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Review

From the Hayflick mosaic to the mosaics of ageing. Role of stress-induced premature senescence in human ageing

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Abstract

The Hayflick limit—senescence of proliferative cell types—is a fundamental feature of proliferative cells in vitro. Various human proliferative cell types exposed in vitro to many types of subcytotoxic stresses undergo stress-induced premature senescence (SIPS) (also called stress-induced premature senescence-like phenotype, according to the definition of senescence). The known mechanisms of appearance the main features of SIPS are reviewed: senescent-like morphology, growth arrest, senescence-related changes in gene expression, telomere shortening. Long before telomere-shortening induces senescence, other factors such as culture conditions or lack of 'feeder cells' can trigger either SIPS or prolonged reversible G_0 phase of the cell cycle. In vivo, 'proliferative' cell types of aged individuals are likely to compose a mosaic made of cells irreversibly growth arrested or not. The higher level of stress to which these cells have been exposed throughout their life span, the higher proportion of the cells of this mosaic will be in SIPS rather than in telomere-shortening dependent senescence. All cell types undergoing SIPS in vivo, most notably the ones in stressful conditions, are likely to participate in the tissular changes observed along ageing. For instance, human diploid fibroblasts (HDFs) exposed in vivo and in vitro to pro-inflammatory cytokines display biomarkers of senescence and might participate in the degradation of the extracellular matrix observed in ageing.

Keywords: Senescence; Oxidative stress; Telomeres; Apolipoprotein J; TGF-β1; Ageing

Contents

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1. The Hayflick limit today

In 1961 Hayflick and Moorhead reported that human diploid fibroblasts (HDFs) divide a finite number of times. Hayflick called 'phase I' the primary culture, 'phase II' the many cumulative populations doublings (CPDs) of luxuriant growth after the primary culture, and 'phase III' the subsequent growth arrest [1,2]. In 1974 'phase III' was termed 'the Hayflick limit' [3]. The phrase 'replicative senescence' is now widely used. Medline refers to more than 6100 papers with 'replicative senescence', more than 9200 with 'in vitro ageing', and about 1300 hits with both phrases. A review of the first 80 genes undergoing senescence-related expression changes or post-translational modifications is available [4]. DNA μarrays enhanced this analysis by displaying the relative steady-state mRNA levels of thousands of genes [5]. Today it is unquestionable that telomere shortening is a universal mechanism that limits the proliferative potential of normal cells lacking endogenous telomerase. Most human primary cells do not express high levels of telomerase and are therefore subjected to a progressive erosion of their telomeres with each cell generation. Critically short telomeres are probably mistaken for damaged DNA, which results in telomeric fusion [6]. In mice, the shortest telomere, not average telomere length, is critical for cell viability and chromosome stability in mice [7]. In HDF clones, the onset of replicative senescence is significantly correlated with the mean telomere fluorescence but, strikingly, not with chromosomes with the shortest telomere length [8].

Recently the so-called relationship between the age of the human donor of HDFs and the maximum number of CPDs has been seriously challenged [9]. Further studies are still needed to confirm or invalidate this dogma. The relationship between the life span of species and the maximum number of HDF CPDs (for a review: [10]) also needs reconsideration. Most studies on replicative senescence were performed with cells cultured at 20% O_2, which obviously represents an oxidative stress when compared to the much lower in vivo physiological pressure of O_2 [6,11]. Each cell type or cell strain might have different sensitivities to chronic exposure to O_2 levels higher than those encountered in vivo. For instance, IMR-90 HDFs are notably sensitive to oxidative stress and, in contrast to most other HDFs, cannot be immortalised by telomerisation unless grown in 1% O_2 [11]. On the contrary, BJ HDFs display high antioxidant capacity and slow telomere shortening at 20% O_2 [12]. Mouse fibroblasts are much more sensitive to oxidative stress than HDFs [13] and undergo less CPDs in vitro (for a review: [11]). Unless most of these experiments are carried out again at the respective O_2 tension characterising specific tissues of different species, intraspecies comparisons of the maximum number of CPDs are on shaky grounds.

2. Effects of culture conditions on senescence

The deregulation of the mitotic machinery of HDFs during in vivo ageing of middle-aged and late-aged donors was analysed with DNA μarrays. Many changes were found in the expression level of genes necessary for the cell cycle to proceed [14]. Most of these genes, however, are different from those undergoing expression changes in in vitro replicatively senescent HDFs. This suggests that in vivo senescence is somewhat different from in vitro replicative senescence.

When cultured under 3% O_2, i.e. close to physiological O_2 tensions, HDFs achieve up to 20 more CPDs

than at 20% O_2. Pre-senescent HDFs incubated with the spin trap α-phenyl-*t*-butyl-nitrone (PBN) achieve more CPDs than untreated cells [15]. Under 20% O_2 a significant proportion of dermal HDFs rapidly switch from a mitotic to a post-mitotic phenotype. In contrast, at 4% O_2, the induction of a post-mitotic phenotype is largely prevented [16]. Growth potential of rat mammary epithelial cells is enhanced when superoxide dismutase, catalase, and Vitamin E and/or low O_2 tension are used during the cell dissociation period. The iron chelator desferal reduces lipid peroxidation. It also enhances growth when used during the cell dissociation period. Antioxidants and low O_2 tension in culture after the cell dissociation period fail to improve growth potential [17]. The examination of growth rate, synthesis and composition of proteoglycans, and morphology of chicken chondrocytes cultivated under 20 or 8% O_2 shows slowed senescence under a lower O_2 pressure [18].

A reduced growth rate and maximum number of CPDs is observed in different human cell types like HDFs [19–21], keratinocytes [22], umbilical vascular endothelial cells [23] or melanocytes [24] when exposed to more than 20% O_2. The growth rate of tumor cells appears to be inversely correlated with the level of peroxidation of membranes [25]. The theory of the dilution of damage [26,27] explains that, as long as the growth rate is sufficient, as in rapidly growing tumor cells, no damage accumulates. As corollary events that favour a decrease of growth rate, like 20% O_2, favour damage accumulation. Lipofuscin accumulation in cultured non-dividing cells is a function of time and O_2 pressure [28,29]. Alterations in mitochondrial (mt) membrane fluidity by lipid peroxidation products [30] could be caused by oxidation of serum lipids. Major lipid peroxidation products can lead to inhibition of NADH-linked mt respiration [31] and cell growth [32–34]. Some components of serum could be oxidized. This could affect some extracellular matrix or transmembrane effectors, or could trigger oxidative stress. Other serum components, like amine oxidase, can produce reactive oxygen species (ROS) [35].

Sometimes oxygen is not the (only) culprit. The effects of repeated trypsinisations, lack or surplus of specific cytokines or growth factors, etc. must be added. Wright and Shay [11] proposed that cultures conditions might exist for each cell type that minimise stress and leave telomere shortening as the only barrier against immortalisation [36]. When human keratinocytes are cultured in chemically defined media, most of the reports found numbers of CPDs—15–20 PDs—dramatically less than the 50 CPDs or so described previously for the growth of keratinocytes on feeder layers [37]. Cultures of HDFs in chemically defined medium in the presence of 0.25% serum arrive at a similar scenario: they are growth arrested after about 25 CPDs regardless of telomere length or telomerase overexpression [11,36]. Feeder layers increase greatly the number of CPDs of human mammary epithelial cells. Without feeder layers the replicative potential of human keratinocyte is limited by the cyclin-dependent kinase inhibitor (CDKI) $p16^{ink\text{-}4a}$. The activation of $p16^{ink\text{-}4a}$ can occur independently of telomere length. Abrogation of this mechanism together with telomerase expression immortalises keratinocytes. It does not seem to affect growth or differentiation control [38–40]. A possibility exists that these cells are in phase G_0 of the cell cycle. Indeed their proliferative capacity is rescued after their transfer into adequate culture medium with 10% serum [36]. This does not mean that serum cannot have deleterious effects at later stages of culture. It is also likely the duration of G_0 determines the rescue of the proliferative capacity of a significant portion of the cell population.

In certain conditions telomere-dependent senescence is not even observed. Indeed when appropriate culture conditions are provided, namely the presence of platelet-derived growth factor (PDGF) in a serum-free culture medium, two rat cell types do not undergo replicative senescence neither show signs of transformation or immortalisation, even when cultivated at 20% O_2 [41,42].

3. The Hayflick mosaic

In vivo the proliferative capacity of HDFs is never completely exhausted. HDFs of centenarians are still able to divide in vitro, sometimes for a number of CPDs that renders them undiscriminable from explants of HDFs of young donors [43]. Stem cells are present in the connective tissues of dermis and skeletal muscle derived from geriatric humans. These cells contain lineage-committed myogenic, adipogenic, chondrogenic, and osteogenic progenitor stem cells

as well as lineage-uncommitted pluripotent stem cells capable of forming muscle, adipocytes, cartilage, bone, fibroblasts, and endothelial cells [44]. In other words, only a minority of cells is likely to be senescent in tissues of aged persons. This could be called the 'Hayflick mosaic'. This would be more deferential than the phrase 'replicative mosaicism' coined by Mikhelson [45].

It was proposed that the presence of a minority of senescent cells in a tissue could interfere with the homeostasis of somatic tissues, thereby participating in tissular ageing [3]. Many papers argue that the biomarkers of in vitro replicative senescence of HDFs and other cell types appear during in vivo ageing. These biomarkers are for instance an enlarged cell size [46,47], senescence-associated β-galactosidase activity (SA β-gal) [48], deletions in the mt DNA [49–54], lower induction of c-*fos* proto-oncogene [55], decreased induction of heat shock proteins [56–60], and increased metalloproteinase activity degrading the extracellular matrix (for a review: [10]). The main issue raised by these claims is that most of these 'biomarkers' can also appear after exposure to subcytotoxic oxidative stress and DNA damaging agents.

4. The mosaics of ageing

The narrowest definition of senescence is irreversible growth arrest triggered by telomere shortening, which counts cell generations [11] (definition 1). Other authors enlarged this definition to a functional definition encompassing all kinds of irreversible arrests of proliferative cell types including that induced by damaging agents (definition 2) [6]. Irreversible growth arrest of proliferative cell types induced by damaging agents may be called stress-induced senescence-like phenotype, according to definition 1, or stress-induced premature senescence, according to definition 2. These phrases are both abbreviated as SIPS [61,62] herein. According to definition 2, telomere-dependent replicative senescence is one of the types of cellular senescence. Telomere-dependent replicative senescence has often been described as the ultimate barrier against cell immortalisation. All other forms of senescence may also represent barriers against immortalisation as long as irreversible growth arrest is observed. Therefore, a common trait between the different forms of senescence would be based on irreversible growth arrest of proliferative cell types and subsequent phenotypic changes. It might be too simplistic and misleading to consider that the stress response of all the cells of a cell culture (or a tissue) is an all-or-nothing process. One must discriminate between a situation where all cells of a given population are only transiently growth arrested; and another situation where a major proportion of a cell population remains irreversibly growth arrested, as observed in SIPS.

A population of cells of a given type is heterogeneous enough so that these cells become gradually affected by oxidative stress, and are subsequently growth arrested (see data in [20]). Considering definition 1 of senescence, any HDF that irreversibly stops dividing cultivated under 20% O_2 before its telomeres reach a minimal length, is a stress-induced senescence-like phenotype. Therefore the post-mitotic HDFs of a culture under 20% O_2 become a mosaic made of two kinds of growth arrested cells: HDFs in stress-induced senescence-like phenotype, and HDFs in telomere-dependent replicative senescence. In other words in vitro senescence under 20% O_2 could be due partly to a 'culture shock' (for a review: [63]). The proportion of cells in SIPS-like phenotype should be higher in a culture that has been exposed to increased O_2 partial pressure. Inversely, the proportion of cells in telomere-dependent replicative senescence due to the end-replication problem should be lower in cultures exposed to increased stress. Another possibility is that increased stress causes accelerated telomere shortening due to DNA single strand breaks.

5. SIPS in vitro

Many proliferative cell types (lung and skin HDFs, human melanocytes, endothelial cells, human retinal pigment epithelial cells, human erythroleukemia cells, etc.) exposed to subcytotoxic stress (UV, organic peroxides, H_2O_2, ethanol, mitomycin C, hyperoxia, γ-irradiations, homocysteine, hydroxyurea, *tert*-butylhydroperoxide (*t*-BHP) etc.) undergo SIPS. SIPS can be defined as the long term effects of subcytotoxic stress on proliferative cell types, including irreversible growth arrest of (a majority of) the cell population. Repeated stress allows the dose of the stressor to decrease [50]. The induction of SIPS can be studied

independently, to some extent, from purely adaptative responses if the cells are allowed to recover for several days after the last stress and only then analyse the biomarkers of senescence (for a review: [64]).

Among the numerous features they share with senescent cells, cells in SIPS display a stable senescent-like morphology (for a review: [64]). Several clues exist to explain these morphological changes. First, the presence of the retinoblastoma protein (Rb) is necessary for the appearance of a senescent morphology after subcytotoxic H_2O_2 stress. HDFs overexpressing the papilloma virus E7 protein, which facilitates the proteolytic degradation of Rb namely, were used to study the role of Rb. These cells do not adopt a senescent morphology after exposure to H_2O_2. This morphology reappears when HDFs overexpressing mutated E7 cDNAs that do not bind Rb are stressed. An increase of stress fibers is observed in H_2O_2-treated HDFs, which participates in the development of the senescent-like morphology. The appearance of these stress fibers can be linked with a redistribution of vinculin and paxallin after stress [65,66]. Interestingly, transforming growth factor-β1 (TGF-β1) is overexpressed after subcytotoxic H_2O_2 stress. Incubations of H_2O_2-treated HDFs with antibodies against TGF-β1, or against TGF-β receptor II abrogate the appearance of the senescent morphology. The overexpression of TGF-β1 disappears in HDFs overexpressing E7 [67].

6. Molecular mechanisms of SIPS

6.1. Growth arrest

HDFs in H_2O_2-induced SIPS cannot launch a mitogenic response when stimulated with serum or usual growth factors [68]. Most of HDFs in H_2O_2-induced SIPS are growth arrested in the G_1 phase of the cell cycle [65]. Hyperoxia under 40% O_2 also leads to growth arrest of HDFs in the G_1 phase [69].

The proportion of HDFs positive for SA β-gal activity correlates with CPDs. It also increases in SIPS, induced by *tert*-butylhydroperoxide (*t*-BHP) and H_2O_2 (for a review: [64]). SA β-gal activity is rather a marker of growth arrest than a specific marker of telomere-dependent replicative senescence [36]. A recent study attributed this activity to an increase in lysosome size [70].

The steady-state mRNA level of the immediate early proto-oncogene c-*fos* is sharply diminished in senescent HDFs and in HDFs in SIPS induced by hyperoxia, mitomycin C, and H_2O_2. This may contribute to a decrease in quantity and activity of AP-1 transcription factor in its c-Fos/c-Jun dimeric complex [55,71–73].

CDKI $p21^{waf\text{-}1}$ protein expression level increases dramatically during the two to three last passages before senescence. This level declines when senescence is reached. During this period, CDKI $p16^{ink\text{-}4a}$ protein level gradually rises and is multiplied nearly by 40-fold in senescent HDFs when compared to HDFs at early passage. In senescent HDFs, $p16^{ink\text{-}4a}$ is a major inhibitor of CDK4 and CDK6 kinase activities [74]. Rb is crucial for growth suppression by $p16^{ink\text{-}4a}$ [75]. $p16^{ink\text{-}4a}$ and $p21^{waf\text{-}1}$ CDKIs induce several facets of the senescent phenotype when ectopically expressed in HDFs at early CPDs. These facets include a reduced proliferative capacity, an altered cell size and cell shape, an underphosphorylation of Rb, an increased expression of plasminogen activator inhibitor (PAI-1) and SA β-gal activity [76]. $p21^{waf\text{-}1}$ remains overexpressed for at least three weeks after subcytotoxic stress with H_2O_2 [65]. $p21^{waf\text{-}1}$ is overexpressed in HDFs at 72 h after repeated subcytotoxic *t*-BHP stress [50]. CDKIs inhibit the phosphorylation of Rb in senescent cells (for a review: [10]) and in *t*-BHP and H_2O_2–induced SIPS. Hypophosphorylated Rb is known to inactivate the E2F transcription factor family (for a review: [10]).

When IMR-90 HDFs are treated for 2 h with 50–200 μM H_2O_2, a dose-dependent fraction of HDFs detach at 16–32 h after the treatment. The cells that remain attached are growth-arrested and develop SIPS. The detached cells show dose-dependent caspase-3 activation. They display the typical morphology associated with apoptosis. Apoptotic cells are mainly distributed in the S-phase of the cell cycle. Growth-arrested cells exhibit G_1 and G_2/M-phase distributions. Reduction of p53 levels with human papillomavirus E6 protein prohibits the activation of caspase-3. This decreases the proportion of apoptotic cells. Growth arrested cells have elevated $p21^{waf\text{-}1}$ levels. $p21^{waf\text{-}1}$ is absent in the apoptotic cells [77].

Within 4 h after exposure to low doses of UVB, human melanocytes induce an overexpression of p53 and $p21^{waf\text{-}1}$. At higher doses, these cells become irreversibly growth arrested and acquire a senescent

morphology [78,79]. Premature skin ageing is a prominent side-effect of psoralen photoactivation. This photoactivation is used as therapy against a variety of skin disorders, including psoriasis. Treatment of dermal HDFs with 8-methoxypsoralen and UVA results in a permanent growth arrest, a senescent morphology, and an upregulation of $p21^{waf-1}$ and matrix-degrading metalloproteinases. SA-β gal positive cells are also detected in the treated HDFs [80].

Etoposide, doxorubicin, *cis*-platin, and phleomycin D1 cause DNA double-strand breaks (DSBs). These molecules induce a permanent cell cycle arrest in normal HDFs. Camptothecin, an agent that causes DNA single-strand breaks converted to DSBs during phase S, is unable to induce permanent cell cycle arrest [81].

Exposure of bovine aortic endothelial cells to γ-rays results in a dose and time-dependent senescent morphology several days after irradiation. The

Table 1
Conditions leading to cellular senescence of proliferative cell types

Conditions	References
Serial subcultivations in vitro	[10]
SIPS in vitro after exposures of different cell types to subcytotoxic conditions (for a review: [64])	
H_2O_2	[67,68]
Hyperoxia	[21]
Tert-butylhydroperoxide	[50]
Homocysteine	[97]
Mitomycin C	[124]
Etoposide, doxorubicin, phleomycin	[81]
Cis-platin	[125]
5-Bromodeoxyuridine	[126]
Bleomycin	[127]
Hydroxyurea	[128,129]
γ-Rays	[82]
UV	[130]
UVB on melanocytes	[79]
UVB on skin fibroblasts (this issue)	[131]
UVA + phleomycin 8-methoxypsoralen	[80]
Strong electromagnetic fields ethanol	[132]
Ethanol	[133]
Deficiency in glucose-6-phosphate dehydrogenase	[134]
Stimulation with cytokines	
TGF-β1	[67,135]
Repeated stimulation with IL-1α and TNF-α	[111,113]
Overexpression of proto-oncogenes	
raf-1	[136]
ras	[137]
E2F1	[138]
c-*rel*	[139,140]
JunB	[141]
Overexpression of tumor suppressors	
Overexpression of $p16^{ink-4a}$ and $p21^{waf-1}$	[76]
Deficiency in *JunD*	[142]
Premature senescence in vivo observed in:	
Venous ulcers	[114]
Distal lower extremities of patients with venous insufficiencies	[104]
Arteries subjected to balloon angioplasty	[106]
Tissue surrounding liver carcinomas	[107]
Prostatic hyperplasia	[108]

majority of these cells stain positively for SA β-gal. A prolonged overexpression of $p21^{waf-1}$ protein is noted. Phenotypically altered cells are present as long as 20 weeks after irradiation [82] (Table 1).

6.2. TGF-β1 and the mechanisms of irreversible growth arrest in SIPS

The steady-state mRNA level of several genes is increased both in replicative senescence and in *t*-BHP, hyperoxia or H_2O_2-induced SIPS, like osteonectin, apolipoprotein J, fibronectin and α1(I)-procollagen [50]. IMR-90 HDFs developing the H_2O_2-induced SIPS overexpress TGF-β1. In addition, stimulation of IMR-90 HDFs with TGF-β1 triggers the appearance of SA β-gal activity. It induces a senescent morphology and growth arrest. It increases the mRNA steady-state level of the senescence-associated genes fibronectin, osteonectin, apolipoprotein J, and SM22. Antibodies against TGF-β1 or TGF-β1 receptor II abrogate the overexpression of these genes after subcytotoxic H_2O_2 stress. They abrogate the appearance of the senescent-like morphology and SA β-gal activity after this stress [67]. Within 8 h, TGF-β1 induces the release of H_2O_2 from IMR-90 HDFs. Diphenyliodonium, an inhibitor of the NADPH oxidase complex and other flavoproteins, inhibits this TGF-β1-induced H_2O_2 production [83]. Therefore constant oxidative stress might be generated once TGF-β1 is overexpressed, which constitutes a closed regulatory loop explaining why cells in SIPS are maintained in a state of irreversible growth arrest.

Stimulation of dermal HDFs with TGF-β1 results in increased expression of type I collagen and osteonectin [84]. TGF-β1 induces the overexpression of fibronectin and osteonectin, both at the mRNA and protein levels in human pulp cells [85]. Osteonectin-null mesangial cells have decreased levels of TGF-β1 mRNA and secreted TGF-β1 protein. These cells have a decreased steady-state levels of α1(I)procollagen mRNA and protein, as compared to wild-type cells. Addition of recombinant osteonectin to osteonectin-null cells restores the transcription of α1(I)procollagen and TGF-β1 mRNA [86]. Thus, in different systems, TGF-β1 regulates the expression of fibronectin, osteonectin and α1(I)procollagen mRNA. In return, osteonectin regulates the expression of TGF-β1 mRNA.

Apolipoprotein J is overexpressed in several models of apoptosis. It is overexpressed in *t*-BHP or H_2O_2-induced SIPS. A retrovirus-mediated stable overexpression of apolipoprotein J in WI-38 HDFs increases cell survival after exposure to *t*-BHP and ethanol at cytotoxic concentrations. In addition, this ectopic overexpression decreases the induction of two biomarkers of SIPS after exposure to subcytotoxic concentrations of ethanol or *t*-BHP. This ectopic overexpression also triggers the overexpression of osteonectin. Osteonectin decreases the mitogenicity of PDGF [87,88]. Apolipoprotein J overexpression also triggers the overexpression of fibronectin [87].

7. Is telomere shortening involved in SIPS?

WI-38 HDFs kept under 40% O_2 for 3 CPDs undergo SIPS. An accelerated telomere restriction fragment (TRF) shortening (500 bp/PD) is observed [69]. Forty percent O_2 induces single-strand breaks and accelerated TRF shortening of human retinal pigment epithelial cells [89]. WI-38 HDFs undergo accelerated TRF (490 bp/stress) and irreversible growth arrest after four exposures to subcytotoxic *t*-BHP stress, with a stress at every 2 CPDs. After these stresses, the cells stop proliferating. The control cells keep on proliferating for more than 20 extra CPDs. Both stressed and control cultures stop growing when the mean TRF length reaches 5 kbs [90]. When subcytotoxic H_2O_2 stress or five repeated subcytotoxic *t*-BHP stresses were performed along the same PD, respectively a 322 ± 55 and 381 ± 139 bp-TRF shortening was observed, only during the first PD after stress [90]. HDFs at early CPDs exposed to 150 mM H_2O_2 once or 75 mM H_2O_2 twice in 2 weeks display long-term growth arrest, enlarged morphology, increases in proportion of SA β-gal activity positive cells, and overexpression of apolipoprotein J mRNA. Weekly treatment with 75 mM H_2O_2 fails to induce significant TRF shortening [91]. This suggests at least that SIPS can emerge without telomere shortening. It is most probable that no telomere shortening occurred in these cells since telomeric attrition requires cell division [92]. We already published an integrative interpretation of these results. This interpretation is based on the calculation of the TRF shortening due to the compensatory cycling of the cells which go on dividing despite adverse stress

conditions. Depending on the models of SIPS studied, one shows either that TRF shortening occurs before growth arrest takes place (models where cells are stressed over several population doublings, whether continuously or discretely) or that SIPS occurs before significant TRF shortening takes place (models where cells are repeatedly stressed during a given population doubling) [93]. Since IMR-90 HDFs in H_2O_2-induced SIPS overexpress TGF-β1 [67], which triggers an overproduction of H_2O_2 [83], it might be that once in SIPS cells keep dealing with DNA single-strand breaks, telomeric or not, without TRF shortening.

The expansion of human β-cell-enriched pancreatic islet cultures is limited by growth arrest after 10–15 CPDs. SA β-gal activity, increased $p16^{ink-4a}$ levels, and decreased telomere lengths are observed. The senescent phenotype is not prevented by retroviral transduction of the telomerase *hTERT* gene, although telomerase activity is induced [94]. Protection by antioxidants during the period of cell dissociation from explants or during the subcultures might avoid this form of premature senescence of cultivated cells. In comparison, it is interesting to note that the rate of telomere loss decreases by half in HDFs treated with PBN [95], or with a Vitamin C derivative [96]. Exposure of cultured endothelial cells to homocysteine leads to stress-induced premature endothelial senescence. Homocysteine leads to TRF shortening. These effects of homocysteine are inhibited by the H_2O_2 scavenger catalase [97]. The mean TRF length in human osteoblasts undergoing in vitro ageing decreases from an average of 9.3 kbp in middle-aged cells to an average of 7.8 kbp in senescent cells. The rate of TRF shortening in these cells is around 100 bp/PD, as reported for HDFs [98]. These cells stop growing long before their telomeres reach a minimal length of 5 kbp. This suggests again that ameliorations of the culture conditions of human osteoblasts (decrease of O_2 partial pressure, addition of antioxidants, etc.) could increase the maximum number of CPDs obtained so far with osteoblasts.

TRF shortening in normal liver was found to be around 120 bp per year. This is similar to the TRF shortening observed in HDFs (around 100 bp/PD). The replacement rate of human liver cells, once a year, also reinforces these results. The mean TRF length was around 10 kbp in normal livers from 80-year-old individuals. In chronic hepatitis and in liver cirrhosis, the TRF length was lower when compared to that found in normal livers of the same age. In all samples taken from patients with chronic liver disease, with the exception of two samples, TRFs were not shorter than 5 kbp, which is assumed to be the inferior limit in untransformed cells [99].

8. Hayflick mosaic or mosaics of ageing?

From definition 1 of senescence, the fact that HDFs can endure more PDs at physiological low O_2 partial pressures decreases the probabilities to find senescent cells based on the end-replication problem in vivo. Theoretically, starting from the two first telomerase-negative cells that appear during in vivo differentiation, and that will become fibroblasts (in the case of symmetric divisions) 2^{50} cells ($>10^{15}$ cells) must be produced before the first telomere-dependent replicatively senescent HDFs appear, after 50 PDs under 20% O_2. If the cells make 30 more PDs at physiological low O_2 concentrations, this number goes up to 2^{80} fibroblasts ($>10^{24}$ cells) which represents cubic kilometric volumes of cells. Of course tissular turn-over and asymmetric division processes must be considered, which decrease these figures. In vivo, non proliferative periods might favour the appearance of single-strand breaks as observed in vitro after long periods of confluence [100]. These cells undergo telomere shortening when resuming mitosis. On the other hand, there are many other proliferative cell types in a mammalian organism which should also divide a similar number of times before all to them become telomere-dependent replicatively senescent cells. This increases these cubic kilometric volumes of cells. This also increases the chances that the irreversibly growth arrested HDFs found in vivo represent cells in SIPS rather than cells in telomere-dependent replicative senescence. Stress occurs in vivo under multiple forms. In vivo, cells can be exposed to abnormal oxidative stress in certain locations (pneumocytes facing tobacco smoke, ozone, urban smog, etc.) circumstances (hepatocytes facing ethanol; endothelial cells facing inflammation, ischaemia–reperfusion, hypertension, oxidised LDL and cholesterol; keratinocytes, skin fibroblasts and melanocytes facing long sunbaths; enterocytes facing food oxidants; muscle under excessive exercice, etc.)

[101] and pathologies such as age-related neurodegenerative diseases, diabetes, atherosclerosis, Down syndrome, to enumerate only but a few.

Individual variations due to various stresses, pro-inflammatory histories, and pathologies make it difficult to compare the proliferative in vitro life span of a given cell type between individuals. In this respect, it is not surprising that the relationship between the age of the donor and the proportions of cells positive for the SA β-gal activity has been challenged [9]. Along the same line, it is not surprising that the proliferative life span in vitro and the proteasome activity of some explants of centenarian cells are undistinguishable from those of cells of young donors [43,102,103]. Perhaps we should talk in terms of individual-specific mosaics of proliferative cell types with a different story to tell about their remaining proliferative potential and their past exposures to stress. Are there in vivo situations where such mosaics of cells might exist? HDFs cultured from distal lower extremities of patients with venous insufficiencies display cellular characteristics of senescence. These changes precede ulcer formation and suggest a mechanism intrinsically related to venous reflux [104]. Another study demonstrated a clinical correlation between quantitative in vitro senescence and time-to-healing of leg ulcers [105]. SA β-gal activity positive cells are also found in arteries subjected to balloon angioplasty [106], tissue surrounding liver carcinomas [107] and prostatic hyperplasia [108].

Cytokines are secreted proteins that trigger global cellular responses like mitosis, apoptosis, differentiation, etc. When cells are environmentally challenged (cuts, injuries, burns, mechanical stress, hypoxia, xenobiotics, systemic stress, etc.) cytokines induce tissue repair, struggle against bacterial invaders, etc. Stimulations with cytokines such as the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) or interleukin-1α (IL-1α) result in a transient intracellular production of harmful ROS. At first glance, these low levels of ROS were considered only as secondary messengers [109]. It was soon conceived, however, that low levels of ROS might become harmful if repeatedly or constantly present, and might trigger SIPS [110]. WI-38 HDFs repeatedly exposed to non-cytotoxic and non-proliferative concentrations of TNF-α and IL-1α display a senescent morphology and SA β-gal activity [111,112]. HDFs excised from gastric venous ulcers display several features of senescent cells: reduced proliferative capacity, enlarged size, SA β-gal activity, overexpression of fibronectin. TNF-α is a major component identified in the fluid of these ulcers [113,114].

Several reports show that pro-inflammatory cytokines can induce the degradation of the extracellular matrix. HDFs of the synovial membrane of patients struck by rheumatoid arthritis overexpress several enzymes degrading the extracellular matrix, such as metalloproteinases MMP-1, -2, -3, -9 and -10. Exposure of synovial HDFs to TNF-α triggers the expression of MMP-3 [115]. IL-1α induces the synthesis of MMP-1 in skin HDFs [116]. Exposure of cardiac fibroblasts to TNF-α leads to the overexpression of fibronectin and several metalloproteases. Degradation of collagen is observed [117]. These data are puzzling since the overexpression of several metalloproteinases is also observed in senescent HDFs (for a review: [4]). It must also be noted that human ageing is accompanied by an elevation of the circulating levels of TNF-α and IL-1 (for a review: [118]). Recent data suggest that those individuals who are genetically predisposed to produce high levels of IL-6 during ageing, i.e. −174 locus GG homozygous men, are disadvantaged for longevity [119].

9. Conclusions

The models of SIPS are already used in toxicology to seek possible long term effects of molecules in R&D. One can also detect possible anti-ageing effects of molecules in human cells in SIPS. Automation of the models of SIPS will lighten the budgetary and ethical burden of in vivo tests [120]. These systems give more useful mechanistic information compared to the information gained when using lower invertebrate animals as toxicological models.

The appearance of SIPS could be due to exacerbated modifications of a limited number of parameters that also undergo, to a more limited extent, age-related changes, among multiple other age-related changes. Irreversible changes in gene expression take place when SIPS becomes established: genes become permanently underexpressed while others become overexpressed. A change in the cellular targets under positive feed-back is operated by the establishment of

cascades of new regulatory loops, eventually locking the system in a new attractor [121]. Common and different pathways are induced after exposure to different kinds of subcytotoxic stress, changing the level of expression of common and different genes. Some of these pathways seem to share common portions with telomere-dependent replicative senescence. Abnormal oxidative stress is involved in many inflammatory processes, pathologies and intoxications. It would be worth examining whether cells taken from inflammatory sites are more prone to SIPS, thereby favouring the "inflamm-ageing" theory of ageing [122].

Last but not least, research on the mechanisms triggering SIPS is also of primary importance for inducing SIPS in cancer cells and not in normal cells. Complex interactions might exist between senescent cells and surrounding normal or cancer cells, whether fibroblasts or not. On one hand, the senescence of fibroblasts suppresses their own tumorigenesis. On the other hand, senescent fibroblasts, whether in telomere shortening-dependent senescence, oncogene overexpression-dependent senescence or H_2O_2-induced SIPS, were shown to promote growth and tumorigenesis of neoplastic and proneoplastic epithelial cells, and not of normal epithelial cells [123].

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