might result from localised bursts of cell division in response to damage or infection. The subsequent reduction in mitotic potential could be relevant to human tissues where a high level of cell division occurs throughout life (e.g., the epidermis and the lining of the gut) or tissues which normally have a low baseline rate of cell division (e.g., dermal fibroblasts and endothelium) but retain the capacity to upregulate division in certain circumstances, such as when damaged.

TABLE 1. Selected Alterations in Cell Phenotype with the Onset of Senescence

Phenotypic alteration in senescence[a]	Cell type	Ref.
Permanent growth arrest	All	13, 19, 64, 65
Repression of c-*fos*	Fibroblasts, T lymphocytes	66, 67
Repression of cyclins A and B	Fibroblasts	68
G_2 arrest on restimulation without division	Fibroblasts, T lymphocytes	69, 37
Elevated collagenase	Fibroblasts	42
Elevated TIMP-2	Fibroblasts, endothelial cells	44, 70
Elevated PAI-1	Fibroblasts, endothelial cells	44
Elevated ceramide	Fibroblasts	71
Transcriptional repression of IGF-1	Fibroblasts	72
Induction of Ws3.10 inhibitor of Ca^{2+}-dependent membrane currents	Fibroblasts	73
Elevated IL-1α expression	Fibroblasts	56
Decreased IL-6 expression	Fibroblasts	74
Senescence-associated β-galactosidase	Fibroblasts, keratinocytes, mammary epithelial cells, endothelial cells, neonatal melanocytes	23
Induction of SAG gene	Fibroblasts	75
Repression of 17α-hydroxylase	Adrenocortical cells	11
Elevation of cytochrome *b* and NADH 4/4L subunit	Fibroblasts	76
Elevated *hic-5* expression	Fibroblasts	77

[a]TIMP-2, tissue inhibitor of metalloprotein 2; PAI-1, plasminogen activator inhibitor 1; IGF-1, insulin-like growth factor 1; IL, interleukin.

One such example is provided by the immune system. The demonstration of the senescence of T lymphocyte cultures overturned a prevailing preconception among immunologists that normal T lymphocytes were immortal.[10,19,37] A decline in T cell proliferative response in vivo has been reported with ageing and correlates with an increasing fraction of nonmitogen-responsive cells and a fraction of G_2-arrested cells with similar characteristics to those of restimulated cultures of senescent T cells.[37–39] There is also evidence for a population of ''presenescent'' T cells with reduced division potential

in aged individuals.[40] Theoretical calculations indicate that if the proliferative potential of T cells is the same within the body as it is ex vivo, then the number of expansions which a particular cell clone can undergo in response to antigen stimulation is not indefinite and is probably less than ten.[41]

The potential effect of senescent cells is not limited to a reduction in division potential. The presence of senescent cells could also have an effect as often they overexpress proteins that act at a distance, such as the classic example of collagenase overexpression by senescent dermal fibroblasts.[42] In this final section, we review how the known changes in cell biology of senescent cells in culture might have an impact on the ageing of various human tissues.

Effects of senescence on the vasculature

Vascular disease, particularly arteriosclerosis, is a major killer of the elderly. The disease results from a progressive thickening of the arterial intima, which leads to a gradual narrowing of the lumen of the vessel. Approximately 60% of men surviving to the sixth decade show 75–100% stenosis of at least one coronary artery.[43]

A variety of known senescence-specific changes have the potential to contribute to this disease. The cell types involved within the vascular wall are smooth muscle cells (in larger vessels) or fibroblasts (in the capillary vasculature) and an overlying vascular endothelium, which regulates haemostasis. Senescent HUVECs, commonly used as a simple approximation for adult vascular endothelium, upregulate urokinase-type plasminogen activator (u-PA) and plasminogen activator inhibitor-1 (PAI-1) more than 50-fold relative to growing controls.[44] Elevated PAI levels in vivo are a major risk factor for myocardial infarction and deep vein thrombosis, and in transgenic studies PAI-1 overexpression leads to thrombotic disease.[45,46] This may be made worse by a senescence "bystander effect" on the endothelium.[47] Under normal conditions, PAI-1 activity in endothelial cells is downregulated by factors produced by the attendant smooth muscle cells or fibroblasts. However, if cultured senescent cells of either type are substituted for their young counterparts, the downregulation of PAI-1 activity either disappears or is replaced by a significant upregulation.

Similar cross-talk may occur in vivo with respect to the regulation of vascular tone. Endothelial cells relax contracted smooth muscle cells via nitric oxide production. Senescent HUVECs in culture lack the ability to produce nitric oxide, and thus senescent endothelial cells may be unable to modulate vascular tone in vivo. Other changes potentially relevant to arteriosclerosis include the reduced angiotensin-converting enzyme activity of senescent bovine endothelial cells and the elevated production of elastase by senescent smooth muscle cells and of thrombospondin by senescent fibroblasts, which may contribute to vascular degeneration.[48]

Senescence and dermal wound healing

Poor wound healing is an almost axiomatic feature of old age. Much of the problem stems from indirect effects of age, such as poor nutrition, infection, and drug side effects. Because of such complications, data regarding a direct effect of age in healthy individuals on wound healing are less coherent than often supposed.[49,50] Indeed, given these qualifications, it is encouraging that the evidence for age-related alterations in wound healing is as robust as it is.

There is a well-characterised change in the steady-state extracellular matrix of elderly individuals which is independent of environmental insults. In particular, the proportions of collagen, elastin, and glycosaminoglycans alter in a manner reminiscent of changes in extracellular matrix production seen between senescent and growing fibroblasts in vitro.[50,51] This coupled with the direct observation of senescent fibroblasts in the ageing dermis[30] strongly suggests that the tissue balance in aged skin is altered towards a more catabolic state.

The healing of dermal wounds is a complex process involving inflammation, cellular proliferation, and tissue remodelling.[49] Although one obvious effect of senescence on the fibroblasts which subsequently infiltrate the wound would be their inability to proliferate, secondary effects include a decreased movement rate,[52,53] increased latent time,[54] and reduced overall ability of the population to contract the extracellular matrix components.[55]Various matrix metalloproteinases, such as collagenase, stromelysin, and elastase,[6,42,56] are overexpressed by senescent fibroblasts. This may adversely affect matrix resynthesis and lead to the decreased tensile strength of the closed wound seen in the elderly.[57] Furthermore, terminal differentiation of senescent keratinocytes occurs slower than for their growing counterparts,[8] which when coupled with their inability to divide could contribute to reduced rates of epithelialisation.[49]

Senescence and wound healing in the eye

A common denominator of a range of age-related ocular problems is the need for surgical intervention to correct them, usually involving incision surgery of the cornea. Compromised wound healing by the corneal fibroblasts (keratocytes) has been documented in both WS[58] and normal ageing.[59] The keratocyte population appears potentially rather prone to the production of senescent cells. Following surgical removal of the overlying epithelium, anterior keratocytes respond by entering apoptosis, followed by a wound healing and repopulation cycle by the unaffected keratocytes in the posterior layers of the stroma.[60] It is unlikely that this process evolved simply to irritate ophthalmologists trying to carry out reproducible photorefractive keratectomy. Rather, the current theory is that this represents a "scorched earth" mechanism by which viruses that infect the corneal epithelium (such as herpes simplex virus and smallpox) are prevented from invading the

deeper stromal layers and entering the eye.[61] This is supported by the observation that herpes simplex infection of rabbit corneal epithelium triggers keratocyte apoptosis.[61]

Surgery is a common treatment for age-related cataract. One serious potential complication is damage to the highly specialised endothelium which regulates corneal transparency. Cataract surgery can remove up to 20% of the endothelium in a single operation and greatly increases the cell loss rate in subsequent years.[62] Although the principle repair mechanism within the endothelium is cell enlargement to reform a contiguous cell sheet, these cells can proliferate for up to 30 PDs in vitro before undergoing senescence[18] and mitoses are observed within the monolayer.[63] Endothelial cell number undergoes a gradual decline with advancing age and the number of senescent cells within the layer (visualised as label unincorporating) increases.[20] Thus, an aged endothelium appears less able to utilise whatever residual mitotic healing it possesses precisely when its need to undertake repair is greatest.

Conclusion

With the development of modern molecular and cell biology techniques, the mechanism of the ageing process has moved from the status of an ''unsolvable'' problem to that of an ''attackable'' one.[4] The demonstration that senescent cells do indeed occur in vivo has finally moved the field on from a time when a link with human ageing was predominantly an act of faith. The time is now ripe to ask which of the various changes in cell physiology that have been documented in the in vitro culture systems have a significant, quantitative contribution to age-related degenerations. The challenge for the future will be to determine not simply that such altered cell biology could have an effect but that their effects are large enough to make a significant contribution to age-related changes.

Although the cell hypothesis of ageing is not concerned with the mechanisms leading to the appearance of senescent cells, understanding this aspect may ultimately allow extremely powerful interventional tests of the hypothesis. Linking senescence and ageing yields two predictions. First, that some factors which increase life span should reduce the appearance of senescent cells and, second, that reducing the rate of senescence should alter physiological parameters relevant to life span. Recent work suggests that the first prediction may hold true.[29] The second, and perhaps most exciting, test of the hypothesis remains to be undertaken.

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References

1. Technology Foresight Panel on Health and Life Sciences. Progress through partnership. Technology foresight 4. London: HMSO; 1995. p 20–22.
2. Gompertz B. On the nature and function expressivity of the law of human mortality and on a new mode of determining life contingencies. Phil Trans R Soc Lond 1825;115:513–585.
3. Finley MI. Ancient Sicily. Chatto and Windus London; 1979.
4. Kirkwood TBL. Human senescence. Bioessays 1996;18:1009–1016.
5. Hayflick L. The limited in vitro lifespan of human diploid cell strains. Exp Cell Res 1965;37:614–636.
6. Campisi J. The biology of replicative senescence. Eur J Cancer 1997;33: 703–709.
7. Macieira-Coelho A, Ponten J, Philipson L. The division cycle and RNA synthesis in diploid human cells at different passage levels in vitro. Exp Cell Res 1966;42:673–684.
8. Norsgaard H, Clark BFC, Rattan SIS. Distinction between differentiation and senescence and the absence of increased apoptosis in human keratinocytes undergoing cellular aging in vitro. Exp Gerontol 1996;31:563–570.
9. Hornsby PJ, Gill GN. Loss of division potential in culture: aging or differentiation? Science 1980;208:1482–1483.
10. Perillo NL, Walford RL, Newman MA, Effros RB. Human T lymphocytes possess a limited in vitro lifespan. Exp Gerontol 1989;24:177–187.
11. Hornsby PJ, Hancock JP, Vo TP, Nason LM, Ryan RF, McAllister JM. Loss of expression of a differentiated function gene, steroid 17α-hydroxylase, as adrenocortical cells senesce in culture. Proc Natl Acad Sci USA 1987;84:1580–1584.
12. Wille JJ, Pittelkow MR, Shipley GD, Scott RE. Integrated control of growth and differentiation of normal human prokeratinocytes cultured in serum-free medium: clonal analysis, growth kinetics and cell cycle studies. J Cell Physiol 1984;121:31–44.
13. Adams JC, Watt FM. An unusual strain of human keratinocytes which do not stratify or undergo terminal differentiation in culture. J Cell Biol 1988;107:1927–1938.
14. Martin GM, Sprague CA, Epstein CJ. Replicative lifespan of cultivated human cells. Effects of donor's age, tissue and genotype. Lab Invest 1970;73:3584–3588.
15. Lipman RD, Taylor A. The in vitro replicative potential and cellular morphology of human lens epithelial cells derived from different aged donors. Curr Eye Res 1987;6:1453–1457.
16. Gilchrest BA. Relationship between actinic damage and chronologic aging in keratinocyte cultures in human skin. J Invest Dermatol 1983;81: 184s–189s.
17. Blake DA, Yu HN, Young DL, Caldwell DR. Matrix stimulates the proliferation of human corneal endothelial cells in culture. Invest Ophthalmol Vis Sci 1997;38:1119–1129.
18. Engelmann K, Böhnke M, Friedl P. Isolation and long term cultivation of human corneal endothelial cells. Invest Ophthalmol Vis Sci 1988;29:1656–1662.
19. Effros RB, Walford RL. Neonatal T cells as a model system to study the possible in vitro senescence of lymphocytes. Exp Gerontol 1987;22:307–316.
20. Hoppenreijs VPT, Pels E, Vrensen GFJM, Treffers WF. Effects of platelet derived growth factor on endothelial wound healing of human corneas. Invest Ophthalmol Vis Sci 1994;35:150–161.
21. Van Hinsburgh VWM. Arteriosclerosis: impairment of cellular interactions in the arterial wall. Ann NY Acad Sci 1992;673:321–330.
22. Kumazaki T, Wadhwa R, Kaul SC, Mitsui Y. Expression of endothelin, fibronectin, and mortalin as aging and mortality markers. Exp Gerontol 1997;32:95–103.
23. Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelly C, Medrano EE, Linskens M, Rubelj I, Periera-Smith OM, Peacocke M, Campisi J. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci USA 1995;92:9363–9367.
24. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 1997;88:593–602.
25. Watanabe Y, Lee SW, Detmar M, Ajioka I, Dvorak HF. Vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) delays and induces escape from senescence in human dermal microvascular endothelial cells. Oncogene 1997;14:2025–2032.
26. Maier JAM, Voulalas P, Roeder D, Maciag T. Extension of the life-span of human endothelial cells by an interleukin-1α antisense oligomer. Science 1990;249:1570–1574.

27. Kipling D. Telomere structure and telomerase expression during mouse development and tumorigenesis. Eur J Cancer 1997;33:792–800.
28. Rohme D. Evidence for a relationship between longevity of mammalian species and life spans of normal fibroblasts in vitro and erythrocytes in vivo. Proc Natl Acad Sci USA 1981;78:5009–5013.
29. Li Y, Yan Q, Wolf NS. Long-term calorie restriction delays age-related decline in proliferation capacity of murine lens epithelial cells in vitro and in vivo. Invest Ophthalmol Vis Sci 1997;38:100–107.
30. Lane MA, Ingram DK, Cutler JJ, Bernard DE, Roth RG, Knapka GS. Dietary restriction in nonhuman primates: progress report on the NIA study. Ann NY Acad Sci 1992;673:36–45.
31. Tollefsbol TO, Cohen HJ. Werner's syndrome: an underdiagnosed condition resembling premature aging. Age 1984;7:75–88.
32. Faragher RGA, Kill IR, Hunter JAA, Pope FM, Tannock T, Shall S. The gene responsible for Werner syndrome may be a cell-division counting gene. Proc Natl Acad Sci USA 1993;90:12030–12034.
33. Kill IR, Faragher RGA, Lawrence K, Shall S. The expression of proliferation-dependent antigens during the lifespan of normal and progeroid human fibroblasts in culture. J Cell Sci 1994;107:571–579.
34. Schulz VP, Zakian VA, Ogburn CE, McKay J, Jarzebowicz AA, Edland SD, Martin GM. Accelerated loss of telomeric repeats may not explain accelerated replicative decline of Werner syndrome cells. Hum Genet 1996;97:750–754.
35. Suzuki N, Shimamoto A, Imamura O, Kuromitsu J, Kitao S, Goto M, Furuichi Y. DNA helicase activity in Werner's syndrome gene product synthesized in a baculovirus system. Nucleic Acids Res 1997;25:2973–2978.
36. Fukuchi K, Martin GM, Monnat RJ. Mutator phenotype of Werner syndrome is characterised by extensive deletions. Proc Natl Acad Sci USA 1989;86:5893–5897.
37. Perillo NL, Naeim F, Walford RL, Effros RB. In vitro aging in T lymphocyte cultures: analysis of DNA content and cell size. Exp Cell Res 1993;207:131–135.
38. Kubbies M, Schindler D, Hoehn H, Rabinovitch PS. BrdU-Hoechst flow cytometry reveals regulation of human lymphocyte growth donor-age related growth fraction and transition rate. J Cell Physiol 1985;125:229–234.
39. Inkeles B, Innes JB, Kuntz MM, Kadish AS, Wekslers ME. Immunological studies of aging III: cytokinetic basis for the impaired response of lymphocytes from aged humans to plant lectins. J Exp Med 1977;145: 1176–1187.
40. Pawelec G, Adibzadeh M, Solana R, Beckman C. The T cell in the ageing individual. Mech Ageing Dev 1997;93:35–45.
41. Adibzadeh M, Mariani E, Bartoloni C, Beckman I, Ligthart G, Remarque E, Shall S, Solana R, Taylor GM, Barnett Y, Pawelec G. Lifespans of T lymphocytes. Mech Ageing Dev 1996;91:145–154.
42. Millis AJT, Sottile J, Hoyle M, Mann DM, Diemer V. Collagenase production by early and late passage cultures of human fibroblasts. Exp Gerontol 1989;24:559–575.
43. Lakatta EG. Health in an Older Society. Institute of Medicine and National Research Council: Committee on Aging Society (ed.), Washington DC: National Academic Press; 1980. p 73–104.
44. West MD, Shay JW, Wright WE, Linskens MHK. Altered expression of plasminogen activator and plasminogen activator inhibitor during cellular senescence. Exp Gerontol 1996;31:175–193.
45. Auwerx J, Bouillon R, Collen D, Geboers J. Induction of tissue-type plasminogen activator and plasminogen activator inhibitor in diabetes mellitus. Arteriosclerosis 1988;8:68–72.
46. Erickson LA, Fici GJ, Lund JE, Boyle TP, Polites G, Marrotti KR. Development of venous occlusions in mice transgenic for plasminogen activator inhibitor. Nature 1990;346:74–76.
47. Zhang JC, Fabry A, Paucz L, Wojta J, Binder BR. Human fibroblasts downregulate plasminogen activator inhibitor type-1 in cultured human macrovascular and microvascular endothelial cells. Blood 1996;88:3880–3886.
48. Thweatt R, Goldstein S. Werner syndrome and biological ageing: a molecular genetic hypothesis. Bioessays 1993;15:421–426.
49. Goodson H, Hunt TK. Wound healing and aging. J Invest Dermatol 1979;73:88–91.
50. Ashcroft GS, Horan MA, Ferguson MWJ. The effects of ageing in cutaneous wound healing in mammals. J Anat 1995;187:1–26.
51. Passi A, Albertini R, Campagnari F, De Luca G. Modifications of proteoglycans secreted into the growth medium by young and senescent human fibroblasts. FEBS Lett 1997;402:286–290.
52. Kondo H, Yonezawa Y. Changes in the migratory ability of human lung and skin fibroblasts during in vitro aging and in vivo cellular senescence. Mech Ageing Dev 1992;63:223–233.
53. Schneider EL, Mitsui Y. The relationship between in vitro cellular aging and in vivo human age. Proc Natl Acad Sci USA 1976;73:3584–3588.
54. Muggleton-Harris AL, Reisert PS, Burghoff RL. In vitro characterisation of response to stimulus (wounding) with regard to ageing in human skin fibroblasts. Mech Ageing Dev 1982;19:37–43.
55. Bell E, Ivarsson B, Merrill C. Production of tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro. Proc Natl Acad Sci USA 1979;76:1274–1278.
56. Zeng GY, Millis AJT. Differential regulation of collagenase and stromelysin mRNA in late passage cultures of human fibroblasts. Exp Cell Res 1996;222:150–156.
57. Morocutti A, Earle KA, Rodemann HP, Viberti GC. Premature cell ageing and evolution of diabetic nephropathy. Diabetologia 1997;40:244–246.
58. Jonas JB, Ruprecht KW, Schmitzvalckenberg P, Brambring D, Platt D, Gebhart E, Schachtschabel DO, Naumann GOH. Ophthalmic surgical complications in Werner's syndrome: report of 18 eyes of nine patients. Ophthalmic Surg 1987;18:760–764.
59. Faragher RGA, Mulholland B, Tuft SJ, Sandeman S, Khaw PT. Aging and the cornea. Br J Ophthalmol 1997;81:814–817.
60. Wilson SE, He YG, Weng J, Li Q, McDowall AW, Vital M, Chwang EL. Epithelial injury induces keratocyte apoptosis: hypothesised role for the interleukin 1 system in the modulation of corneal tissue organisation and wound healing. Exp Eye Res 1996;62:325–337.
61. Wilson SE. Molecular cell biology for the refractive corneal surgeon: programmed cell death and wound healing. J Refract Surg 1997;13:171–175.
62. Bourne WM, Nelson LR, Hodge DO. Continued endothelial cell loss ten years after lens implantation. Ophthalmology 1994;101:1014–1022.
63. Mishima S. Clinical investigations on the corneal endothelium. Am J Ophthalmol 1982;93:1–29.
64. Smith JR, Periera-Smith OM. Replicative senescence: implications for in vivo aging and tumor suppression. Science 1996;273:63–67.
65. Cristofalo VJ, Pignolo RJ. Replicative senescence of human fibroblast-like cells in culture. Physiol Rev 1993;73:617–638.
66. Sikora E, Kaminska B, Radziszewska E, Kaczmarek L. Loss of transcription factor AP1 DNA binding activity during lymphocyte aging in vivo. FEBS Lett 1992;312:179–182.
67. Seshadri T, Campisi J. Repression of *c-fos* and an altered genetic programme in senescent human fibroblasts. Science 1990;247:205–209.
68. Stein GH, Drullinger LF, Robetorye RS, Periera-Smith OM, Smith JR. Senescent cells fail to express cdc2, cycA & cyc B in response to mitogen stimulation. Proc Natl Acad Sci USA 1991;88:11012–11016.
69. Kill IR, Shall S. Senescent human diploid fibroblasts are able to support DNA synthesis and to express markers associated with proliferation. J Cell Sci 1990;97:473–478.
70. Zeng GY, Millis AJT. Expression of 72Kd gelatinase and TIMP2 in early and late passage human fibroblasts. Exp Cell Res 1994;213:148–155.
71. Venable ME, Lee JY, Smyth MJ, Bielawska A, Obeid LM. Role of ceramide in cell senescence. J Biol Chem 1995;270:30701–30708.
72. Ferber A, Chang CD, Sell C, Ptasznik A, Cristofalo VJ, Hubbard K, Ozer HL, Adamo M, Roberts CT, Leroith D, Dumenil G, Baserga R. Failure of senescent human fibroblasts to express the insulin-like growth factor 1 gene. J Biol Chem 1993;268:17883–17888.
73. Liu S, Thweatt R, Lumpkin CK, Goldstein S. Suppression of calcium dependent membrane currents in human fibroblasts by replicative senescence and forced expression of a gene sequence encoding a putative calcium-binding protein. Proc Natl Acad Sci USA 1994;91:2186–2190.
74. Goodman L, Stein GH. Basal and induced amounts of IL6 mRNA decline with age in human fibroblasts. J Biol Chem 1994;269:19250–19255.
75. Wistrom C, Villeponteau B. Cloning and expression of SAG: a novel marker of cellular senescence. Exp Cell Res 1992;199:355–362.
76. Kodama S, Yamada H, Annab L, Barrett JC. Elevated expression of mitochondrial cytochrome b and NADH dehydrogenase subunit 4/4L in senescent human cells. Exp Cell Res 1995;219:82–86.
77. Shibanuma M, Mochizuki E, Maniwa R, Mashimo J, Nishiya N, Imai SI, Takano T, Oshimura M, Nose K. Induction of senescence-like phenotype by forced expression of *hic-5* which encodes a novel LIM motif protein in immortalised fibroblasts. Mol Cell Biol 1997;17:1224–1235.