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Comprehensive Invited Review

Cellular Senescence: Molecular Mechanisms, *In Vivo* Significance, and Redox Considerations

Michael Muller

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Centre for Education and Research on Ageing, ANZAC Research Institute, University of Sydney, Concord RG Hospital, Concord, Sydney, Australia.

Abstract

Cellular senescence is recognized as a critical cellular response to prolonged rounds of replication and environmental stresses. Its defining characteristics are arrested cell-cycle progression and the development of aberrant gene expression with proinflammatory behavior. Whereas the mechanistic events associated with senescence are generally well understood at the molecular level, the impact of senescence *in vivo* remains to be fully determined. In addition to the role of senescence as an antitumor mechanism, this review examines cellular senescence as a factor in organismal aging and age-related diseases, with particular emphasis on aberrant gene expression and abnormal paracrine signaling. Senescence as an emerging factor in tissue remodeling, wound repair, and infection is considered. In addition, the role of oxidative stress as a major mediator of senescence and the role of NAD(P)H oxidases and changes to intracellular GSH/GSSG status are reviewed. Recent findings indicate that senescence and the behavior of senescent cells are amenable to therapeutic intervention. As the *in vivo* significance of senescence becomes clearer, the challenge will be to modulate the adverse effects of senescence without increasing the risks of other diseases, such as cancer. The uncoupled relation between cell-cycle arrest and the senescent phenotype suggests that this is an achievable outcome. *Antioxid. Redox Signal*. 11, 59–98.

I. Introduction

THE PIONEERING WORK of Hayflick and Moorhead (170, 171) in the early 1960s established the concept that primary cells isolated from tissues and grown in culture possess a finite capacity to undergo cellular replication. On reaching their replicative limit, such cells were termed senescent and were viewed as aged cells. Subsequently, senescent cells have been considered as a model of mammalian cellular aging, and it has been popular to believe that if cellular aging can be understood, it may provide insight into organismal aging (171, 364, 447). Others have viewed senescence as a crossroad for cells in crisis. From an evolutionary perspective, cellular senescence is thought to have evolved as a mechanism to prevent damaged DNA being replicated and passed on to future generations of cells (49, 163, 315). With the recognition that a diverse range of stimuli can induce cells to senesce prematurely, it became evident that senescence may play a role in disease states (59, 93, 390, 477).

Although substantial progress has been made in our understanding of the molecular mechanisms underlying senescence (51, 390, 392, 394, 451), a good deal of uncertainty remains as to the *in vivo* relevance of senescence and the role it plays in the normal aging process and various pathologic conditions. In particular, the linkages between cellular senescence at the two levels of organization of cell and organism remain poorly understood. Consequently, a need exists to understand the underlying factors that drive cells into senescence, how senescent cells survive *in vivo*, the roles they play in altering their local environment, and their effects on organisms as a whole. Recent research indicates that senescent cells are not a population of innocent-bystander cells; rather, the evidence suggests they play an active role in inducing change and accelerating changes in normal surrounding cell populations. Thus, the concept of cellular senescence has emerged from that of an interesting laboratory curiosity to a mechanism that is now able to provide some insight and explanation for the myriad complexities associated with advancing age and pathologic processes. This review examines the molecular mechanisms associated with the induction and maintenance of senescence and the characteristics and biologic behavior of senescent cells. A particular focus is on the role of senescence in aging and age-related diseases. Furthermore, the evidence for oxidative stress and cellular redox balance as critical mediators of senescence is examined.

A. Historical perspective

August Weismann (455) proposed in 1889 that cells should have a finite proliferative potential based on the consideration that older organisms would be detrimental to the survival of the species. Natural selection, he argued, would favor organisms whose somatic cells had a limited capacity for replication, and by this means, they would be deleted from the gene pool, ensuring that younger organisms had a chance to contribute to the genetic diversity of the species. This idea was challenged

and soon discarded at the beginning of the 20th century with the development of *in vitro* tissue-culture techniques for mammalian cells, when it became widely believed for a time that primary (diploid) cells isolated from tissues and organs could be propagated indefinitely, provided the right culture conditions could be supplied (53). It soon became apparent, however, that serially propagated nonmalignant cells in culture would develop abnormalities that included morphologic changes, polyploidization, and cessation of growth. This failure of diploid cells to undergo indefinite proliferation was ascribed to numerous causes, including a failure to provide one or more essential nutrients or growth factors, infection with Mycoplasma or viruses, or other unknown factors then to be discovered. In contrast, cells that developed a heteroploid karyotype were found to propagate for indefinite periods, reinforcing the view that the indefinite proliferation of normal somatic cells may be possible.

The work of Hayflick and Moorhead (170, 171) led to the development of the idea that somatic cells with replicative potential possess a "mitotic clock" that fixes their maximum lifespan (164). Concurrent with the work on *in vitro* senescence (171), Krohn (219) demonstrated that dermal tissue serially transplanted from a donor animal to younger animals retained the aging characteristics of the donor rather than acquiring those of the younger hosts. It thus became evident that the aging of cells was an intrinsic phenomenon associated with each cell type *in vitro* and *in vivo*; all that remained was to elucidate the mechanisms involved.

Further supporting the concept of senescence as a suitable model of *in vitro* aging was a series of findings demonstrating an inverse relation between human donor age and the number of *in vitro* population doublings isolated cells could achieve before replicative senescence developed (60, 138, 265, 364, 382). This relation was further supported by data from several other mammalian species (364). However, a common characteristic of these studies was the finding of substantial variations in the number of population doublings before the onset of senescence from fibroblasts of different donors. Although many reasons are found for such variability, including a difference in culture media, serum lot, incubation conditions, and tissue-culture surfaces, to name a few, the results cast some doubt as to the robustness of the relation between *in vitro* senescence and organismal aging.

Several years before Hayflick and Moorhead announced their findings, Harman (166) proposed the free radical theory of aging, implicating reactive oxygen species (ROS) and redox reactions in the aging process. This theory was to become particularly relevant four decades later, when it was discovered that mild oxidative stress induces a wide variety of cell types to become prematurely senescent (66, 123, 224). Cellular senescence was thus found to be a complex phenomenon incorporating both genetic and environmental components acting through convergent pathways. Our increased understanding of the molecular mechanisms associated with senescence and the application of new techniques for identifying senescent cells have provided us with a new perspective on the role of cellular senescence *in vivo*.

B. Nomenclature

The word senescence is derived from the Latin *senescere*, meaning to grow old or to wane. Although the term had been in use previously in the biologic sciences to describe the general deterioration of cells and lower organisms grown in culture, it was Hayflick and Moorhead (170, 171) who first used it to describe the finite replicative potential of normal somatic mammalian cells in culture, a phenomenon that has become known as the "Hayflick limit." Since then, the terms replicative senescence, cellular senescence, and cellular aging have become synonymous. The concept of replicative senescence is limited to cells capable of mitotic division and refers to cells that have undergone cell-cycle arrest as a result of telomere attrition. Telomeres are repetitive lengths of DNA and associated proteins that cap and stabilize the ends of chromosomes, preventing end fusions and genomic instability and, in vertebrates, have the base sequence of 5′-TTAGGG-3′ (298). Telomere attrition occurs with each round of cell division (165) because of incomplete DNA replication associated with the end-replication problem (discussed in Section II). It has since been recognized that oxidative stress can induce telomeric single-strand breaks that accelerate telomere loss (334, 335, 340, 442, 444, 446). Differentiated cells, such as neurons, are postmitotic and by definition cannot undergo replicative senescence. Nonreplicating, diploid cells in G_0 phase are technically outside of the cell cycle and are variously termed quiescent, presenescent, or young cells (Fig. 1). The term premature senescence was introduced to describe cells that had been induced to become senescent independent of their biologic or *in vitro* culture age. Premature senescence, also referred to as stress-induced senescence, can be induced by physical and chemical stresses and can involve telomere-dependent or -independent mechanisms. Furthermore, premature senescence can be induced in telomerase-competent cells, such as tumor cells.

To make meaningful numeric comparisons between cell populations with different replicative capacities, the concept of population doublings is used for senescence studies. The

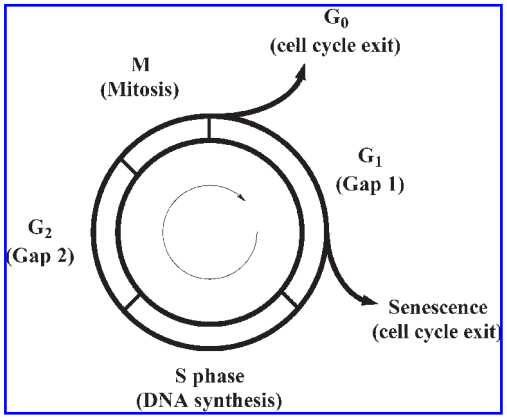

FIG. 1. The cell cycle. The cell cycle represents an ordered progression of events that result in cell replication with the production of two offspring cells. On entering the cycle, cells progress through phases G_1 (Gap1), S (synthesis), G_2 (Gap2), and M (mitosis) with check-points at the G_1/S and G_2/M interfaces to ensure that genomic integrity is maintained. DNA replication occurs during the synthesis (S) stage. Mitosis encompasses chromosomal separation and cytokinesis. After mitosis, cells reenter G_0 and leave the cell cycle. Failure to pass the G_1/S check-point results in exit from the cycle and the onset of cellular senescence or, in some cases, apoptosis.

population doubling (PD) is determined by the following formula:

$$PD = \log(N_t/N_0)/\log 2$$

where N_0 and N_t are the number of viable seeded and harvested cells, respectively. This equation derives from the principle that cells replicate geometrically (*i.e.*, by binary fission) by following the sequence: 2^0, 2^1, 2^3...2^n, where n is the number of generations or population doublings. Such a sequence is described by the equation

$$N_t = N_0 2^n$$

Therefore

$$2^n = N_t/N_0$$

and

$$n = \log_2(N_t/N_0)$$

or

$$n = \log(N_t/N_0)/\log 2$$

as

$$\log_2 n = \log_{10} n/\log_{10} 2$$

C. Characteristics of senescent cells

As originally defined, cellular senescence is the inability of normal diploid cells to undergo further replication (170, 171). However, it is now recognized that senescence involves altered gene expression, giving rise to a senescent phenotype, and cell-cycle arrest is but one manifestation of that altered phenotype. Senescence also confers an altered responsiveness to apoptotic stimuli, morphologic transformation, and altered protein expression (Fig. 2). As is discussed subsequently, replicative arrest and the senescent phenotype are uncoupled responses, and features of the senescent phenotype may result from mechanisms other than cell-cycle arrest. The use of the term senescence in the context of cell biology is perhaps something of a misnomer for, as this present work will endeavor to show, senescent cells, despite their inability to replicate, are otherwise very active.

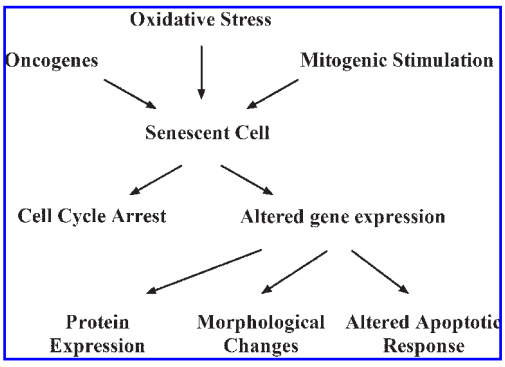

FIG. 2. Characteristics of cellular senescence. On becoming senescent as a result of various stresses, cells exhibit altered behavior, with cell-cycle arrest the defining characteristic. The senescent state is further characterized by altered gene expression that includes increased expression of structural proteins that allow cell enlargement and secretion of proinflammatory mediators. Different cell types acquire altered responses that may result in a loss of responsiveness, or confer increased sensitivity, to apoptotic stimuli.

1. Cell-cycle arrest. The inability to replicate and lack of responsiveness to physiologic mitogenic stimuli are the hallmarks of senescence. Successful progression through the cell cycle involves the synthesis of new DNA that can be quantified by using 5-bromo-2′-deoxyuridine or [^{3}H]-thymidine–incorporation assays. Senescent cells fail to incorporate either compound because of halted DNA synthesis, with growth arrest occurring predominantly at the G_1/S interface (2, 142, 398, 448). Immunologic techniques that detect replicative markers, including proliferating cell nuclear antigen (PCNA) or Ki-67, have been used to identify senescent cells *in vivo* (116). Human diploid fibroblasts (HDFs) in early passage proliferate in response to epidermal growth factor (EGF), tumor necrosis factor-alpha (TNF-α), fibroblast growth factor (FGF), or interleukin-1 (IL-1). In contrast, late-passage cells that have reached senescence fail to respond to these stimuli, even though they retain normal numbers of surface receptors (3, 176, 359) and exhibit normal binding affinity (3). The nonresponsive nature of senescent cells to mitogenic stimuli is related to the upregulation of caveolin-1 protein (see Section II for detailed discussion). In senescent, but not in quiescent, HDFs, caveolin protein binds to EGF receptors, preventing phosphorylation of Erk-1/2 after exposure to the EGF (332). It has been shown that expression of caveolin-1 in CHO cells prevents EGF signaling from EGF receptor to Erk-2, Raf, and MEK-1 (110). Similarly, senescent human mesenchymal stem cells show reduced adipogenic potential toward insulin because of upregulation of caveolin-1 (331).

2. Altered responsiveness to apoptotic stimuli. Replicative senescent cells can survive for several months in culture and display variable sensitivity to apoptotic stimuli. Quiescent WI-38 fibroblasts are sensitive to serum withdrawal and become apoptotic within 2 weeks, whereas senescent WI-38 fibroblasts are insensitive to serum withdrawal and display increased levels of the antiapoptotic bcl2 protein (450), but caspase-3, a key mediator of apoptosis, is downregulated (261). Others have found limited caspase activation present in subpopulations of replicative and prematurely senescent fibroblast cultures (260, 316). Nevertheless, replicative senescent HDFs are sensitive to Fas L (418), ceramide, TNF-α, and okadaic acid (89), compounds that induce apoptosis through different signaling pathways. In contrast to HDFs, replicative senescent human umbilical vascular endothelial cells (HUVECs) have a high rate of spontaneous apoptosis (157, 429, 448). These diverse findings indicate that the ability of senescent cells to undergo apoptosis is dependent on the maintenance of signaling pathways and expression of the apoptotic machinery.

3. Altered gene expression. Various cell growth–regulatory proteins, including p53, $p21^{CIP1}$ (p21), $p16^{INK4a}$ (p16), p38 mitogen-activated protein kinase ($p38^{MAPK}$) and the retinoblastoma protein (Rb), exhibit altered activation states or are upregulated with senescence and are involved with cell-cycle arrest. Others [for example, transforming growth factor-β1 (TGF-β1), and caveolin-1], regulate aspects of the

senescent phenotype. These are discussed further in Sections II and III.

4. Cell morphology. A hallmark of senescent cells in culture is their ability to undergo remarkable morphologic transformations. The most obvious in vitro changes that accompany the onset of senescence are the notable increase in cell size and flattened or thin cytoplasmic appearance that occur, compared with the presenescent state (Fig. 3). Underlying the shape change is an extensive reorganization of the cytoskeleton. In replicative senescent human fibroblasts, the expression of actin and tubulin is downregulated, whereas synthesis of vimentin dramatically increases, with cells having three- to fourfold higher levels of the protein compared with those in presenescent cells (311). Others have reported an increase in the prominence of actin stress fibers with redistribution of focal adhesion proteins (67). These morphologic changes are dependent on activation of proteins of the Rb family and require de novo protein synthesis (67). The cytoplasm becomes highly vacuolated (225), and the nucleus frequently becomes centrally located within the cytoplasm, developing morphologic changes that, under light microscopy, include gross enlargement, loss of internal nuclear structure, and a refractile appearance (279, 290). Senescence may result in genomic instability with the development of abnormal nuclei with binucleate and, less commonly, multinucleate cells developing. When seeded at low densities, where cells are sufficiently separated from each other, it can be demonstrated that multinucleation does not arise from fusion of adjacent cells, but rather from chromosomal abnormalities. Replicative senescent HDFs growth arrested in G_1 phase display normal 2N DNA content, whereas HUVECs, although arrested in G_1, exhibit 4N DNA content, indicative of polyploidization (448). Whereas quiescent cells display diffuse staining for actin and vimentin, senescent cells stain prominently for these proteins with many fiber bundles and express more caveolin-1 than their quiescent counterparts (441). It has been proposed that caveolin-1 plays an important role in senescence-associated morphologic changes by regulating focal adhesion kinase activity and actin stress fiber formation. Downregulation of caveolin-1 alone is sufficient to disrupt focal adhesion complexes, actin fibers, and abolish the characteristic senescence morphology (70, 71).

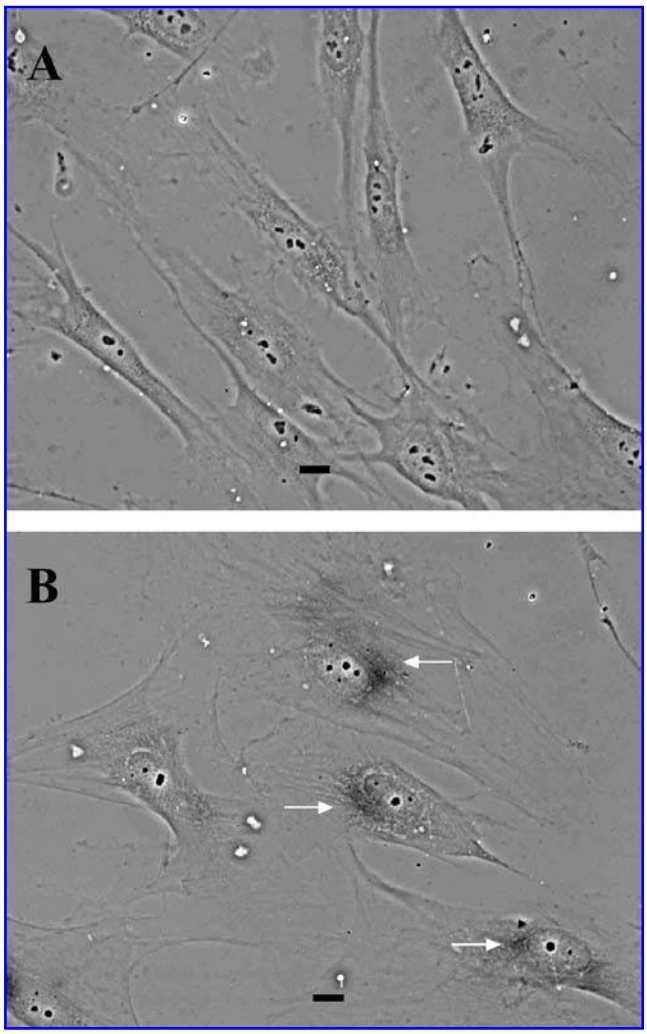

FIG. 3. Morphology of senescent human fibroblasts. Normal human dermal diploid fibroblasts exhibit a typically thin, elongated appearance with dense cytoplasm when subconfluent in culture (**A**). Senescent fibroblasts become much larger with diffuse, thin cytoplasm with prominent stress fibers becoming visible under phase contrast (**B**). *Arrows*, Areas of dark perinuclear staining due to SA β-gal activity (scale bar = 10 μm).

5. Senescence-associated β-galactosidase activity. The most widely used biomarker for determining cellular senescence is the senescence-associated β-galactosidase (SA β-gal) assay (99), which produces a blue perinuclear staining in positive cells (Fig. 3). Since the introduction of the SA β-gal assay, its use has been controversial (79, 391), and as many reports rely substantially on the SA β-gal assay to demonstrate *in vivo* senescence in tissue sections, some consideration of the assay and what it detects is warranted.

It was recognized quite early that 5-fluorouracil, an antineoplastic agent, induced a senescent phenotype in fibroblasts that was associated with altered enzyme activities, including those associated with β-galactosidase (182). β-Galactosidase is a ubiquitous hydrolase localized to lysosomes and functions to cleave β-D-galactose residues from β-D-galactosides. Maximal activity of the enzyme is species dependent but typically occurs in the acidic range (pH 3–5). First reported in 1995 as a biomarker for *in vitro* and *in vivo* cellular senescence, the SA β-gal assay is a simple qualitative histochemical method reported to detect a β-galactosidase at pH 6 in senescent but not presenescent or immortalized cells (99). Although the precise biochemical basis for the SA β-gal reaction was not identified at the time, the authors put forward several possibilities, including that the increase in lysosomal activity associated with senescence might increase lysosomal β-galactosidase protein (pH 4.5) so that its activity becomes detectable at pH 6. The authors concluded that expression of SA β-gal was in response to senescence and not its cause. Thereafter, several groups reported the detection of senescent cells *in vivo* by using the SA β-gal assay (289, 401, 430), and variations of the assay were reported, enabling quantitative results to be obtained (218, 466). Concurrently, the validity of SA β-gal as a biomarker

for cellular aging was under challenge. Krishna *et al*. (218) reported SA β-gal activity in immortalized lung carcinoma (A549), colon carcinoma (Caco2-TC7), promyelocytic leukemia (HL60), and hepatoma (HepG2) cells. With more-sensitive quantitative HPLC methods, β-galactosidase activity was detected at pH 4.5 and pH 6 in all cells, irrespective of senescence status; thus, the activity of β-galactosidase at pH 6 is not a specific marker of senescence. It was subsequently demonstrated that on becoming senescent, cells increase their lysosomal mass with a consequent increase in β-galactosidase activity, which becomes detectable at suboptimal pH (225). Furthermore, fibroblasts from patients with autosomal recessive G_{M1}-gangliosidosis, a lysosomal disorder in which β-galactosidase is defective, do not express SA-β-gal on becoming senescent, thus demonstrating that the enzyme is encoded by the lysosomal β-galactosidase gene, and it is not required for senescence (235).

After the onset of replicative senescence by normal human MRC5 fibroblasts, an increase is found in the presence of autophagic vacuoles and accumulation of lipofuscin (133). *In vivo*, the association between increased lipofuscin, SA β-gal expression, and age has been observed (280). Furthermore, TGF-β1 induces epithelial cells to express SA β-gal activity without arresting cell growth (428) and inhibition of $p38^{MAPK}$ by SB203580, a specific inhibitor, whereas producing only a modest decrease in cell-growth inhibition resulted in a significant decrease in SA β-gal expression (194). Factors other than aging have been found to increase the expression of SA β-gal that may result in reversible or irreversible expression. The use of serum starvation and the growth of cells to confluence produce reversible expression, whereas growth to replicative senescence or treatment with H_2O_2 results in irreversible expression (391, 466). It must be concluded, therefore, that, based on the available data, an increase in SA β-gal activity is not specific for senescence, either *in vitro* or *in vivo*. Nevertheless, expression of SA β-gal is a useful nonspecific marker of cellular senescence when supported by other independent determinants of senescence, such as DNA-damage foci (177, 200, 384, 385).

II. Molecular Mechanisms

From the observations of Hayflick and Moorhead (171), Olovnikov (320) proposed the theory of marginotomy to account for the limited replicative potential of cells. This theory stated that each copy of replicated linear DNA would be shorter than its template due to the inability of DNA polymerase to replicate fully the ends of linear DNA, the end-replication problem, and identified telogenes (telomeres) as the sites of DNA loss. The problem of incomplete DNA strand replication does not occur with bacteria or viral genomes because they possess circular DNA, which is fully replicated, providing an explanation as to why those organisms replicate indefinitely and appear not to age. Subsequently, experimental evidence was produced demonstrating that telomere length and the total amount of telomeric DNA is reduced during the serial propagation of HDFs, with the mean telomere length decreasing by ~50 base pairs per mean population doubling (165). It was subsequently proposed that the exit of HDFs from the cell cycle was due to telomere erosion (6, 241).

The discovery that telomeres shorten with each cellular replicative cycle appeared to provide a mechanism to explain the limited replicative capacity of somatic cells (165). However, it soon became evident that more-complex processes were at work. It was observed that events that induce DNA damage, such as oxidative stress, also result in cell-growth arrest in G_1 phase (126), and senescence could be induced by serum deprivation, excess glucose, or other environmental stresses that were independent of telomere erosion or DNA damage (37, 65, 127, 224, 450). Further, primary cultures of mouse embryonic fibroblasts (MEFs) typically progress to senescence after only several population doublings (396, 397), despite having relatively long telomeres of ~60 kb in length. In contrast, human dermal fibroblasts with telomeres ~12 kb in length are capable of ~50–60 population doublings. Rodent cells also express telomerase, an enzyme responsible for maintaining telomere length; therefore, they should retain their replicative competency for many more population doublings than human cells (465). Their failure to do so led to the realization that factors other than telomere erosion could activate the senescence program. As a model of cellular senescence, the human diploid fibroblast has been studied more than any other cell type. A number of other cells of different origins, for example, HUVECs, have been investigated and yield generally similar results; however, differences exist between various cell types as do interspecies differences.

A. Two pathways to senescence

Detailed investigations at the molecular level have revealed two major pathways for the induction of cellular senescence. Replicative senescence is telomere dependent and is mediated by the p53-p21 pathway (394, 404) and also encompasses the DNA-damage response (DDR) mechanism. In contrast, a telomere-independent mechanism, typically activated by oxidative stress, is mediated by the Erk-$p38^{MAPK}$ pathway (51, 194, 224, 392, 451). Both of these pathways converge on Rb, the retinoblastoma gene product, which is responsible for allowing progression from G_1 to S phase.

Control of cell proliferation involves two checkpoints within the cell cycle: one at the G_1/S interface and one in the G_2 phase of the cycle (Fig. 1). It was noted very early that WI-38 fibroblasts at later population doublings exhibited an increase in cell-cycle time because of a longer residency in the G_1 phase of the cycle (147). Further analyses of senescent WI-38 (142), IMR-90 (398), and MRC5 fibroblasts (2) or HUVECS (448) have confirmed that senescent cells accumulate in late G_1 phase and, on reaching this point, they become unresponsive to mitogenic stimuli.

B. The p53-dependent pathway

A linear sequence of events has been elucidated involving the transcriptional activator and tumor-suppressor protein, p53; the cyclin-dependent kinase inhibitor, p21; and the cell-cycle regulator, retinoblastoma protein (Rb), as the principal players in telomere-dependent senescence (Fig. 4). p53 plays a pivotal role in regulating several aspects of cell function, including control of cell growth, DNA repair, apoptosis, and senescence. The sensing of DNA damage, including telomere shortening, and activation of p53 in G_1 prevents abnormal DNA being replicated in S phase. The functions of cell-growth arrest and apoptosis are therefore critical roles of the protein, for they limit the ability of damaged cells to pass on

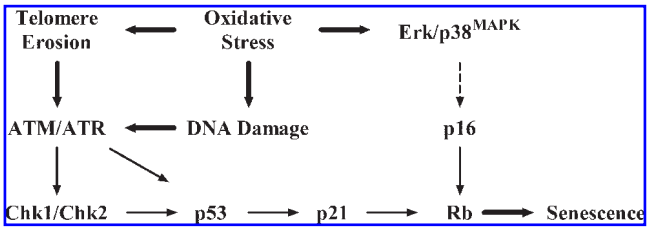

FIG. 4. Pathways to senescence. Senescence is mediated by either the p53/p21 or the Erk/p38^{MAPK} pathways, with both converging on Rb. The p53/p21 pathway is sensitive to telomere attrition, whereas Erk/p38^{MAPK} is activated by various stresses, including oxidative stress. Oxidative stress may cause strand breaks that activate the DNA-damage response through p53. p21 and p16 are cyclin-dependent kinase inhibitors that cause the retinoblastoma protein (Rb) to become hypophosphorylated, leading to cell-cycle arrest.

aberrant DNA or to develop neoplastic potential. Indeed, many human tumors possess mutated p53 protein that is deficient in activity (144, 186, 305), whereas suppression of p53 expression results in emergence of cells from senescence, resumption of replication, and immortalization (102). Similarly, MEFs derived from *p53*$^{-/-}$ animals do not become senescent (169, 327). The reversibility of senescence due to telomere dysfunction on inactivation of p53 is dependent on low levels of p16 and is cell-type dependent. Furthermore, senescent cells expressing human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase, without p53 inactivation do not resume replication (25). In contrast, mice in which p53 has been mutated to express the activated form of the protein develop a premature aging syndrome suggesting that p53 plays a role in organismal aging (426).

The simian virus 40 (SV40) large T-antigen and its mutated forms have played an important role in elucidating the functions of p53 and Rb in senescence, as have the human papillomavirus E6 and E7 genes. Whereas the large T-antigen has binding domains for both p53 and Rb, the E6 and E7 proteins bind only p53 and Rb, respectively (394). On binding with T-antigen, p53 is unable to bind further to DNA, thus inhibiting the cell-cycle arrest mechanism and resulting in a limited extension of the replicative lifespan of cells. With the E6 and E7 proteins, it has been established that although p53 initiates the steps leading to senescence, it must also act with Rb to arrest the cell cycle (394). Initial evidence for the transcriptional regulation of p53 during replicative senescence was provided by Kulju and Lehman (221), who found p53 mRNA and p53 protein levels in the IMR-90 diploid fibroblast were increased severalfold in near-senescent cells compared with younger cells. They further noted an increase in phosphorylated p53 in cells nearing senescence. Other studies, however, have not reported an increase in cellular p53 mRNA or p53 protein levels on induction of senescence, but rather, p53 becomes activated by posttranslational modification with subsequent induction of growth arrest (2, 13, 438).

With WI-38 human fibroblasts, it has been found that p53 activity is higher in quiescent and senescent cells compared with proliferating cells. Cells entering into G_0 on growth factor withdrawal increase their p53 mRNA levels and show a transient increase in p53 protein, with increased transcription of p21 mRNA and protein. Thus, p53 plays a key role in initiating and maintaining the growth arrest of quiescent (G_0) cells (189). The recently discovered microRNAs, miR-34a, miR-34b, and miR-34c, have been identified as direct transcriptional targets of p53 and play key roles in tumor suppression. Induced by DNA damage and oncogenic stress, the miR-34 class induces apoptosis and senescence (77, 173, 416).

C. Activation of p53

How is p53 activated in response to telomere shortening or DNA damage? The mechanisms by which telomere-shortening or DNA-damage signals are transduced to the p53 protein remain to be fully elucidated; nevertheless, much has been learned in recent years. The association of p53 with DNA damage and cell-cycle arrest in G_1 was noted when increased levels of the protein were found in ML-1 cells after gamma irradiation or treatment with the genotoxin, actinomycin D (209). Injection into the nucleus of linearized plasmid DNA or circular DNA with a large gap induces p53-dependent arrest (185). Within the telomere-dependent pathway, the activation of p53 occurs when telomeres become critically short. Human telomeres are ~10–15 kb long and are heterogeneous in length (232), although senescence occurs when the mean telomere length is reduced to 4 to 7 kb (6, 165). However, as terminal restriction fragment (TRF) analysis is used as a measure of telomere length, and TRF analysis reports mean telomere length, it may be sufficient for only one critically short telomere to trigger the senescence pathway. Support for this proposal was provided by nuclear microinjection titration experiments with normal HDFs, in which the insertion of DNA with a single irreparable double-strand break results in p53 activation (185). The connection between telomeres and senescence may be due to the formation of dicentric chromosomes as a result of recombination of fused telomeric ends. Benn (26) reported an increase in the number of dicentric chromosomes in human fibroblasts with increasing population doublings, and it has been proposed that recombination of critically short telomeres results in dicentric chromosomes that break during mitosis, thus signaling DNA damage and activation of the senescence pathway (436). The shortening of telomeres eventually leads to telomere uncapping, resulting in exposure of the DNA end strands (34). Such an exposure of uncapped ends may lead to homologous recombination and nonhomologous end joins, thus forming dicentric chromosomes. The ends of intact telomeres terminate in a large duplex loop, the "T-loop" [reviewed extensively in (36, 440)], which is thought to protect the 3′ overhang that consists of single-stranded DNA several hundred bases in length (145). The T-loop stabilizes and protects the ends of telomeres, as uncapped telomeres may also be sensed as double-stranded DNA breaks (407). Maintenance of the T-loop is dependent on the DNA-binding proteins telomeric-repeat-binding factor-1 and -2 (TRF1, TRF2) and protection of telomere 1 (POT1; 28, 172). Inactivation of TRF2 (207), or its displacement (81, 178), results in telomere uncapping and senescence. These lines of investigation have led to a new unresolved question as to how a change to telomere structure induces senescence: is senescence due to telomere shortening or to telomere un-

capping? Comparison of shortening rates of the double- and single-strand ends of telomeres indicates that the single-strand end is subject to erosion, as is the whole telomere (407). Ectopic expression of hTERT in quiescent fibroblasts results in extension not only of the double-stranded region of the telomere but also of the 3′ overhang (407). Thus, senescence may be dependent on critically short telomere 3′ overhangs that are sensed as uncapped telomeres rather than on overall telomere length. Oxidative stress accelerates the erosion of telomeres and hastens the onset of senescence both *in vitro* and *in vivo* (208, 334, 335, 442, 443, 444). The telomeric 3′ overhang ends in a G triplet that is particularly susceptible to oxidative cleavage, which may be detected as single-strand breaks (175, 317, 446).

Other studies have identified DNA-damage foci that are located at the telomeres of senescent cells, and these have been implicated in the induction of senescence (82, 134, 178, 445). ATM (ataxia–telangiectasia mutated) belongs to the phosphatidylinositol 3-kinase family of proteins and plays a direct role in maintenance of telomere length (285) and DNA repair of double-strand breaks (399). It was identified in the autosomal recessive disorder ataxia–telangiectasia (AT), a disorder that has shed much light on the mode by which DNA-damage signals are transduced to p53. Fibroblasts from individuals with AT senesce prematurely in culture and exhibit enhanced telomere erosion with increased p53 activation (438). By a complex series of signals that remain to be fully elucidated, ATM and ATR (ATM and RAD3-related), activate p53 by phosphorylation (178). ATM and ATR translocate to sites of DNA damage where the histone H2AX is phosphorylated to γ-H2AX. γ-H2AX is thought to induce changes in the local chromatin structure, thus facilitating in the focal assembly of DNA-repair proteins and the checkpoint proteins Chk1 and Chk2. ATM and ATR are then able to phosphorylate Chk1 and Chk2, which subsequently activate p53 (82, 134, 178, 445). ATM can phosphorylate p53 directly or indirectly by phosphorylation of Chk2 at Thr68 (274), which in turn directly phosphorylates p53 (62, 413). Thus, DNA damage or telomere uncapping due to shortened telomeres appears to be sufficient to initiate senescence.

Poly(ADP-ribose) polymerase (PARP) is another of several enzymes activated in response to DNA damage that interacts with p53. PARP is an abundant nuclear protein that catalyzes the NAD^+-dependent transfer of ADP-ribose monomers to nuclear acceptor proteins and is activated in response to single- and double-strand breaks. Co-immunoprecipitation experiments have revealed that PARP associates with p53, both *in vitro* and *in vivo*, and inactivation of PARP results in loss of p21 and the upregulation of MDM2 (murine double minute-2) in response to DNA damage (438). In other experiments, inhibition of endogenous PARP by 1,5-dihydroxyisoquinoline or by trans-dominant inhibition by overexpression of the PARP-1 DNA-binding domain abrogated p53 activity, attenuated p21 expression, and suppressed p53-mediated G_1 arrest in response to DNA damage induced by ionizing radiation (457).

D. Regulation of p53

Because of the critical role p53 has in controlling cellular activities, the mechanisms regulating p53 are necessarily complex and consist of a range of posttranslational changes to the protein. These include phosphorylation (13, 453), acetylation (191, 438), and redox control (197). The activity of p53 is tightly controlled by MDM2, a negative regulator molecule. The crucial *in vivo* role of MDM2 in regulating the activity of p53 was demonstrated in *mdm2*-deficient mice, in which inactivation of p53 resulted in rescue of embryonic survival (294). In unstressed cells, p53 has a half-life of a few minutes (259) and is maintained at low levels in an inactive form by direct binding to MDM2 [reviewed in depth (120)]. Indeed, p53 induces transcription of the *Mdm2* gene through an autoregulatory feedback loop, resulting in MDM2-p53 binding that promotes p53 degradation by the ubiquitin proteasome pathway (367). By this means, cellular levels of p53 and MDM2 are precisely regulated. In addition, an alternative ubiquitin-independent degradation pathway has been reported (12).

In response to various stress signals, p53 becomes activated by posttranslational modifications (198). Phosphorylation may occur at several sites, and phosphorylation differences have been discerned between replicative and stress-induced senescent cells (453), although some have found no correlation between p53 and MDM2 binding (13). Acetylation of p53 by the p300/CBP transcriptional coactivator occurs in response to several activators, including UV irradiation, hypoxia, hydrogen peroxide, and the antineoplastic DNA-damaging agents, camptothecin and cisplatin. MDM2 suppresses p300/CBP acetylation of p53 both *in vitro* and *in vivo* (191). PML (promyelocytic leukemia protein) is also known to regulate p53 activity by posttranslational acetylation in response to oncogenic RAS (338). These processes are illustrated in Fig. 5.

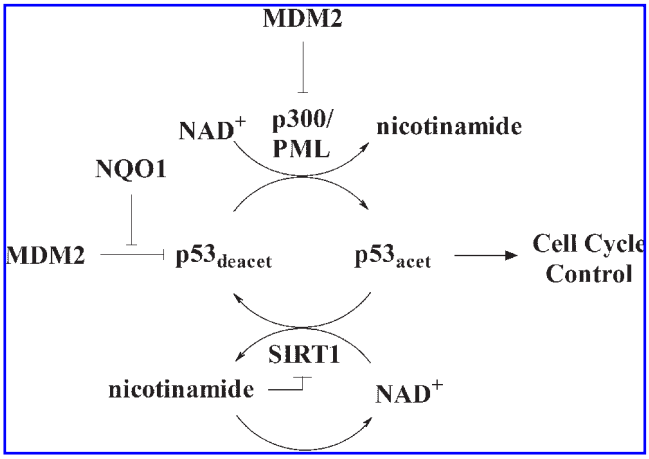

FIG. 5. Regulation of p53. The p53 protein controls several aspects of cell function, including cell-cycle progression, apoptosis, and senescence. The availability of p53 is tightly controlled by binding with the negative regulator, MDM2. Posttranslational acetylation of p53 occurs by p300/CBP or promyelocytic leukemia protein (PML) in response to various stresses, including hydrogen peroxide and oncogenic RAS. MDM2 suppresses the acetylase activity of p300/CBP. NQO1 has been reported to stabilize p53 by preventing its association with MDM2. SIRT1 is a NAD^+-dependent deacetylase belonging to the sirtuin family of proteins that inhibits p53. SIRT1 is inhibited by nicotinamide, the metabolite of NAD^+, enabling precise regulation of p53.

E. Role of p21, p16, and Rb

On becoming activated because of sensing critically short telomeres or DDR signals, p53 initiates a series of downstream transcriptional events. An immediate target is the $p21^{WAF1}$ gene (108, 313), which encodes the potent cyclin-dependent kinase (Cdk) 2 inhibitor, p21 (168). Increased levels of p21 mRNA (313) and p21 protein (5, 406, 407) are found in HDFs as they approach senescence. Moreover, p21 is overexpressed in senescent cells (313), and overexpression of p21 results in cell-growth arrest in G_1 when the $p21^{WAF1}$ gene is transfected into quiescent cells (467). The disruption of the p21 gene in otherwise normal HDFs causes senescence to be bypassed (45). Nevertheless, once senescence has been accomplished, p21 levels decline markedly (5, 406). It is potent inhibitor of Cdk2; immunoprecipitation experiments have revealed that p21 complexes with cyclin A, cyclin D1, cyclin E, and Cdk2 (168).

The second Cdk inhibitor to play a role in the induction of senescence is p16 (190, 357, 389, 406), which is under the control of Bmi-1 (190). Bmi-1 belongs to the Polycomb group gene family and acts as a transcriptional repressor (431). Bmi-1 is a direct transcriptional target of c-Myc, and diminished c-Myc signaling is associated with increased expression of p16 and senescence (151). In contrast, increased Bmi-1 levels extend the replicative lifespan of cells (190). After the decline in p21 levels with the onset of senescence, the level of p16 dramatically increases, and it is this protein that assumes the long-term role of inhibitor for the Rb kinases, CDK4 and CDK6, which is achieved by complexation between kinase and p16 (5, 43, 276, 389, 406). Before the onset of senescence, p16 is maintained at a low level by miR-24, a negative-regulator microRNA that suppresses the translation of p16. As cells enter senescence, the level of miR-24 diminishes while p16 increases (229). It is noteworthy that the timeframe for the increase in p16 expression correlates with phenotypic changes, including increased cell size and the expression of SA β-gal activity (406).

Rb is a cell-cycle regulator, one function of which is to control progression from G_0 and G_1 to S phase (162, 397). Cell-cycle progression is dependent on hyperphosphorylation of Rb by cyclin-dependent kinases in association with cyclins to allow transcription of genes that permit G_1- to S-phase progression (Fig. 6). Rb is regulated by phosphorylation with Cdk2, Cdk4, and Cdk6 acting as Rb kinases, whereas p21 and p16 are Cdk inhibitors that act by forming complexes with Rb (5).

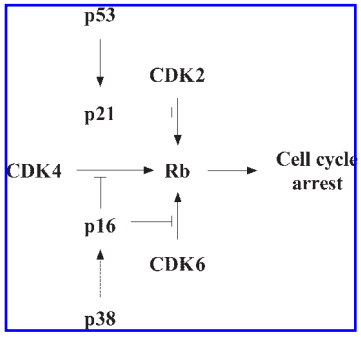

FIG. 6. Regulation of Rb. The phosphorylation status of the retinoblastoma protein (Rb) determines the course of cell-cycle progression. Activation of the cyclin-dependent kinases (CDK), CDK2, CDK4, and CDK6, results in hyperphosphorylation of Rb with cell-cycle progression. Inhibition of the cyclin-dependent kinases by p21 and p16 result in Rb becoming hypophosphorylated, and cell-cycle progression is halted.

In quiescent and senescent HDFs, the Rb protein is hypophosphorylated, and Cdk2 protein levels are low. However, Rb becomes hyperphosphorylated, and Cdk2 levels increase before entry into S phase after challenge of quiescent HDFs with serum. In contrast, serum challenge of senescent cells does not induce Rb phosphorylation, Cdk2 upregulation, or S-phase progression (107). Further, both SV40 T-antigen and E7 proteins bind Rb, preventing phosphorylation, and allow progression into S phase (394). Thus, it has been established that an inability to phosphorylate Rb prevents cells from traversing the G_1/S barrier and promotes their entry into senescence (404, 405). Hypophosphorylation of Rb inactivates the E_2F transcription factor, which then no longer transactivates the promoter gene required for progression through S phase (65).

A number of important differences exist between the senescence pathways of mouse and human cells when grown under normal laboratory conditions. Despite having telomeres ~60 kb long and expressing telomerase activity, MEFs grown in culture and exposed to atmospheric oxygen (20% O_2) senesce after 10 to 20 population doublings and express elevated levels of p19/ARF, p53, and p21 (397). MEFs derived from *p53*-null or *ARF*-null mice do not senesce (169), whereas those from *p16*-null or *Rb*-null animals do (480). When grown at physiologic oxygen tensions (3% O_2), MEFs replicate indefinitely (333). Thus, for MEFs, the p16/Rb pathway appears to contribute less to senescence than in HDFs.

The ability of senescent cells to remain in a permanent state of replicative arrest and to maintain the stability of the senescent phenotype is believed to be related to epigenetic regulation because of changes in chromatin dynamics (128, 131), including the formation of senescence-associated heterochromatin foci (SAHFs; 309). Although not specific to senescence, SAHFs have been detected using cytohistochemical techniques (309). Staining of senescent cell nuclei with 4′,6-diamidino-2-phenylindole (DAPI) reveals the presence of DAPI positive foci, whereas the nuclei of young cells show a more-diffuse staining pattern. SAHFs consist of several chromatin proteins, including the heterochromatin markers K9-methylated histones H3 and HP1, the macroH2A histone variant, and HMGA structural proteins (308). These foci appear predominantly in response to p16/Rb-dependent senescence rather than by p53 activation and are associated with the repression of proliferation-promoting genes, such as E_2F (309).

F. SIRT1 as a p53 regulator

The sirtuins are a family of highly conserved proteins with NAD^+-dependent protein deacetylase activity that have been extensively implicated in aging processes (74, 270). After it was first identified in yeast as a transcriptional regulator, silent information regulator 2 (Sir2) upregulation was shown to result in lifespan extension for *Saccharomyces cerevisiae* (249) and the nematode *Caenorhabditis elegans* in response to calorie restriction (421). In mammalian cells, silent information regulator two ortholog 1 (SIRT1), a human Sir2 homologue of yeast, binds to, and deacetylates p53, thus antagonizing PML/p53-mediated senescence (217, 231). Over-

expression of SIRT1 results in inhibition of hydrogen peroxide–induced senescence biomarkers, including changes to cell morphology and expression of SA β-gal (322). Inhibition of SIRT1 by the specific inhibitor, sirtinol, induces senescence in HUVECs by increasing p53 acetylation (322), and in breast tumor cells, by attenuating Ras/p38^{MAPK} signaling (323). Furthermore, depletion of NAD^+ prevents deacetylation and maintains cells in a senescent state (129). SIRT1 further promotes cell survival by deacetylating the DNA-repair factor Ku70 resulting in sequestration of the proapoptotic Bax protein, thus inhibiting stress-induced cell death (74). Whereas SIRT1 is NAD^+ dependent, the by-product of deacetylation, nicotinamide, is a noncompetitive inhibitor of Sir2 and SIRT1, acting as a negative regulator (32). However, whereas nicotinamide reduces the lifespan of yeast by inhibition of Sir2, it extends the replicative lifespan of mammalian cells in culture rather than inducing senescence (205, 246). This effect may be due to differences in the metabolism of nicotinamide by yeast and mammalian cells, particularly by the NAD^+-salvage pathway. Alternatively, yeast lack a p53 orthologue, and Sir2 acts as a transcriptional regulator by modulation of chromatin structure, which may be inhibited by nicotinamide (203). Furthermore, nicotinamide may act independent of SIRT1 by inhibiting cellular production of reactive oxygen species and reducing telomere erosion (205).

G. The Erk-p38^{MAPK} pathway

It is apparent that mechanisms other than telomere erosion induce cellular senescence. HDFs exposed to oxidative stress, in the form of repeated treatments with sub-cytotoxic concentrations of hydrogen peroxide or *tert*-butyl hydroperoxide, become growth arrested and develop senescence characteristics (65, 224). Similarly, serum withdrawal provokes senescence (450), as does prolonged expression of oncogenic *ras* in human IMR-90 or primary mouse embryo fibroblasts, resulting in permanent cell-cycle arrest with accumulation of p53 and p16 (94, 390). Moreover, several groups have found antioxidants to be useful in preventing the senescence-inducing effects of oxidants (14, 15, 208, 302) and extending the replicative potential of cells in culture (14, 15, 208, 277, 326).

The *ras* protooncogene family encodes small GTP-binding proteins that transduce growth signals from cell-surface receptors in response to extracellular stimuli (278). Induction of oncogenic *ras* results in premature senescence executed by the Ras-Raf-mitogen–activated protein kinase kinase-Erk-p38 pathway (194, 224, 451). Senescence is induced by the sequential activation of MEK-extracellular signal–regulated kinase (Erk) followed by the accumulation of the MAP kinase kinases, MKK3 and MKK6, and p38^{MAPK} activation (Fig. 7; 194, 451). As with the signaling processes involved with p53 activation, much remains to be determined with respect to the events surrounding the transduction of senescence-inducing signals from the plasma membrane. Cellular responses to external stimuli are transduced *via* intracellular signaling pathways, key components of which are the mitogen-activated protein kinases (MAPKs) that act to couple extracellular signals to changes in gene expression. Several isoforms have been identified, each having differing substrate specificities, giving effect to different physiologic responses. Activation of p38^{MAPK} occurs in response to many external

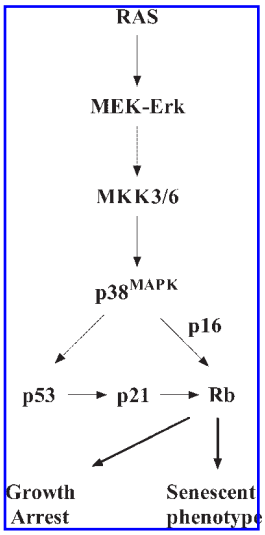

FIG. 7. The p38^{MAPK} pathway to senescence. Premature senescence can result from sustained activation of p38^{MAPK} by oncogenic signaling. Activation of MEK-extracellular signal-regulated kinase (Erk) is followed by accumulation of the mitogen-activated kinases (MKK3 and MKK6), followed by p38^{MAPK} activation of the cyclin-dependent kinase inhibitor, p16. This results in hypophosphorylation of Rb and cell-cycle arrest. Indirect activation of the p53/p21 pathway can occur because of upregulation of NAD(P)H oxidase with subsequent DNA damage due to oxidant production (*dashed arrows*, indirect pathways).

stimuli that include growth factors (118), cytokines (236), microbial toxins (159), and osmotic changes (158). The role of p38^{MAPK} in senescence was discovered after microinjection of a p38-encoding plasmid into NIH 3T3 fibroblasts, which downregulated cyclin D1 expression and arrested cell growth in G_1 phase (291). It was subsequently shown that low-level oxidative stress activates p38^{MAPK}, causing cell-cycle arrest (224).

Support for the role of p38^{MAPK} in the induction of premature senescence is provided by U937 human lymphoid cells, which lack functional p53 (104). Treatment of U937 cells with a low concentration of hydrogen peroxide (20 μM) induces cell-cycle arrest in M phase (224). Early responses (within 10 min) are the phosphorylation of p38^{MAPK} without change in protein level, phosphorylation of the p38^{MAPK} activators, MKK3/MKK6, and phosphorylation of ATF-1 and ATF-2. Furthermore, p38^{MAPK} remains activated for 24 h after exposure. Inhibition of these several phosphorylation events can be achieved by the antioxidant, *N*-acetylcysteine, indicating a role for ROS, and possibly, reactive nitrogen species. Based on flow-cytometric analysis of cellular DNA content, and in contrast to p53-mediated senescence, it appears that the p38^{MAPK} pathway allows progression through the S phase but does not allow M-phase completion in U937 cells, as indicated by the development of a high number of cells exhibiting polyploidy. Inhibition of p38^{MAPK} with the specific inhibitor, SB203580, demonstrated the key role of the kinase by preventing polyploidy after oxidant exposure (224). Since then, a role for p38^{MAPK} has been demonstrated in telomere-dependent and telomere-independent senes-

cence mechanisms (161, 194, 451). Activation of $p38^{MAPK}$ is by MAP kinase kinases (MKKs), with selectivity being achieved by different MKK isoforms, in particular, MKK3 and MKK6. Stable expression of activated MKK6 in U2SO cells results in activation of $p38^{MAPK}$ and the development of senescent characteristics (161).

How is p53 activated through the Erk-$p38^{MAPK}$ pathway? Although this pathway is independent of the p53–DNA-damage pathway, constitutive activation of Raf-MEK-Erk induces p53 and upregulation of p21 and p16 (247), which is likely due to a Ras-induced increase in oxidative stress, resulting in DNA damage and activation of the DNA-damage response (234). In normal IMR-90 lung fibroblasts, activation of $p38^{MAPK}$ results in increased oxidative stress due to upregulation of a specific membrane-bound NADH oxidase that generates hydrogen peroxide (420). The increased availability of this oxidant is thought to result in increased DNA damage, which is then sensed, resulting in p53 activation and upregulation of p21. Supporting evidence for this view is provided by the observation that senescent HDFs excise and excrete fourfold more 8-oxoguanine per day from DNA and have ~35% higher steady-state levels of 8-oxo-2′-deoxyguanosine in DNA compared with presenescent cells (66). However, oxidative base modifications to DNA alone appear to be insufficient to activate p53, as no correlation has been found between p53 activation and steady-state levels of 8-oxo-2′-deoxyguanosine after oxidant exposure (65). However, with single-cell gel electrophoresis (the Comet assay), Boots *et al*. (40) reported increased DNA damage in rat lung epithelial cells in the form of strand breaks in response to treatment with hydrogen peroxide concentrations known to induce senescence.

H. Role of caveolin in senescence

Caveolin-1 has been identified as an important mediator in cellular signaling processes. Caveolin-1 is a 21- to 24-kDa integral membrane protein and a principal component of caveolae, which are 50- to100-nm vesicular invaginations of the plasma membrane (353, 355). The caveolins act as scaffolding proteins to concentrate and functionally regulate signal-transduction molecules, including EGF receptor, G proteins, Src-like kinases H-Ras, protein kinase C, endothelial nitric oxide synthase, and integrin, and are involved in vesicular trafficking (216, 242, 319, 354, 355). Subjecting 3T3 fibroblasts to serum starvation (130) or oxidative stress with hydrogen peroxide (441) results in increased expression of caveolin-1 and subsequent cell-cycle arrest in G_0/G_1. Moreover, the expression of caveoloin-1 is associated with a two- to threefold increase in p53 activity and increased p21 expression (130).

Sp1 belongs to a family of proteins involved in oxidative stress–induced gene transcription (378). Sequential activation of $p38^{MAPK}$ and Sp1 after oxidative stress results in activation of the caveolin-1 gene-promoter elements and transcription of the caveolin-1 gene (85). The *in vivo* expression of caveolin-1 through stable transgenic expression causes an increase in mouse embryonic fibroblasts in G_0/G_1 phase and reduces cell proliferation. Targeted downregulation of caveolin-1 by antisense oligonucleotides or small interfering RNA allows cells to exit G_0/G_1 and resume replication, indicating a central role for caveolin-1 in signal-transduced pathways to senescence, particularly the p53/p21 pathway (71, 130, 250). Caveolin-1 and p53 may act in a synergistic manner, as p53 is a positive regulator of the caveolin-1 gene (353), and caveolin-1 increases p53 activity (130). Two antioxidants, quercetin, a common dietary polyphenol found in fruit and vegetables, and vitamin E, inhibit the upregulation of caveolin-1 by hydrogen peroxide and the development of the senescent phenotype (441). In other studies, quercetin has been shown to protect cells from DNA damage due to hydrogen peroxide at the expense of intracellular GSH (40). Caveolins have been implicated in the senescence processes *in vivo*. Caveolin protein is markedly upregulated in the brains, spleen, and lungs of rats aged 27 months compared with animals 2 months of age (332).

I. Cell-cycle arrest and the senescent phenotype: uncoupled responses

The seemingly paradoxic situation whereby senescent cells lack replicative competence yet exhibit increased synthesis of specific proteins and continue to enlarge in size and mass indicate that cell-cycle arrest and the development of the senescence phenotype are uncoupled responses. A number of regulatory events downstream of Rb activity have been identified in producing the senescent phenotype.

1. Role of TGF-β. TGF-β1 plays a central role in the development of the senescent phenotype. TGF-β isoforms are involved in downregulating cell proliferation both *in vitro* and *in vivo* (358, 369). The expression of senescent cell morphology, SA β-gal, and increases in steady-state fibronectin, apolipoprotein J, osteonectin, and SM22 mRNA levels can be induced in HDFs by TGF-β1 (124). Furthermore, exposure to sublethal oxidant stress in the form of hydrogen peroxide causes TGF-β1 secretion to be increased some 36 h after exposure, and neutralizing antibodies to TGF-β1 or blockade of TGF-β receptor II (TGF-β RII) diminishes the appearance of senescent biomarkers (124). Rb activates TGF-β1 expression in conjunction with the ATF-2 transcription factor (124, 214). Thannickal and Fanburg (420) identified an inducible plasma membrane–bound NADH oxidase as the enzyme responsible in lung-derived HDFs that generates H_2O_2 on exposure to TGF-β1. Unlike the classic NADPH oxidase of phagocytic cells, this inducible oxidase is an NADH-specific flavoprotein that mediates the two-electron reduction of molecular oxygen directly to hydrogen peroxide rather than superoxide.

Two discrete phases, early and late, occur in the development of the senescent phenotype (Fig. 8). The mechanism, elucidated by Frippiat *et al*. (125), requires the phosphorylation of $p38^{MAPK}$ during stress that allows activation of ATF-2. Phosphorylated ATF-2 promotes the overexpression of TGF-β1, which is subsequently released to activate TGF-β1 rII. Activation of TGF-β1 rII provides for the sustained phosphorylation of $p38^{MAPK}$. It is only when Rb is hypophosphorylated, some 24 h after stress, that it binds with phosphorylated ATF-2, and the senescent phenotype can be expressed.

Stimulation of A549 lung adenocarcinoma cells or human prostate basal cells with TGF-β1 results in the development of a senescent phenotype characterized by cell enlargement and expression of SA β-gal activity but without immediate

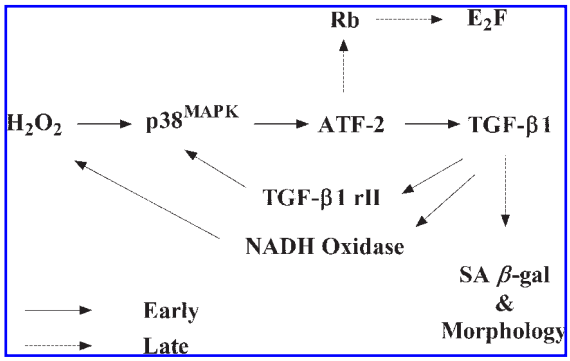

FIG. 8. Oxidative stress as a regulator of fibroblast senescence. Treatment of fibroblasts with hydrogen peroxide results in the immediate phosphorylation of $p38^{MAPK}$, which subsequently phosphorylates ATF-2. ATF-2 promotes the overexpression of TGF-β1, inducing expression of NADH oxidase and TGF-β1 rII plasma membrane receptors. After a 24-h delay, ATF-2 interacts with hypophosphorylated Rb, and full expression of the senescent phenotype appears. *Solid arrows*, Immediate responses; *dashed arrows*, delayed responses.

cell-cycle arrest (210, 428). Moreover, in the continual presence of TGF-β1, A549 cells exhibit progressively reduced telomerase activity with eventual telomere shortening and the onset of replicative senescence (210).

2. Role of phosphatidylinositol 3-kinase. Many of the biologic effects of TGF-β1 are mediated *via* the phosphatidylinositol 3-kinase (PI3K) pathway (245, 248, 348, 363). Increased cell size is dependent on *de novo* protein synthesis and requires activated p70 S6 kinase to phosphorylate 40S ribosomal S6 kinase, a key component in the transcription of mRNAs (105). PI3K is an upstream regulator of p70 S6 kinase (425), and exposure of various cell types to sublethal concentrations of hydrogen peroxide activates PI3K (347, 393, 425, 452). Activation of PI3K, together with p70 S6 kinase, leads to increased synthesis of a range of proteins and cell enlargement (Fig. 9; 425). Selective inhibition of PI3K with LY294002 or wortmannin prevents cell enlargement, reduces SA β-gal expression, but does not prevent cell-cycle arrest (452). In contrast, HDFs expressing E6 protein develop senescence characteristics, including cell enlargement and SA β-gal expression, while maintaining the ability to replicate (106). Thus, with either prematurely or replicative senescent cells, the development of the typical senescent phenotype is not directly linked to cell-cycle arrest. The uncoupled nature of cell-growth arrest and the senescent phenotype is of considerable importance to potential therapeutic approaches to senescence-related pathologies and is discussed subsequently.

III. *In Vivo* Significance

A. Senescence as a proinflammatory state

The ultimate fate of senescent cells *in vivo* is not known. Senescent cells tend to be resistant to apoptosis (450) and can persist for several months under culture conditions, although not all exhibit this trait (157, 429, 448). Resistance to apoptosis in human fibroblasts appears related to the down-regulation of caspase-3 (261), a pivotal early mediator of apoptosis. It has been appreciated for many years that senescent cells can influence the behavior of quiescent cells in culture. Early studies involving the fusion of young and senescent fibroblasts resulted in DNA synthesis being switched off in the younger nuclei (254, 339). More-recent studies identified abnormal protein synthesis by senescent cells of various lineages (1, 139, 184, 223, 267, 287, 395, 475). Studies, including microarray and RNA-profiling analyses, have identified many differences in gene expression between quiescent and senescent cells (24, 96, 395, 469). Overall, changes identified in senescent fibroblasts and endothelial and epithelial cells indicate a change to a more proinflammatory phenotype and exhibit changes associated with wound-repair and tissue-remodeling behavior compared with their presenescent counterparts (271, 287, 395). Unlike normal wound healing, no resolution occurs of the initial inflammatory response followed by cell proliferation to restore function to affected tissues. Tissue homeostasis is critically dependent on the normal functioning of cells and the maintenance of balance between various regulatory signaling pathways and redox status (368). Because of their altered gene expression, senescent cells have the capacity to disturb tissue homeostasis by producing an array of molecules capable of sending inappropriate signals to neighboring cells, including ROS, growth factors, growth inhibitors, and proteases. Consequently, it is likely that the presence of senescent cells *in vivo* may influence local tissue behavior through paracrine signaling, and in this way, contribute to the general and progressive deterioration recognized as organismal aging and promoting age-related diseases.

Fibroblasts isolated from patients with chronic venous insufficiency, which manifests as poor vascular function of the extremities with venous ulcers, exhibit reduced proliferative capacity (282, 283), display increased SA β-gal expression (349), and, it has been concluded, the course of clinical disease progression correlates with reduced cellular prolifera-

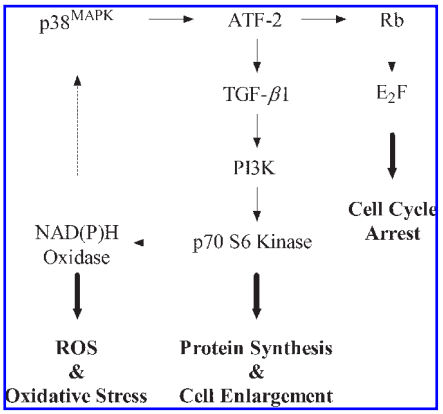

FIG. 9. Cell-cycle arrest and the senescent phenotype are uncoupled responses. In addition to cell-cycle arrest, the onset of premature senescence is accompanied by TGF-β1 production. TGF-β1 upregulates the phosphatidylinositol 3-kinase (PI3K) pathway, leading to *de novo* protein synthesis. Antibody blockade of TGF-β1 or TGF-β1 rII receptors or inhibition of PI3K prevents expression of the senescent phenotype, including cell enlargement, but not cell-cycle arrest.

tion (230). Furthermore, increased pressure has been found to promote senescence in dermal fibroblasts (174). The growth and development of tissues and tissue remodeling after wound repair is dependent on the production of extracellular matrix (ECM) factors. Fibroblasts play a major role in the formation and maintenance of the ECM, including the production of collagen and fibronectin. The collagens are a group of proteins composed of structural units of three chains of ~1,000 amino acids each and forming a triple-stranded helical rod. Collagens exist in several forms, although type I is the most abundant in mammals. Fibronectin belongs to a group of glycoproteins that occur as dimers with subunits of 225 kDa each. Although the regulation of collagen synthesis and its degradation is normally tightly controlled to maintain tissue homeostasis, a decrease in collagen synthesis and an increased rate of degradation with age are found. In rats and humans, *in vivo* aging is associated with reduced dermal collagen content because of increased degradation (275, 293, 379). However, studies of cultured fibroblasts indicate that collagen production by these cells decreases with donor age (201). In contrast, cultured fibroblasts from patients with Werner syndrome, a premature aging disorder, display increased collagen synthesis due to overtranscription of collagen type I and III genes (9). Similarly, senescent pigskin fibroblasts overexpress fibronectin and type III collagen genes, with reduced expression of type I collagen (267), although senescent human fibroblasts are reported to produce less collagen III (100). Fibronectin is also overexpressed by normal senescent fibroblasts from several species and those with Werner syndrome (124, 211, 223, 352, 456). Furthermore, human aortic endothelial cells and dermal fibroblasts *in vivo* and HUVECs and dermal fibroblasts *in vitro* express increased fibronectin mRNA and increased cell-surface area with increasing donor age (222). The interaction of cells with extracellular fibronectin is mediated by fibronectin receptors, members of the integrin family of proteins. Senescence-related changes in integrin expression have been noted with both normal senescent and Werner fibroblasts compared with normal quiescent cells, and the internalization of collagen by fibroblasts is increased because of increased expression of integrin receptors by senescent cells (239).

To maintain tissue homeostasis, ECM production must be balanced by ECM degradation, as excessive ECM deposition results in fibrosis. Two separate mechanisms degrade collagen, an internal phagocytic pathway and an ECM–metalloproteinase pathway. The internal pathway is dependent on α_2-integrin expression, whereas the extracellular degradation of collagen is achieved by collagenases, a group of specific neutral pH proteolytic enzymes. Interstitial collagenase (matrix metalloproteinase-1; MMP-1) and stromelysin (matrix metalloproteinase-3; MMP-3) are synthesized by fibroblasts as procollagenase and prostromelysin, respectively. On activation, collagenase cleaves collagen types I, II, and III (460), whereas stromelysin degrades glycoproteins, including proteoglycans, type IV collagen, laminin, and fibronectin (318). Also produced is tissue-inhibitor metalloproteinase-1 (TIMP-1) as a regulator of matrix metalloproteinase activity. Whereas expression of MMP-1 and MMP-2 mRNAs and protein are increased in senescent fibroblasts and constitutively by Werner fibroblasts (287, 456, 475), TIMP-1 is downregulated with the onset of senescence and in Werner syndrome (287). However, others have reported that TGF-β1 produced by senescent cells (214) induces extracellular matrix TIMP-1 (228) and TIMP-3 production (348). In murine xenograft experiments, senescent HDFs have been shown to have mitogenic effects on co-transplanted tumor cells because of increased production of MMPs (251). Proteomic analysis has revealed a more than fivefold increase in connective tissue growth factor (CTGF) production by senescent fibroblasts (213, 214). Induced by TGF-β1 (188), CTFG is involved in fibroblast proliferation, tissue repair, and remodeling (119). Plasminogen, urokinase plasminogen activator, and plasminogen activator inhibitor type-1 (PAI-1) play central roles in normal wound healing (365), fibrin accumulation (19), tissue remodeling, and fibrosis (19, 52). Both PAI-1 mRNA and protein are overexpressed by normal senescent fibroblasts and those from Werner syndrome patients compared with normal quiescent fibroblasts (179).

B. Cellular senescence and organismal aging

Advancing age is well associated with an increase in systemic levels of oxidative stress, with evidence provided by studies showing increases in markers of oxidative DNA damage (22), protein oxidation (69), lipid peroxidation (284, 321), and accumulation of lipofuscin (91). Similarly, evidence exists for age-related changes in various antioxidant defense systems (7, 39, 150, 262, 284). The hypothesis that cellular senescence contributes to organismal aging and age-related disease is an attractive one, based on the view that exposure to oxidative stress promotes senescence (65, 123, 336, 442). Similarly, conditions that involve constant tissue regeneration will inevitably result in a number of cells reaching their replicative limit. Although a general consensus agrees that cellular senescence plays an important role *in vivo* as a tumor-suppression mechanism (50, 92) and has been implicated as a factor in several age-related pathologies (4, 56, 57, 117, 215, 288, 302, 435), the contribution of the phenomenon to the aging process remains uncertain and the subject of some controversy (28, 50, 75, 92, 253, 337).

Aging may be defined as the progressive loss of physiologic function with time, resulting in an increased susceptibility to disease, injury, and, ultimately, death. Cellular senescence has the potential to contribute in a positive and a negative manner to organismal aging. The positive contribution stems from its tumor-suppressing activity, thus allowing organisms to survive to later life. The negative contribution is twofold. First, senescence can be a limiting factor in tissue renewal because of decreased regenerative capacity and, second, by perturbation of normal tissue homeostasis with possible systemic implications due to aberrant gene expression.

1. Evidence for decreased cellular regenerative capacity *in vivo*.

After the discovery of the finite replicative capacity of somatic cells (171), early research indicated that the "mitotic clock" was operative both *in vitro* and *in vivo*. Thus, the replicative capacity of cells in culture was reported to be inversely related to the ages of donors (265, 364, 382, 403), and cells from patients with premature aging syndromes, such as Werner syndrome, underwent fewer population doublings in culture compared with those from healthy age-matched donors (86, 138, 140, 181, 265). However, much of the data demonstrate a good deal of variability with, in some

cases, only modest levels of statistical significance being achieved, whereas other studies failed to identify any correlation between donor age and cellular replicative potential (80). This has led to a serious questioning of the role of senescence in aging (28, 50, 92, 253, 337). Several explanations have been put forward to account for the apparently variable and conflicting results, including the small number of donors within each study, the health status of the donors (fetal, healthy adult, or postmortem tissue specimens), and the lack of standardized tissue types, culture conditions, and methods for assessing replicative potential. Studies comprising only a few individual donors have very limited power to discern true statistically significant differences between groups and, even so, it is not yet apparent because of the small number of studies conducted whether the reported results are of biologic significance. However, animal studies, in which tissues have been serially transplanted from older donors to younger hosts, provide further support for the limited replicative potential of cells *in vivo*. In such studies, the transplanted tissues retain the proliferative capacity of the original donor and are not rejuvenated by the younger hosts (84, 219).

It is important to note that a basic underlying assumption of these studies appears to have been that a degree of homogeneity exists in the replicative potential of cells derived from individual donors. However, once an organism reaches maturity, only a limited number of cells need to replicate to repair injured tissue or maintain tissue homeostasis while others remain quiescent (G_0 phase), thus preserving their replicative potential. Consequently, a degree of tissue heterogeneity with respect to replicative potential would be expected to develop with advancing age. By this means, the size and mass of organs and tissues remain relatively constant with age, and substantial reserves of cells with replicative potential are maintained. This view is supported by three observations: (a) individual cell clones derived from a common cell mass display considerable heterogeneity in replicative potential (402); (b) in culture, the number of senescent cells increases progressively over time (212); and (c) stochastic variations in telomere shortening rates (269) and oxidative damage to DNA (64) contribute to replicative heterogeneity.

2. Evidence for telomere shortening *in vivo*. Several studies have reported correlations between donor age and reductions in mean telomere length, as determined by TRF analysis, from various tissues, although the estimated rates of shortening are somewhat variable (4, 6, 60, 437). Thus, for human skin biopsies, the reduction in TRF length was ~15 bp per year (6), and for vascular tissue, reductions ranged from 47 to 147 bp per year (60). In the case of vascular tissue, variability resulted from sampling tissues of different vessels, some of which were subjected to greater cellular turnover because of hemodynamic stress. In human livers covering a donor age span of 17–81 years, the reduction rate was 120 bp per year, but even so, the mean telomere repeat length of normal 80-year-old individuals was still 10 kb long (4). Similar relations have been identified with human leukocytes (227, 292). The telomeres of hematopoietic stem cells are subject to erosion, particularly within the period of early development to adulthood. Human stem cells isolated from fetal liver and cord blood have longer telomeres compared with those from adult bone marrow donors. Furthermore, adult stem cell telomere length appears to decrease with donor age (437). In the baboon, longitudinal studies indicate that telomeres shorten predominantly within the first year of life and then stabilize (20). In a further study of leukocyte telomere dynamics, telomere lengths from individuals older than 85 years were found to be unstable and were not predictive of morbidity or mortality (268). A comparison of telomere lengths from human kidneys yielded similar results, although interestingly, shortening occurred at a faster rate for the cortex compared with the medulla. Up to an age of 40 years, the mean telomere length of the cortex remained at ~11.5 kb, whereas after 40 years, it shortened by 80 bp per year (281). However, age-dependent telomere shortening in rat kidneys has not been a consistent finding (280). In addition to age differences in telomere shortening, gender differences have been observed in rats, with the telomeres of males reducing faster than those of females (68). The increased rate at which telomeres shorten in males has been attributed to gender differences in antioxidant status (415).

It is noteworthy that human telomeres are relatively short, ~10–15 kb, compared with those of other species, including other primates that have telomeres ~23 kb in length (204) or mice with 60 kb (397). Does this mean that human tissues are predisposed to a higher incidence of replicative senescence compared with those of other species? The answer would seem to be no, because cellular replicative potential better correlates with species body mass (253), at least for mammals, rather than longevity (364). Furthermore, a good inverse correlation appears between maximal mammalian lifespan and susceptibility to oxidative stress (22, 206) that must be considered when interpreting studies comparing telomere length and replicative potential as cell-culture conditions, such as supraphysiologic oxygen levels, influence *in vitro* replicative potential, and telomere shortening to a considerable extent (66, 325, 370). Other forms of stress have been found to accelerate telomere shortening in a non–age-dependent manner. Thus, psychological stress correlates with increased oxidative stress, reduced telomerase activity, and decreased telomere length in peripheral blood mononuclear leukocytes (111, 112). Similarly, increased pulse pressure is inversely correlated with shortened leukocyte telomeres (199). Although the evidence indicates that telomere shortening does occur *in vivo*, the database is limited, and it remains inconclusive whether it makes a substantial contribution to organismal aging, although an increasing body of evidence suggests it contributes to age-related diseases.

3. Evidence for senescence biomarkers *in vivo*. An examination of other biomarkers of senescence can be informative in assessing their role in aging. However, the evaluation of senescent biomarkers within older age groups, and particularly with individual organs, must take into consideration the possibility of age-related pathology that may bias findings. Several tissues have been investigated by using SA β-gal expression as the main biomarker for aging. Dimri *et al*. (99) reported a positive association between donor age and SA β-gal expression by dermal fibroblasts and epidermal keratinocytes. However, by using the same tissue types and technique, Severino *et al*. (391) found no correlation between donor age and SA β-gal expression. With other tissues and species, the results have been variable. Young

(1–2 years old) rhesus monkey retinal pigment epithelial cells were negative for SA β-gal activity, whereas those of older animals (16–29 years old), although staining positive, demonstrated intermittent expression (289). A comparative study of isolated hepatic sinusoidal endothelial cells from healthy young (8 months old) and old (26 months old) rats identified a significant increase in SA β-gal expression with age. Approximately 6% of sinusoidal endothelial cells from young livers stained positive for SA β-gal expression, whereas with older livers, ~80% were positive (author's unpublished results). Recently, substantial age-related changes in the hepatic microvascular system of otherwise healthy mice were reported, including leukocytic infiltrates, early capillarization, and reduced sinusoidal diameters with consequent reduced sinusoidal blood flow (193). Furthermore, hepatic dysfunction is associated with increased production of TGF-β1 (55), which is involved with the development of the senescence phenotype (124). Taken together, these data suggest that senescence may play an important role in the aging liver.

In the rat, a correlation between increased lipofuscin, SA β-gal expression, and age has been observed, and, although kidney mass and function were preserved during aging, the development and persistence of senescent cells, as determined by p16 expression, was linked to renal dysfunction with resultant nephron shutdown. It is also noteworthy that organs other than the kidneys showed increased expression of p16. However, senescence was not associated with telomere shortening, which suggested to the investigators that cumulative environmental influences were driving renal senescence in the rat (280).

In another study, a positive correlation was found between increased expression of p16 in human skin cells with donor age and an inverse correlation with the *BMI-1* gene, which regulates p16 expression (357).

The localization of DNA-damage foci by using γ-H2AX has provided an important new approach to the identification of senescent cells *in vivo* (177, 200, 384, 385). In baboons, senescent dermal fibroblasts, as determined by telomere damage, active checkpoint kinase ATM, high levels of heterochromatin proteins, and elevated levels of p16, have been reported to increase exponentially in the skin of aged baboons, with >15% of fibroblasts showing evidence of damaged telomeres (177, 200). Similarly, in the mouse, γ-H2AX foci were found to accumulate with age (384, 385). However, others have reported the eventual loss of DNA-damage foci from senescent cells, although the machinery associated with cell-cycle arrest remains active (63), indicating that senescence processes, and their biomarkers, are not immutable.

4. Evidence for aberrant gene expression by senescent cells *in vivo*. Does an association exist between elevated pro-inflammatory responses in the elderly and cellular senescence? Advancing age in humans is associated with an increase in chronic systemic inflammation that is independent of overt disease states (42, 143, 153, 328). This is a consistent finding from several studies based on age-dependent elevations in plasma cytokine levels, particularly TNF-α (103, 328) and IL-6 (103, 454). Plasma levels of IL-6, although low to undetectable in the majority of healthy young people, begin to increase at ~50–60 years of age, with the response becoming pronounced after 70 years (328). In senescent cells, polynucleotide phosphorylase, a 3′,5′-exoribonuclease, is upregulated and induces ROS production with activation of nuclear factor-κB (NF-κB), promoting increased cytokine production (373). Increased expression of IL-6 and IL-8 has been detected from various senescent cell types (373, 381, 469), although the data from fibroblasts is conflicting (141). Pro-inflammatory cytokines, such as TNF-α and IL-1, induce oxidative stress by upregulating NAD(P)H oxidase, resulting in increased production of ROS that potentially contribute to further senescence. *In vivo*, TNF-α induces premature senescence in hematopoietic stem and progenitor cells and other tissues in a murine model of the Fanconi anemia mutation, a premature aging syndrome. Interestingly, senescence in this model was due to TNF-α–induced ROS generation that caused DNA damage, thus activating the DNA damage–response pathway to senescence (476). A study of Werner syndrome, a premature aging disease, supports the association of enhanced nonspecific inflammation with aging. Werner syndrome is due to a mutation to the *WRN* gene that encodes a RecQ helicase (471) involved in DNA replication, recombination, and repair. *In vivo*, plasma levels of plasminogen activator inhibitor-1 (PAI-1), intracellular adhesion molecule-1 (ICAM-1), and TNF-α from Werner patients are elevated (304, 468). Senescent fibroblasts from Werner patients similarly show increased production of these factors (179, 304), and normal senescent endothelial cells produce increased levels of IL-8, ICAM-1, and PAI-1 (464).

5. Conclusions. Does senescence drive the aging process? It is well accepted that aging is associated with a general increase in systemic oxidative stress (48, 284, 473), and oxidative stress is considered to be a major component in the aging process at the cellular level (48, 166, 350). As oxidative stress is also an inducer of premature senescence, it is likely that organismal aging is driven by a positive-feedback loop involving an escalating cycle of oxidative stress and senescence. An examination of the data linking *in vivo* senescence with aging indicates that reduced replicative potential does occur, although the association is not strong for a direct link with aging. Similarly, the data support the hypothesis that telomeres shorten with age, although even in older individuals, the average telomere length indicates considerable remaining replicative capacity. However, it appears likely that current analytic techniques based on remaining replicative potential or telomere length lack sufficient sensitivity and specificity to identify the true extent of senescent cell populations *in vivo*. The evidence is somewhat more robust for specific markers such as p16, although more studies must be completed to assess its true significance. Although senescent cells produce pro-inflammatory cytokines that likely contribute to the elevated systemic levels reported in the aged, it remains uncertain as to the extent of that contribution. Furthermore, increasing oxidative stress with activation of NF-κB likely contributes to cytokine increases, particularly TNF-α, promoting further oxidative stress and conditions that favor the development of senescent cells, thus further contributing to the aging process. For humans, environmental factors may be of particular relevance to inducing premature senescence *in vivo*. Thus, diet, smoking, alcohol, excess exposure to sun, and exhaust emissions are but a few of the environmental components that may contribute to *in vivo* senescence, either by caus-

ing cell death with resultant compensatory cellular proliferation, as with chronic liver injury (330, 386, 458), or by the induction of premature senescence, as may occur with excess exposure to ultraviolet light (59, 88).

Based on currently available data, it cannot as yet be concluded that aging is directly due to senescence processes, but rather, senescence contributes to the process of aging (Fig. 10). The extent of that contribution remains to be determined. However, current information indicates that cellular senescence is not a major driving force in the early aging process, although it may become more important toward the latter part of life. The senescence of individual cells *in vivo* appears to be a response to stress and, once formed, it has the capacity to influence adversely neighboring quiescent cells. The contribution of senescence to aging may therefore be disproportionate, with a relatively small number of growth-arrested cells exerting a substantial influence through abnormal paracrine signaling.

C. Cellular senescence in disease states

The evidence supporting a role for cellular senescence in a number of pathologic conditions is relatively robust, particularly for cancer, vascular, hepatic, and prostatic diseases. For other pathologies, a mounting body-of-evidence indicates that senescence may play a role. Generally, data on senescence are available for those pathologies that are age related. However, non–age-related diseases have been relatively neglected, possibly because of a lack of recognition that premature senescence may result from non–age-related pathologic processes. Cellular proliferation is a general response to tissue injury in disease states. Therefore, it would be expected that increased cell proliferation might correlate with decreased telomere length in affected tissues. Similarly, diseased tissue is a microenvironment associated with inflammation and increased oxidative stress, conditions likely to promote the formation of prematurely senescent cells. Data from several studies tend to support this view, although the precise role that senescent cells play in the disease process remains uncertain. In particular, no causal link has, as yet, been identified between senescence and a disease state. The frequency with which senescent cells occur in lesions is typically low, and thus it may be argued that their role is necessarily relatively minor. Nevertheless, the altered gene expression of senescent cells, particularly in relation to their production of growth factors, may allow them an influence beyond what their numbers might suggest. The ability of senescent cells to act with a paracrine function in particular has been implicated in localized pathologic changes to tissues and, possibly, disease progression.

1. Cancer. It has been recognized for many years that normal diploid cells in culture are resistant to experimentally induced tumorigenesis and rarely develop spontaneously into tumor cell lines; instead, cells typically become senescent or apoptotic. The concept that cellular senescence, immortality, and tumorigenesis are connected originated in the 1980s, when it was noted that virally transformed fibroblasts underwent senescence, thus providing resistance to tumor formation (315). Subsequently, Harley (163) proposed that senescence is an evolutionary adaptation to inhibit tissue hyperplasia and the development of tumors (163). In addition to normal cells in culture becoming senescent, rather than tumorigenic, several lines of evidence support the view that

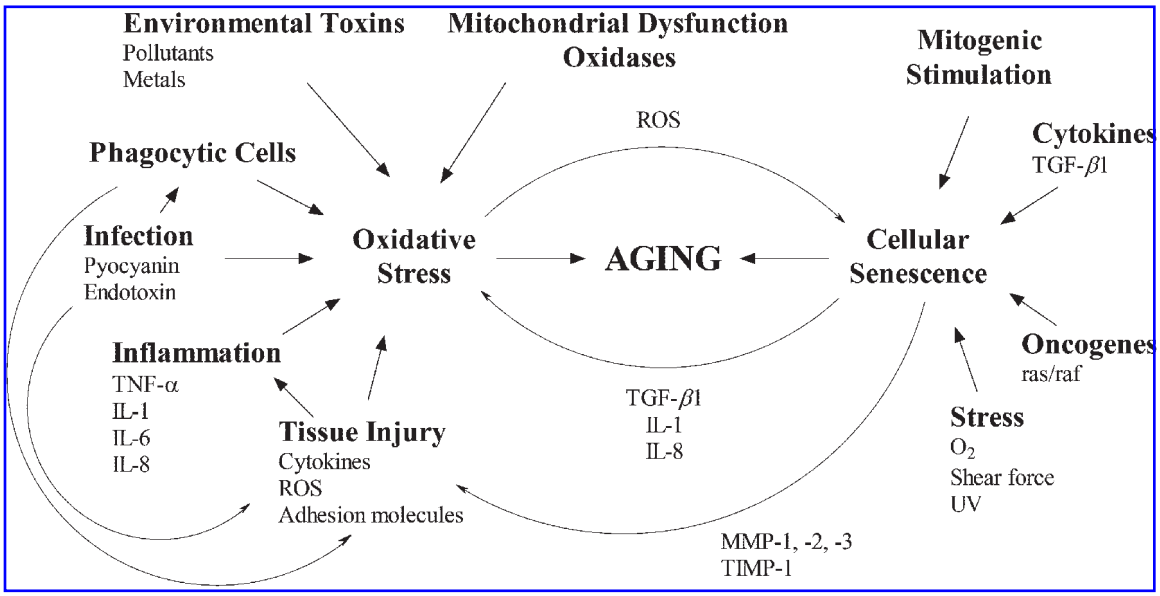

FIG. 10. Senescence in the aging process. Aging is the result of multiple processes acting at the cellular level. Oxidative stress is a key process in aging that can alter cellular function by causing damage to DNA, proteins, and lipids, and activates redox-sensitive pathways, resulting in cytokine production, and promotes cellular senescence. The cumulative effect is impaired cellular function that results in subsequent organ dysfunction and physiologic decline. Cellular senescence is likely to contribute to the aging process by limiting the capacity for tissue replacement and by aberrant paracrine signaling to neighboring cells that disturbs tissue homeostasis. Increased reactive oxygen species (ROS) produced by senescent cells further contribute to aging by creating a positive-feedback loop that progressively escalates, resulting in impaired physiologic function.

senescence is an antitumor mechanism. The replicative lifespan of fibroblasts is extended by inactivation of p53 or Rb by viral oncoproteins (394). Disruption of p53 function together with constitutive telomerase expression in mice results in an increase in cancers (426). Indeed, repression of telomerase activity can force immortal tumor cells to enter replicative senescence because of telomere attrition (324) and, *in vivo*, telomerase deletion in mice with short telomeres suppresses the incidence of tumors in a p53-dependent manner (78, 115, 233). Furthermore, the majority of human tumors are associated with mutations to p53, p16, or Rb (144, 186, 305, 433), or polymorphisms to p21 (297). Loss of p53 activity has been proposed to provide a permissive environment that may initiate tumor development because of end-to-end fusions of critically shortened telomeres, resulting in changes in gene copy number (10). *In vitro*, oncogenic stimulation results in cell-cycle arrest in G_1 due to sustained hypophosphorylation of Rb (390, 479). SAHFs are believed to be involved in intrinsic tumor-suppressor activity, and their presence has been detected by using several *in vivo* models (41, 75).

In evaluating the relative contribution of senescence and apoptosis to tumor suppression in murine models of tumorigenesis, the senescence pathway was found to play a more significant role than apoptosis (78, 115).

Senescence is now seen as a double-edged sword in the control of tumorigenesis, as it is recognized that, while acting as an antitumor mechanism, it can also promote tumor development. This has resulted in senescence being viewed as an example of antagonistic pleiotrophy (49), that is, a function that evolved to protect an organism in the early developmental and reproductive stages of growth but becomes detrimental in later life (459). Senescence of preneoplastic cells in early life allows survival during the reproductive years and hence, from an evolutionary perspective, confers a survival advantage. However, in the latter stages of life, senescent cells may disrupt tissue homeostasis and promote age-related disorders, including cancer. The ability of senescent cells to display paracrine function and to alter their local microenvironment is an area of increasing interest in senescence biology, as it may be a major factor in the development of tumors. The elevated production of growth factors by senescent cells can induce mitogenic proliferation in neighboring cells, resulting in hyperplasia and tumor formation. Senescent human fibroblasts have been shown to stimulate premalignant and malignant epithelial cell growth *in vitro* and to contribute to tumor development in mice (220). Furthermore, the increased production of matrix metalloproteinases by these cells may promote tumor development by stimulating the growth of neoplastic lesions, disrupting intercellular communication and playing a role in the formation of metastases (97, 251, 264).

A limiting factor in the growth of solid tumors is the supply of oxygen and other nutrients for their rapid growth and increased metabolic demands. To supply these nutrients, angiogenesis or vascularization of the newly formed tissues is an important component of tumor growth. Angiogenesis occurs in tissues in response to vascular endothelial growth factor (VEGF), and hypoxia is the primary mechanism by which vascularization occurs. Low oxygen tensions are sensed by hypoxic-inducing factor-1α transcription factor, resulting in increased expression of VEGF (299). *In vitro*, senescent fibroblasts produce increased amounts of VEGF, independent of the hypoxic response, and coinjection of senescent fibroblasts with malignant epithelial cells increases vascularization of tumors in mice (76).

Much evidence suggests that tumor cells exposed to sublethal doses of anticancer agents will senesce rather than become apoptotic. Indeed, cellular senescence is increasingly recognized as an important factor in cancer therapy with ionizing radiation and several drugs, including doxorubicin (Adriamycin), camptothecin, cisplatin, and daunomycin, inducing premature senescence in tumor cells both *in vitro* and *in vivo* (109, 160, 361, 417, 477). As the induction of senescence was thought to result in irreversible growth arrest, treatment-induced senescence has been considered to contribute to a positive cancer-therapy outcome by blunting tumor growth (356, 380). More recently, this view has been questioned, as it has been reported that drug-induced senescence is not an irreversible process. Roberson *et al*. (361) demonstrated that senescent H1299 lung carcinoma cells can escape senescence to resume normal replication and that clones of these escaped cells overexpress cyclin-dependent kinase Cdc2/Cdk1 (361). The ability of a subpopulation of tumor cells to escape therapy-induced senescence may be a contributing factor in the reemergence of some tumors, patient relapse, and resistance to further treatment.

2. Vascular disease. Advancing age is now recognized as the major risk factor for vascular disease. An assessment of risk factors for vascular disease from the Framingham study found aging to be a risk more than two- to threefold greater than that of smoking, hypertension, diabetes mellitus, or hypercholesterolemia (149). Throughout life, the vascular system is constantly exposed to a variety of stresses, including hemodynamic stress due to pulsatile blood flow with varying pressure gradients and oxidative stress due to luminal exposure to oxidized lipids, ROS, and reactive nitrogen species (470). These constant processes give rise to endothelial dysfunction, recognized as a key step in the development of atherosclerotic plaques. Indeed, the proliferation of vascular cells is greatest at vessel junction and bifurcations, where physical stresses are greatest (461), and these are also the sites of atherosclerotic plaque formation (136). Vascular smooth muscle cells cultured from atherosclerotic lesions of normal patients undergo fewer population doublings when compared with those of cells from healthy vessels (27, 244). Similarly, cells isolated from human coronary artery lesions have shortened telomeres and stain positive for SA β-gal activity, suggesting involvement in the atherogenic process (288, 435). Endothelial cells, very much enlarged and displaying multiple nuclei, have been detected in vascular biopsies from healthy aged and atherosclerotic patients (422). When grown to senescence in culture, HUVECs similarly develop a much enlarged and multinucleated appearance although they do form spontaneously in younger cultures (Fig. 11). Studies of premature aging syndromes further indicate that cellular senescence is linked to vascular disease. In individuals with Werner syndrome or Hutchinson-Gilford progeria, premature atherosclerosis with a high incidence of myocardial infarction develops, and their vascular cells senesce earlier in culture compared with those of age-matched controls (113, 137).

Evidence for the role of hemodynamic stress as a contributor to *in vivo* vascular aging was provided by Chang and

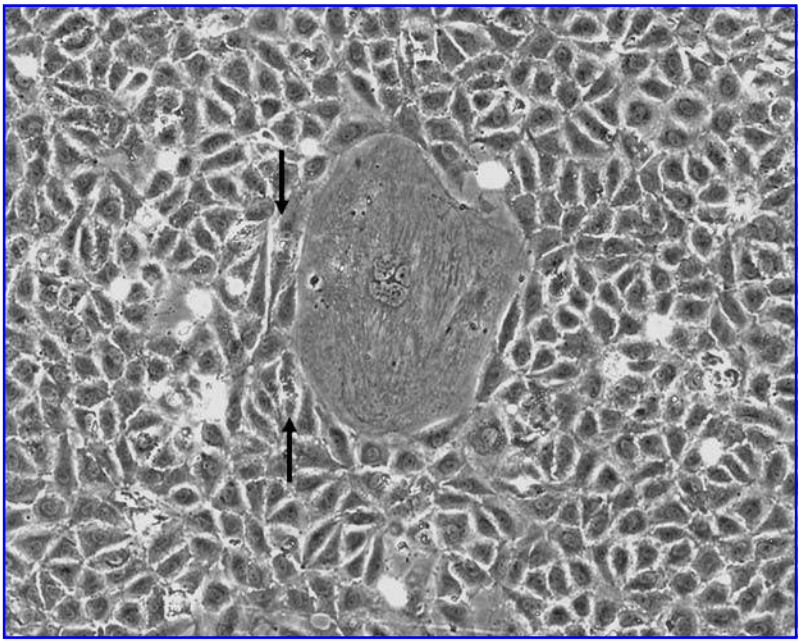

FIG. 11. Replicative senescent endothelial cell. Senescent human umbilical vein endothelial cells (HUVECs) spontaneously emerge in culture, becoming much larger than neighboring quiescent cells, and typically display abnormal nuclear structure. Although few in number, senescent cells exhibit an increased capacity to secrete cytokines and reactive oxygen species, enabling them to influence the behavior of neighboring cells. Quiescent endothelial cells typically adopt a "cobblestone" appearance, but the expansion of the senescent cell has compressed cells on its periphery. *Arrows*, Cells that have become morphologically altered. *Upper arrow*, A newly emergent senescent cell; *lower arrow*, a cell showing karyorrhexis, which is indicative of apoptosis (original magnification, ×100).

Harley (60) by comparing the rates of telomere loss (mean TRF lengths) from the intima of the iliac and thoracic arteries, which are subject to high and low hemodynamic stress, respectively. The mean TRF length of intimal tissue from both arteries decreased as a function of donor age, and telomere shortening of tissue from the iliac artery was consistently greater than that of the thoracic artery. Furthermore, comparison of iliac arterial and venous tissue from the same individuals demonstrated the rate of decrease in mean TRF length over the age range of 20–60 years was significantly greater for the artery than for the vein. The importance of mechanical damage to vascular senescence *in vivo* has been highlighted by studies of surgical procedures performed on vessels. Neointimal lesions induced by balloon-catheter denudation of arteries resulted in endothelial and vascular smooth muscle cell proliferation for several weeks after the procedure (17). Increased staining for SA β-gal activity of endothelial and vascular smooth muscle cells was detected after single or double balloon-catheter denudations of rabbit arteries, with greater numbers of positive cells detected after a second procedure. Increased levels of proliferating cell nuclear antigen confirmed increased cell proliferation, which continued to be detected at the sites of injury for up to 6 weeks (116).

Monocytes play an important role in the etiopathogenesis of atherosclerosis, and the presence of inflammatory infiltrates in atherosclerotic lesions was recognized early in the study of the disease, eventually leading to the recognition of atherosclerosis as an inflammatory process (303). Endothelial cell expression of adhesion molecules (ICAM-1) increases with senescence (288, 395), resulting in increased monocyte binding (255). Monocyte-derived proinflammatory cytokines, such as TNF-α and IL-1, induce oxidative stress by upregulating endothelial NAD(P)H oxidase, resulting in increased production of ROS, thus creating a positive-feedback loop further promoting endothelial senescence. *In vitro*, chronic oxidative stress results in increased cellular turnover that promotes telomere erosion and senescence of endothelial (226) and vascular smooth muscle cells (273). Furthermore, in studies involving a large number of subjects, a strong correlation was found between reduced leukocyte telomere length and risk of premature myocardial infarction that was independent of other risk factors or markers of inflammation (44, 371). Shortened leukocyte telomeres were associated with an approximate threefold increase in risk of an infarct, and the association was particularly strong for those with a family history of the disease, suggesting that telomere dysfunction, rather than individual genes, may be a predisposing risk factor for premature myocardial infarction (44). Other studies have linked accelerated leukocyte telomere shortening to psychological stress, possibly due to elevated plasma levels of stress hormones (111, 112) and increased pulse pressure (199).

RNA profiling of senescent endothelial cells in culture has identified changes that are likely to be adverse *in vivo*. These changes include increased levels of vascular endothelial growth inhibitor, IL-8, and the IGF-binding proteins 3 and 5. Increased IL-8 levels (up to 50-fold increase in secreted protein) have been identified and may account for the increased apoptotic rate of senescent endothelial cells (429, 448). Unlike senescent fibroblasts, senescent endothelial cells readily undergo apoptosis (157, 448, 429) and, *in vivo*, this has the potential to result in denuded areas exposing smooth muscle cells, thus contributing to atherosclerosis and thrombosis (257). Furthermore, senescent human endothelial cells downregulate nitric oxide synthase (NOS) expression, reducing nitric oxide (NO) production, a key regulator of endothelial function (272, 288). Cytokines and ROS, through transcription-factor regulation, induce expression of adhesion molecules that further amplify the inflammatory response (387).

Hyperhomocysteinemia is a well-recognized independent risk factor for vascular disease and has been implicated in endothelial cell senescence *in vitro* and *in vivo*. Homocysteine

is an endogenously derived amino acid, and high plasma levels most commonly result from a deficiency of B-group vitamins, specifically folic acid (vitamin B_9) and cobalamin (vitamin B_{12}), which are required for the further metabolism of homocysteine (310). HUVECs exposed to homocysteine developed a four- to fivefold increase in the percentage of cells staining positive for SA β-gal activity *in vitro*. In addition, TRF analysis showed an accelerated reduction in telomere length (464). The *in vivo* significance of these findings is uncertain; Xu *et al*. (464) reported in the same work, abrogation of homocysteine-induced SA β-Gal activity and telomere shortening by the addition of extracellular catalase (464). As with many thiols, homocysteine will autoxidize in aqueous solution, generating ROS, including hydrogen peroxide (180), and hydrogen peroxide is an inducer of premature senescence. Nevertheless, hyperhomocysteinemia has been reported to induce oxidative stress *in vivo* (16) and impairs the ability of cells to produce NO, a key regulator of endothelial function (409).

Taken together, the development of the senescent endothelial cell phenotype is associated with decreased NO production, together with increased levels of ROS and expression of adhesion molecules (ICAM-1), changes that are likely to adversely affect the endothelium, resulting in dysfunction, giving rise to decreased vascular compliance and increased atherogenesis and thrombogenesis.

3. Prostatic disease. Aging is well recognized as the major risk factor for diseases of the prostate. Benign prostatic hyperplasia (BPH) is an important age-related condition that is rarely seen in men younger than 40 years but increases in incidence significantly at age 50 or older, and at age 85 or older, the incidence increases to 90%. In a study of men with BPH, Choi *et al*. (73) observed a highly significant correlation between increased prostate weight and expression of SA β-gal activity, as observed in frozen tissue sections from patients after radical prostatectomies. Furthermore, expression of the enzyme was limited to epithelial cells, with stromal cells remaining negative for SA β-gal activity. The expression of SA β-gal further correlates with the severity of prostatic disease, as determined by prostate weight, prostate specific antigen, and tissue levels of IL-1α and IL-8 (56, 57).

In culture, replicative senescent prostatic epithelial cells expressing increased SA β-gal and p16 protein levels also exhibit upregulated IL-1α and IL-8 expression. IL-1α acts as a paracrine inducer of fibroblast growth factor (FGF) 7 by prostatic stromal fibroblasts, whereas IL-8 induces FGF_2 production. FGF_2 is a growth factor for stromal fibroblasts, whereas FGF_2 and FGF_7 are potent epithelial growth factors, both of which are upregulated in prostatic hyperplasia (366). Microarray analysis has identified altered gene expression in prostatic fibroblasts that are likely to contribute further to the development of prostate cancer. In addition to FGF_7, the production of the paracrine-acting proteins hepatocyte growth factor and amphiregulin are increased by senescent prostate fibroblasts (24). These findings suggest a strong link between the development of senescent cells and an age-related prostatic disease. A scheme for senescence-driven prostatic hyperplasia is presented in Fig. 12.

TGF-β1 is thought to play a role in prostatic tissue modification by causing prostatic epithelial cells in culture to take on senescence characteristics, including enlarged flattened morphology and the expression of SA β-gal activity without complete inhibition of cell proliferation (428). Similarly, TGF-β1–induced fibroblast-to-myofibroblast transdifferentiation and not senescence may contribute to prostatic hyperplasia (427).

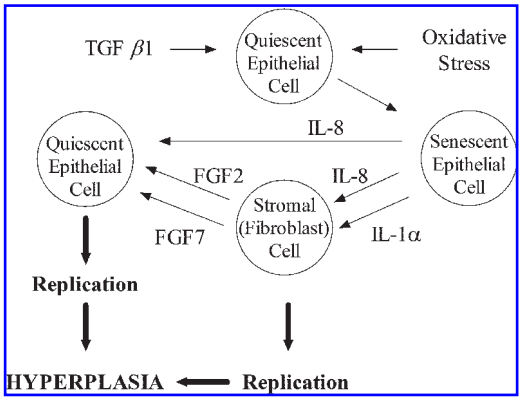

FIG. 12. Senescence in prostatic hyperplasia. The development of senescent prostatic epithelial cells may influence neighboring stromal cells. Senescent epithelial cells produce IL-1α, an inducer of fibroblast growth factor (FGF) 7, and IL-8, which induces FGF2. These growth factors have the potential to induce sustained replication of epithelial and stromal cells, resulting in hyperplasia.

4. Liver disease. Chronic liver diseases are associated with increased hepatocyte proliferative activity. This is particularly true of cirrhosis, a common liver disease caused by a broad range of hepatotoxins, including excess alcohol consumption, viral infection (chronic hepatitis A and B), cholestasis, autoimmune disease, and excess iron or copper. The disease is characterized by the excessive deposition of extracellular matrix proteins into the interstitial space, predominantly due to activation of hepatic stellate cells (122). The cirrhotic pathology develops slowly over many years, starting with a reversible fibrosis and progressing to irreversible cirrhosis. Chronic death and compensatory regeneration of hepatocytes are characteristic features of the pathology, as is activation of hepatic stellate cells (121). In the early stages of cirrhosis, hepatocyte proliferation is initially increased but is reported to decline as the disease progresses (90). Several groups have reported accelerated telomere shortening and the presence of senescent hepatocytes associated with chronic hepatitis and liver cirrhosis compared with those in normal age-matched livers (4, 215, 330, 386, 458), and a good correlation exists with the expression of SA β-gal activity (330, 458). Similar results with telomere shortening have been obtained from the examination of the livers of patients with nonalcoholic fatty liver disease (306). In addition to telomere-dependent replicative senescence, the decline in hepatocyte proliferation has been attributed to the growth-inhibitory effects of TGF-β1 (307), which is markedly increased in the plasma of patients with chronic liver disease (55, 400) and produced by activated hepatic stellate cells (Fig. 13). TGF-β1 is an inducer of senescence (124) and is produced by senescent cells (214). Human stellate cells that have undergone replicative senescence *in vitro* are re-

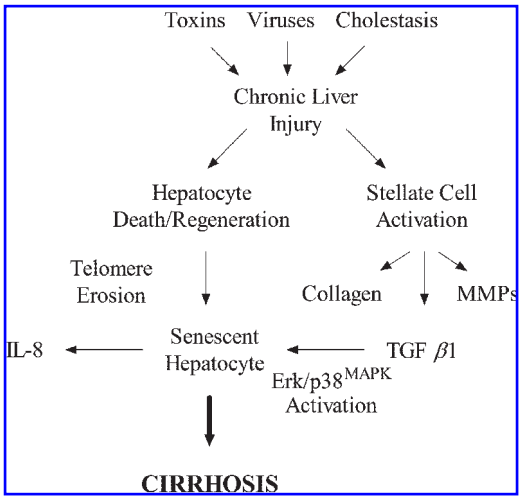

FIG. 13. Senescence as a contributor to hepatic cirrhosis. Liver injury, as a result of prolonged exposure to toxins or infection, causes multiple rounds of hepatocyte death and regeneration, leading to the erosion of telomeres. Hepatic stellate cells become activated and produce TGF-β1, which activates the Erk/p38^{MAPK} pathway, further inhibiting hepatocellular growth.

ported to express a less-fibrogenic but more-proinflammatory phenotype (381).

Donor age and the accumulation of senescent cells may be a factor in the success of liver transplants. Human hepatocytes are reported to be capable of between 69 to 86 population doublings (114), but replicative senescence may be of importance in liver transplantation, as older livers tend to graft less successfully than those from younger donors (263), although this view is not supported by all studies (295, 449).

5. Dementia. Little is known of the role cellular senescence plays in the development and progression of dementia, although advancing age is the major risk factor for the disorder. Paracrine signaling plays a vital role in the normal functioning of the basic neurovascular unit of the brain that comprises neuronal, glial, and endothelial cells. Changes in paracrine signaling within this unit have been implicated in the development of dementia (478), the two most common forms of which are Alzheimer disease and vascular dementia. Alzheimer disease is characterized by the formation of extracellular amyloid plaque deposits and intracellular neurofibrillary tangles with associated neuronal and synaptic loss, whereas vascular dementia is characterized by various changes to the large and small blood vessels of the brain. Two proteins relevant to Alzheimer disease are β-amyloid and apolipoprotein J. A self-aggregating protein, β-amyloid is a major component of plaque deposits and is associated with increased microglial cell activation, whereas the role of apolipoprotein J remains uncertain. *In vivo*, rat and human microglial cells exhibit age-dependent reductions in telomere length that, in humans, appear to be exacerbated by the presence of β-amyloid deposits (117). It is noteworthy that on becoming senescent, human fibroblasts produce increased amounts of both β-amyloid and apolipoprotein J (1, 341). Treatment with colostrinin is reported to improve cognitive status in Alzheimer patients and may function by decreasing β-amyloid fibril production (30, 383). *In vitro*, however, colostrinin significantly extends the proliferative lifespan of murine diploid fibroblasts, possibly because of decreased intracellular generation of ROS (18). Although neuronal cells are differentiated postmitotic cells and do not undergo replicative senescence, they are subject to DNA damage with activation of the DNA-damage–response mechanism involving ATM signaling, in a manner similar to replicating cells (31). It is not known, as yet, if neuronal cells develop a proinflammatory senescent phenotype after DNA damage. IL-1β produced by stressed microvascular endothelial cells of the brain causes neuronal cells to secrete β-amyloid, which in turn leads to microglial cell activation and telomere attrition (117).

Vascular dysfunction involving both the large and small vessels of the brain is a further cause of dementia. Although the presence of senescent vascular cells has not yet been reported from affected brains, it has been noted that bovine microvascular endothelial cells undergo greater morphologic transformation on becoming senescent compared with larger aortic vessels, in addition to loss of angiogenic potential (58).

6. Respiratory disease. Because of the high oxygen tensions in the lung and coupled with the presence of phagocytic cells that elaborate ROS on activation by microorganisms and environmental pollutants (*e.g.*, dust particles and noxious gases), the lung is a suitable environment for the development of premature cellular senescence. Lung fibroblasts isolated from patients with emphysema (183, 314) or from smokers (312) display reduced rates of proliferation compared with cells from control patients. Lung fibroblasts isolated from patients with moderate to severe emphysema are reported to exhibit increased staining for SA β-gal (16%) compared with controls cells (4.4%), although no difference was found in mean telomere length between the two groups (300). With quantitative PCR, expression of insulin-like growth factor (IGF)-binding protein-3 was found to be upregulated, and cell-culture supernatants showed increased concentrations of the protein. Expression of IGF-binding proteins has been reported to be upregulated by senescent cells (24, 271, 395, 469). In another study, alveolar epithelial and endothelial cells from patients with chronic obstructive pulmonary disease (COPD) expressed more p16 and p21 protein with telomere shortening than did matched controls, thus identifying senescence as a limiting factor in alveolar tissue regeneration (423).

7. Osteoarthritis. *In vivo*, oxidative stress is considered to be an important contributor to osteoarthritis and synovial joint degeneration, with matrix metalloproteinases (MMPs) playing a major role in the remodeling of cartilage. Articular cartilage chondrocytes isolated from patients with osteoarthritis show evidence of reduced replicative capacity, telomere shortening, increased expression of caveolin-1, and SA β-gal expression, compared with matched control cartilage (83, 266, 344, 472). Chondrocyte senescence has been linked to ROS derived from mitochondrial dysfunction (83, 266), and isolated chondrocytes, in response to mild oxidative stress, exhibited the same spectrum of senescence changes observed *in vivo* (472). However, no correlation ap-

pears to exist between MMP expression from osteoarthritic lesions and sites distal to the senescent chondrocytes (344). Cellular senescence is also associated with age-related intervertebral disk degeneration with, in some cases, up to one third of disk cells expressing SA β-gal activity (148). Disk cells isolated from patients with intervertebral disk disease showed decreased replicative potential, shortened telomeres, and upregulation of p16 (148, 240, 362). These cells appear to persist for extended periods in damaged disks and compromise the ability of the disk to repair itself.

8. Infection. A role for senescence in the tissue-repair response to infection is beginning to emerge. Pathogenic bacteria with the capacity to produce redox-active virulence factors have the potential to induce premature senescence at sites of infection. *Pseudomonas aeruginosa* is an important opportunistic human pathogen that produces substantial quantities of pyocyanin, a redox-active phenazine derivative, which induces premature senescence in lung-derived epithelial cells *in vitro* (302). In mammalian cells, pyocyanin undergoes intracellular redox cycling generating superoxide and, by dismutation, hydrogen peroxide (301) and its senescence-inducing effect can be inhibited by maintenance of the intracellular redox status (Fig. 14; 302). *P. aeruginosa* frequently colonizes the airways of patients with cystic fibrosis, and, combined with the host response to infection, extensive tissue damage ensues. In this setting, the ability of pyocyanin to compromise tissue repair by inducing senescence may contribute to the progressive loss of lung function these patients experience. *P. aeruginosa* is also an important pathogen of burn wounds. We recently demonstrated the presence of pyocyanin in the wounds of infected burns patients and, by using an *in vitro* wound-repair model with HDFs, equivalent concentrations of pyocyanin inhibited tissue regeneration by the induction of premature senescence. Furthermore, prior treatment of normal HDFs with the $p38^{MAPK}$ inhibitor, SB203580, inhibited the senescence-inducing effect of pyocyanin, indicating that the toxin operates by oxidant-mediated signals transduced *via* the $p38^{MAPK}$ pathway (302 and author's unpublished data).

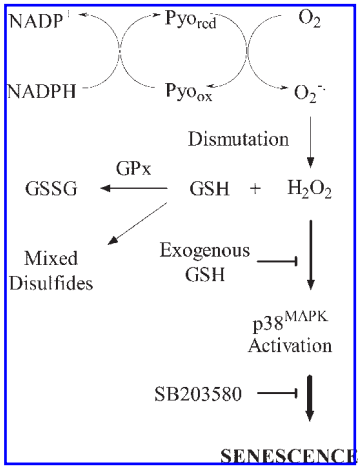

FIG. 14. Premature senescence induced by a redox-active bacterial toxin. The bacterial phenazine toxin, pyocyanin, undergoes intracellular redox reactions with NAD(P)H, providing reducing equivalents. Reduced pyocyanin (Pyo_{red}) reacts with molecular oxygen to yield superoxide and, by dismutation, hydrogen peroxide, while regenerating the oxidized form of the toxin (Pyo_{ox}). Hydrogen peroxide depletes GSH predominantly through *S*-thiolation reactions but also by conversion to GSSG by glutathione peroxidase (GPx). The resulting oxidative stress activates the $p38^{MAPK}$ pathway. Maintenance of the GSH/GSSG redox balance or inhibition of $p38^{MAPK}$ with SB203580 inhibits cell-cycle arrest and the development of senescence characteristics.

It has been recognized for some considerable time that infected wounds require more time to heal compared with noninfected wounds, yet the cellular responses to infection that result in delayed healing remain poorly understood. Similarly, cells with senescence characteristics have been identified in chronic nonhealing wounds, yet the mechanisms associated with their formation are poorly characterized (282, 283, 349). Recent studies have found that chronic nonhealing wounds become colonized by biofilm communities of bacteria, with *P. aeruginosa* one of the most common species isolated (135, 196). It is also well established that the presence of *P. aeruginosa* in chronic leg ulcers results in significantly larger wounds than those in which the organism is not present (135, 156). Consequently, *P. aeruginosa* is now recognized as a major factor in the failure of chronic dermal wounds to heal (33).

Although *P. aeruginosa* is thus far the only bacterial species known to be capable of inducing cellular senescence in human cells, it is likely that other species may inhibit wound healing by inducing senescence. A promising candidate is *Mycobacterium tuberculosis*, which causes chronic infection with progressive tissue injury and is known to inhibit cell growth by producing a redox-active virulence factor (132).

IV. Redox Considerations

A. Oxidative stress and senescence

It is evident that oxidative stress is a central process involved in the induction and maintenance of cellular senescence. Available oxygen tension inversely affects the rate of telomere shortening and the replicative lifespan of cells in culture (333, 334, 335, 442, 444). Approximately 20–30 more population doublings are achievable if the oxygen level is maintained at 2–3%, which is close to physiologic oxygen tensions, instead of the ~20% normally available under tissue-culture conditions (66, 325, 343, 370). Furthermore, antioxidants (ascorbate, tocopherol, and *N-tert*-butyl hydroxylamine) delay the onset of senescence (14, 15, 66, 277, 326) and, in other studies, reduce the rate of telomere loss (208, 334, 360, 372, 334, 388). Moreover, direct exposure of normal HDFs (65) or HUVECs (123) to oxidative stress in the form of subcytotoxic concentrations of hydrogen peroxide result in cell-cycle arrest and the development of the senescent phenotype. It has been further noted that at low population doublings, cells produce constant levels of endogenous ROS, but at later population doublings, the rate increases and correlates with the induction of senescence (152). Thus, either exogenous or endogenous oxidative stress can induce premature senescence by activation of the Erk/$p38^{MAPK}$ pathway (224) or the p53/p21 pathway because of the DNA-damage response mechanism in response to compromised DNA or telomere integrity (Fig. 4) (226). These observations provide general support for the free-radical theory of aging (166), and

oxidative stress in particular, as underlying mechanisms involved in cellular senescence.

ROS are continuously generated by many enzymatic systems, including cellular flavin oxidases [*e.g.*, NAD(P)H oxidases and xanthine oxidase], cytochromes P450, cyclooxygenase, lipoxygenase, and mitochondrial activity. ROS, and particularly hydrogen peroxide, are known to play an important role in intracellular signaling, including cell proliferation (47, 408). Normal intracellular physiologic concentrations of hydrogen peroxide range from ~0.001 to 0.7 μM, with higher concentrations (0.01–1 μM) promoting cellular proliferation (47, 408). Still higher concentrations induce apoptosis and, ultimately, necrosis. Excess levels of intracellular hydrogen peroxide are countered by antioxidant defense systems principally comprising the GSH–glutathione peroxidase system located in the cytosol and mitochondrial matrix and by catalase located in peroxisomes (256). Unlike glutathione peroxidase, catalase is specific for hydrogen peroxide, although it is less effective when the oxidant is present at low micromolar concentrations (256, 411). Thus, the maintenance of adequate GSH levels is critical in regulating sub- to low-micromolar concentrations of hydrogen peroxide. In addition to GSH, further nonspecific protection is provided by low-molecular-weight antioxidants, chiefly comprising urate, ascorbate, and tocopherol.

B. Role of mitochondria in senescence

Mitochondria are viewed as sources of free radicals and the sites at which ROS are continuously produced. Electron leakage from the mitochondrial electron-transport chain brings about the univalent reduction of molecular oxygen. It has been estimated that healthy cells lose up to 1–2% of these electrons to nonspecific processes, but with increasing cell passage in culture, mitochondria become dysfunctional, resulting in substantially increased ROS production (48). Similarly, *in vivo*, mitochondrial function declines with age (329). Harman (166) proposed that free radicals are involved in the aging process and subsequently suggested that mitochondria-derived ROS may contribute to cellular aging (167). Experimental support for mitochondrial involvement in cellular senescence was initially provided by treatment of IMR-90 fibroblasts with *N-tert*-butyl hydroxylamine, an antioxidant that is recycled by the mitochondrial electron-transport chain. *N-tert*-butyl hydroxylamine extends fibroblast replicative capacity and delays age-related changes in mitochondrial function, including reducing ROS production, preservation of mitochondrial membrane potential, and increasing the cellular GSH/GSSG ratio (14, 15). Furthermore, *N-tert*-butyl hydroxylamine was found to improve mitochondrial function and several parameters of oxidative stress in aged animals (15). More recently, it was demonstrated that mitochondria-derived ROS play an important and direct role in the shortening of telomeres and the onset of senescence (252, 372, 442). Mild uncoupling of mitochondrial respiration with 2,4-dinitrophenol results in diminished superoxide production, reduced telomere shortening and accumulation of telomeric γ-H2AX foci, and delayed onset of senescence (335). Passos *et al*. (334) proposed a causal link between mitochondrial DNA damage, which results in mitochondrial dysfunction with loss of mitochondrial membrane potential, increased ROS production, accelerated telomere shortening, and senescence.

After cell-cycle arrest due to exogenous oxidative stress or replicative senescence, mitochondrial mass and mitochondrial DNA increase (237, 238), with dysfunctional mitochondria appearing to become clustered near the nucleus and associated with lysosomes (29). Stimulation of fibroblast mitochondrial function by the addition of pyruvate induces senescence with activation of p53, p21, and p16 and increases ROS production with concomitant reduction of intracellular GSH. Furthermore, increasing mitochondrial numbers in cells by retroviral-mediated expression of mitochondrial biogenesis regulator PGC-1 accelerates the onset of senescence (463). Overstimulation of signaling pathways in cells has also been related to senescence. Ceramide is a lipid second messenger known to disrupt mitochondrial function. Constitutive activation of endothelial cell *rac*1 increases intracellular levels of ceramide, resulting in mitochondrial dysfunction with increased ROS generation and premature senescence (93). It has been proposed that mitochondrial dysfunction induces mitochondrial biogenesis, thus increasing the number of sites in the cell for the production of ROS, which accelerates telomere shortening (336). Despite a clear role having been established for mitochondria-derived ROS in telomere attrition *in vitro*, it remains uncertain as to the *in vivo* relevance of these findings.

C. Role of the GSH/GSSG couple in senescence

GSH is the major endogenous antioxidant for both the intra- and extracellular compartments, and the glutathione/glutathione disulfide (GSH/GSSG) redox couple is the most important cellular system for maintaining the redox status of cells (377). Intracellular GSH concentrations can reach 10 mM and, in conjunction with glutathione peroxidase, it effectively reduces hydrogen peroxide and lipid hydroperoxides to water and lipid alcohols, respectively. Thus, the status of the GSH/GSSG couple is an important determinant of cellular oxidative stress. A study of 122 healthy subjects aged between 19 and 85 years established that although plasma levels of reduced glutathione remain relatively constant during early life, beyond age 45, they begin to diminish steadily. In contrast, evaluation of the cysteine/cystine redox couple from the same subjects found a shift to a more-oxidized state with increasing age (202).

The cellular GSH/GSSG redox balance appears to be an important determinant for senescence. Depletion of HUVEC intracellular GSH by butahionine sulfoximine, an irreversible inhibitor of γ-glutamylcysteine synthase, results in increased intracellular ROS and accelerates the onset of replicative senescence with the development of the senescent phenotype (226). TRF analysis indicates that telomere loss occurs at the rate of ~55 base pairs per population doubling in untreated cells, whereas with GSH depletion, this doubles to 110 base pairs (226). GSH depletion further results in an increase in mitochondrial mass and ROS production (237). Acetaminophen is well recognized for its ability to deplete GSH, and this commonly used analgesic and antipyretic agent accelerates endothelial cell senescence (38). The antitumor agent, busulfan, induces p53-independent senescence in fibroblasts (345) by alkylation of GSH, causing a transient shift in the GSH/GSSG status of cells. This results in in-

creased ROS production in a relatively modest but sustained manner by the upregulation of NAD(P)H oxidase, resulting in activation of Erk/p38^{MAPK} (346). Similarly, pyocyanin, a major virulence factor produced by the human pathogen, *Pseudomonas aeruginosa*, undergoes intracellular redox cycling, generating sustained low levels of hydrogen peroxide, resulting in GSH depletion through *S*-thiolation reactions and inducing senescence (301, 302). Inhibition studies with human epithelial cells and skin fibroblasts have demonstrated that pyocyanin-induced senescence can be abrogated by augmenting GSH levels. In these systems, in addition to a cell-free model, excess GSH appears to act by neutralizing ROS and by the slow formation of a pyocyanin–glutathione conjugate that exhibits reduced redox cycling capacity compared with the parent compound (302; and author's unpublished results).

D. Role of NAD(P)H oxidases in senescence

A constitutive plasma membrane NADH oxidase, designated CNOX, has been identified to contributes to endothelial cell oxidant production and may be an important source of ROS for cells approaching senescence (296). As mitochondrial function declines with increasing population doublings, NADH is produced by compensatory glycolytic ATP production. It has been postulated that CNOX acts as a terminal oxidase, transferring reducing equivalents from cytosolic NADH to extracellular molecular oxygen. NAD(P)H oxidase activity is also inducible during senescence. Fibroblasts induced into premature senescence by oncogenic Ras produce elevated intracellular levels of ROS (187, 234), with associated damage to DNA (single- and double-strand breaks) triggering cell-cycle arrest (23, 98, 258, 412). Furthermore, exposure of fibroblasts to low-level oxidative stress results in upregulation of a plasma membrane–bound NADH oxidase (420, 474). It is not known whether this oxidase is the same as CNOX; however, it is regulated by phosphorylation by a protein tyrosine kinase (419). Unlike the classic phagocytic NADPH oxidase, the TGF-β1–inducible NADH oxidase displays delayed but sustained activation (420). Moreover, the enhanced and sustained generation of ROS by senescent cells contributes to maintenance of the senescent phenotype through secondary signaling pathways involving TGF-β1 (Fig. 8) in a positive-feedback loop (124, 125, 184, 420). More recently, NAD(P)H oxidase activity was identified as a regulator of G_1 to S and, to a lesser extent, G_2 to M phase progression of the cell cycle (439). Inhibition of oxidase activity with diphenyleneiodonium (DPI), a flavin oxidase inhibitor, results in increased proteolysis of cyclin D1, p21, and phospho-p38^{MAPK} and a marked delay in progression time from G_1 to S phase. Furthermore, DPI-induced G_1 delay is ATM and p21 dependent, but independent of p53, indicating that ATM is responsive not only to DNA damage but also to redox changes (439). However, DPI is nonspecific in its action, acting as a general flavoprotein inhibitor, which may influence other cellular functions including cell-cycle progression.

Endothelial cell activity is regulated, in part, by the competing activities of superoxide and nitric oxide (NO) generated by NAD(P)H oxidase and nitric oxide synthase (NOS), respectively. The expression of p22^{phox}, which is part of the catalytic core of NAD(P)H oxidase, is subject to upregulation and positive feedback by hydrogen peroxide through a redox-sensitive mechanism involving p38^{MAPK} and PI3K signaling (101). The reaction of superoxide with NO produces the reactive peroxynitrite radical. The production of NO by endothelial cells diminishes with increasing population doublings in culture (374). Furthermore, senescent endothelial cells exhibit increased expression of caveolin-1, and this protein acts as an inhibitor of NOS (286), whereas caveolin-1 is itself subject to upregulation by oxidative stress (85). Inhibition of endothelial NO production by the endogenous NOS inhibitor, asymmetric dimethylarginine (ADMA), leads to increased ROS formation and accelerates the onset of senescence (38, 375), whereas addition of NO donors delay senescence (434). Similarly, treatment with L-arginine, which increases cellular NO levels, inhibits senescence associated with homocysteine or ADMA (376). Cellular levels of ADMA are controlled by dimethylarginine dimethylaminohydrolase (DDAH), the activity of which is susceptible to oxidative stress (192).

Aspirin has been shown to have an important role in extending the replicative lifespan of endothelial cells. Treatment of HUVECs with aspirin from early until late passage, ~40 cumulative population doublings, results in decreased numbers of cells expressing SA β-gal activity and an apparent increase in telomerase activity (38). The effect is aspirin specific, as two other nonsteroidal antiinflammatory agents, ibuprofen and acetaminophen, do not extend HUVEC replicative capacity. Further, L-NAME, a specific inhibitor of NOS activity, prevents the antisenescence effect of aspirin. NO has been implicated in delaying the onset of senescence by activating telomerase (434). Several studies have attributed antioxidant activities to aspirin both *in vitro* (342) and *in vivo* (462) by reducing NAD(P)H oxidase activity and induction of antioxidant enzymes (146). It is noteworthy that the regular use of low-dose aspirin is associated with improved vascular performance, although no evidence links this clinical outcome with decreased endothelial cell senescence. Another agent in clinical use, atorvastatin, an inhibitor of hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase, also inhibits NAD(P)H oxidase activity, reduces ROS formation, and delays the onset of senescence (152). These data indicate that endothelial NAD(P)H oxidase activity increases with increasing population doublings in a manner similar to that of senescent fibroblasts (420). Coupled with increased ROS and decreased NO production, endothelial cell senescence represents a shift to a more prooxidative state.

E. Redox regulation of protein tyrosine phosphatases and senescence

Mitogen-activated protein kinases are key mediators of senescence, and redox signaling is an important component in the regulation of their activity. The ability of TGF-β1 to upregulate NADH oxidase is dependent on protein tyrosine kinase phosphorylation of two proteins of 103 and 115 kDa before oxidant generation (419). Regulation of protein kinase activity is achieved, in part, by the hydrolysis of phosphotyrosine by protein tyrosine phosphatases (PTPs). PTPs are readily and specifically inactivated by low concentrations of hydrogen peroxide, but not so the serine/threonine protein phosphatases (95). Although the reaction of hydrogen per-

oxide with thiol groups is relatively slow in the absence of transition metal ions or other catalysts, the catalytic cysteine of PTPs is oxidant sensitive and, on oxidation, results in a cysteine sulfonate anion (—SO^-) intermediate (95). This indicates that the critical catalytic cysteine residue likely exists as the thiolate anion (—S^-) at physiologic pH, which reacts with hydrogen peroxide by nucleophilic displacement (54, 95). Reduction of the intermediate sulfonate anion by GSH provides the means to regenerate the active thiolate anion (Fig. 15). Others have proposed a role for reactive nitrogen species, nitrosothiol or peroxynitrite as the active species oxidizing the catalytic cysteine (243, 414). Consequently, changes in the redox buffering capacity of cells, either due to disruption by excess hydrogen peroxide or depletion of GSH, may inactivate PTPs by oxidation of the catalytic site and an inability to regenerate the active thiol.

F. Redox control of p53

The p53 protein contains 10 cysteine residues, eight of which are evolutionarily conserved and are located in the central DNA-binding domain of the protein (198). Mutations associated with these cysteine residues are associated with tumors in humans (155), indicating their importance to p53 tumor-suppressor function. p53 also contains a zinc atom in its central core that maintains the conformation of the molecule for DNA binding. The binding of p53 to DNA is achieved by loops of the DNA-binding surface of the protein that are connected by tetrahedral coordination with the zinc atom and residues Cys 176, His 179, Cys 238, and Cys 242 (72). Oxidation of critical thiol groups result in loss of zinc binding and renders p53 unable to bind to DNA, whereas reducing conditions favor folding of the molecule into the wild-type, DNA-binding conformation (154, 351). Thus, the DNA-binding activity of p53 is directly subject to redox control. However, this behavior by p53 appears contradictory to its role in cellular senescence, as a shift in the redox status of cells, for example, by treatment with sublethal concentrations of hydrogen peroxide, results in activation of p53 and increases its DNA-binding ability. The resolution of this paradox may lie in the extent of oxidative stress and poststress induction of antioxidant responses. Jayaraman *et al*. (197) proposed that Ref-1 is upregulated by oxidative stress, and this protein may maintain p53 in its reduced state. Several oxidoreductase enzymes have also been implicated in the stabilization of p53. Overexpression of WOX1 in L929 cells results in increased levels of p53 (61). Similarly, NAD(P)H: quinone oxidoreductase (NQO1, DT-diaphorase), a ubiquitous cytosolic flavoprotein that catalyzes the obligate two-electron reduction of quinones to hydroquinones, stabilizes p53 in several cell types by redox and nonredox mechanisms. Asher *et al*. (11) reported the stabilization of p53 by NQO1 in a redox-dependent manner. Evidence for redox-dependent stabilization was obtained from experiments in which the enzymatic activity of NQO1 was inhibited with dicumarol, a competitor with the NAD(P)H cofactor. Normal murine thymocytes and M1-t-p53 murine myeloid leukemic cells, a cell line that overexpresses *p53*, produce high levels of p53 when gamma-irradiated. However, in the presence of dicumarol, proteasomal degradation of p53 is enhanced, and cellular levels of the protein decrease. As with p53, expression of the *NQO1* gene is induced by oxidative stress (195), and these experiments indicate that NQO1 regulation of p53 stability and activity is a particularly important response to oxidative stress. Inhibitor experiments using dicumarol and curcumin indicate that displacement of NADH from NQO1 results in a conformational change that regulates its binding to p53 (11, 424). However, another report found NQO1 to associate physically with p53 and to regulate its activity by a noncatalytic redox-independent mechanism, with the NQO1-p53 complex preventing the association of MDM2 with p53 (8). p53 is further regulated by acetylation/deacetylation reactions. Acetylation is achieved by p300/PML, and deacetylation, by SIRT1 (Fig. 5). Treatment of fibroblasts with hydrogen peroxide results in acetylation of p53 and depletion of NAD^+, the critical cofactor for the operation of SIRT1. and so promotes senescence by allowing p53 to remain active (129).

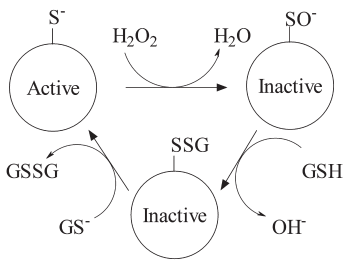

FIG. 15. Redox regulation of protein phosphatase activity. Protein tyrosine phosphatases (PTPs) are key regulators of mitogen-activated protein kinase activity. The catalytic cysteine of PTPs is oxidant sensitive, and the enzymes are susceptible to reversible inhibition by hydrogen peroxide. Oxidation of the catalytic cysteine results in a cysteine sulfonate anion (—SO^-) intermediate. The catalytic cysteine can be regenerated to the active S^- form, provided that intracellular GSH levels are maintained.

G. Redox control of TGF-β1

TGF-β1 plays an important role as a signaling molecule in senescence and especially in developing the senescent phenotype (124, 125, 210, 358, 428). *In vivo*, it is produced in response to inflammatory conditions, particularly those that involve oxidative stress. TGF-β1 is secreted as a latent complex consisting of disulfide-bonded homodimers of growth factor and latency-associated propeptide that requires activation before exerting its actions. Under cell-free conditions, the activity of latent TGF-β1 preparations is increased by metal ion/ascorbate oxidant-generating systems or gamma irradiation but not by hydrogen peroxide alone (21). In contrast, the presence of free reduced thiol compounds (GSH, cysteine, or homocysteine) abolishes TGF-β1 activity, but not in the presence of thiol blockers (35). Thus, nonenzymatic redox reactions regulate TGF-β1 activity. It is noteworthy that *in vivo* conditions that foster TGF-β1 activity tend to be oxidizing environments depleted in GSH.

V. Does a Relation Exist Between Cellular Senescence and Mammalian Lifespan?

A number of correlations have been observed between the maximal lifespan potential (longevity) of species and various biologic parameters. In general, the longevity of mam-

mals positively correlates with body mass and cellular resistance to oxidative stress (22, 206). Furthermore, a linear correlation has been reported between longevity and the *in vitro* replicative capacity of fibroblasts (364), whereas others have found that cellular replicative capacity better correlates with body mass rather than with maximal lifespan (253). However, several independent studies have demonstrated that culture conditions, particularly oxygen tension, play an important role in determining the replicative capacity of isolated cells (325, 333, 343, 370, 397), casting doubt on the relevance of the *in vitro* studies (253, 364). Longevity has also been reported to correlate inversely with mitochondrial production of ROS (22). As telomere dysfunction has been linked to mitochondria-derived ROS (252, 372, 442), it could be concluded that longevity is dependent on maintaining telomere function. However, mice, which have telomeres longer than those of most other species and exhibit telomerase activity, have a short lifespan compared with other mammals. Moreover, despite the shortening of mammalian telomeres throughout life, substantial reserves of cells with replicative potential remain in later life (4, 6), suggesting that remaining cellular replicative potential is unlikely to be a limiting factor for longevity.

Whereas organismal aging is determined by a combination of genetic and environmental influences, with the latter subject to stochastic processes, the longevity of individual species is determined by their genes and is subject to the accuracy and robustness of the cellular defense and repair mechanisms they encode. Based on an evolutionary perspective, the lifespan of a species can be considered to consist of three phases, a developmental, a reproductive, and a postreproductive phase. As the evolutionary fate of organisms in the postreproductive phase of life is irrelevant, no selective pressure exists to pass on genetic improvements that may extend the postreproductive phase. Mechanisms that contribute to survival during the developmental and reproductive phases of life are critical to the success of a species and, if maintained in the postreproductive phase, will contribute to species longevity. A rate-limiting factor governing the longevity of species is the incidence of tumorigenesis, and it has been established that mutations affecting senescence pathways result in an increase in tumorigenesis (10, 144, 186, 297, 305, 433). Studies involving murine models of tumorigenesis indicate that senescence mechanisms can be more important in suppressing tumor development than apoptosis (78, 115). A comparison of the mouse and the naked mole-rat is an interesting example of the importance of tumor avoidance. Although mice exhibit a particularly high incidence of tumors and have a maximal lifespan of ~4 years, the naked mole-rat of similar body weight remains reproductively competent and relatively free of tumors throughout life and has a maximal lifespan of 28 years (46). Thus, it is likely that cellular senescence plays an important role in contributing to mammalian lifespan by the suppression of tumorigenesis in the developmental and reproductive stages of life. In the postreproductive phase of life, the balance between the beneficial tumor-suppressing role of senescence will be countered by the increasingly adverse effects of abnormal paracrine signaling from accumulating senescent cells that may ultimately limit longevity. In conclusion, although the impact of cellular senescence on the maximal lifespan of mammals remains relatively obscure, it is likely that senescence mechanisms contribute to species longevity by the suppression of tumorigenesis.

VI. Future Directions

The contribution of cellular senescence to aging, age-related diseases, and other clinical conditions remains to be fully elucidated, and further research with an emphasis on *in vivo* studies is essential. The characterization of senescent cell behavior has received attention, although much of the work has focused on fibroblasts and, to a lesser extent, HUVECs. Research into the behavior of other senescent cell types is required to understand better their role *in vivo*, particularly their capacity to produce proinflammatory mediators and how these may affect neighboring tissue. The ability of senescent cells to disturb normal tissue functioning through aberrant paracrine signaling presents challenges that must be addressed. Recent advances in our understanding of the molecular events that initiate and maintain senescence suggest that pharmacologic strategies for the *in vivo* manipulation of senescence may be possible. Thus, new therapeutic options for age-related disorders may be obtainable; the great difficulty will be balancing the beneficial role of senescence as a tumor-inhibition mechanism while addressing the adverse effects of altered gene expression.

Telomerase therapy has been proposed as a possible approach to restore regenerative capacity to diseased tissues. However, in many human tumors, telomerase is upregulated. For example, in 80% of hepatoma patients, telomerase is overexpressed (410). Therefore, therapies designed to upregulate telomerase activity must not increase the risk of tumor development. Modulation of p53 activity is possible, although, given its central role in the regulation of several cell functions and its crucial role in tumor suppression, it may prove problematic. Strategies have been found to modulate the activity of sirtuins (SIRT1), which are known to be involved in lifespan extension because of calorie restriction, and SIRT1 is involved in the regulation of p53 by a deacetylation mechanism (231). NAD^+ facilitates the deacetylase function of SIRT1, whereas sirtinol inhibits it and induces senescence while attenuating Ras/$p38^{MAPK}$ signaling independent of p53 (323). Thus, differential targeting of senescence pathways resulting in selective outcomes appears possible.

The finding that cell-cycle arrest and the development of the senescent phenotype are uncoupled events suggests that it may be possible to manipulate, *in vivo*, the negative aspects of senescence without perturbing its beneficial antitumor function (Fig. 9). Therapeutic strategies aimed at targeting $p38^{MAPK}$, TGF-β1, phosphatidylinositol 3-kinase (PI3K). and NAD(P)H oxidases appear as attractive possibilities. Inhibition of $p38^{MAPK}$ by SB203580, or its analogues, suppresses senescence induction (87) without disturbing the tumor-suppressor activity of p53. Selective targeting of TGF-β1 or inhibition of PI3K, although not inhibiting cell-cycle arrest, has been shown to abrogate cell enlargement and abnormal protein expression.

Oxidative stress is of central importance in initiating and maintaining senescence. Although the use of specific low-molecular-weight antioxidants, such as ascorbate, has produced varied results in modulating senescence, the targeted modulation of the sources of ROS in specific cell types may

yield more-promising results. Further characterization of senescent cell NAD(P)H oxidases is of considerable importance, as several studies have identified this class of enzymes as regulators of senescence, and their therapeutic targeting may be particularly useful. Protein tyrosine kinase inhibitors, such as genistein, have been shown to inhibit the activation of senescence-related NADH oxidase (419). Similarly, vascular endothelial cell senescence is amenable to statins, which not only inhibit HMG-CoA reductase but also target NAD(P)H oxidase function (152). The maintenance of intracellular GSH/GSSG redox status appears to be an important key in controlling the onset and course of senescence, and this avenue is worthy of further research. The activity of TGF-β1 is particularly sensitive to changes in GSH/GSSG status, yet GSH can become severely depleted under pathologic conditions.

With increased knowledge as to the mechanisms of senescence induction come the prospects of manipulating the process to extend the replicative potential of cells. This will be a particularly important outcome, as the ability to produce large numbers of cells has implications in the field of restorative surgery and tissue-engineering applications. The provision of adequate numbers of cells with long-term viability to repair burn wounds and other damaged tissue is currently a limiting factor in restorative surgery. Information now available on the mechanisms of senescence induction provides insights into how increased numbers of cells can be cultured for use in tissue-replacement procedures.

The recent finding that long-term treatment with nicotinamide delays the onset of senescence and inhibits the development of phenotypic characteristics typical of senescence warrants further attention. Nicotinamide phosphoribosyltransferase (NAMPT) is the rate-limiting enzyme in the salvage pathway for NAD^+ from nicotinamide. The onset of senescence in smooth muscle cells has been associated with a decline in the activity of this enzyme (432). Furthermore, NAMPT antagonists hasten senescence, whereas introduction of the NPRT gene into aged cells delays senescence. Thus, modulation of NPRT activity may prove to be one of several useful strategies in addressing senescence-related disorders.

Abbreviations

ADMA, asymmetric dimethylarginine; AT, ataxia–telangiectasia; ATF, activating transcription factor; ATM, ataxia–telangiectasia mutated kinase; ATR, ATM and RAD3-related; BPH, benign prostatic hyperplasia; CDK, cyclin-dependent kinase; CNOX, constitutive plasma membrane NADH oxidase; CTGF, connective tissue growth factor; DDR, DNA-damage response; DPI, diphenyleneiodonium; ECM, extracellular matrix; EGF, epidermal growth factor; Erk, extracellular signal-regulated kinase; FGF, fibroblast growth factor; GSH, glutathione; GSSG, glutathione disulfide; HDFs, human diploid fibroblasts; HMG-CoA, hydroxymethylglutaryl coenzyme A; hTERT, human telomerase reverse transcriptase; HUVECs, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; MEFs, mouse embryonic fibroblasts; MDM2, murine double minute-2; NAMPT, nicotinamide phosphoribosyltransferase; NF-κB, nuclear factor-κB; NO, nitric oxide; NOS, nitric oxide synthase; NQO1, NAD(P)H/quinone oxidoreductase-1; PCNA, proliferating cell nuclear antigen; PD, population doubling; PI3K, phosphatidylinositol 3-kinase; POT1, protection of telomere 1; PTPs, protein tyrosine phosphatases; Rb, retinoblastoma protein; ROS, reactive oxygen species; SA β-gal, senescence-associated β-galactosidase; SAHFs, senescence-associated heterochromatin foci; SIRT1, silent information regulator two ortholog 1; SOD, superoxide dismutase; TGF-β, transforming growth factor-β; TIMP-1, tissue inhibitor metalloproteinase-1; TRF, terminal restriction fragment; TRF1, telomeric repeat-binding factor 1; TRF2, telomeric repeat-binding factor 1; VEGF, vascular endothelial growth factor.

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Address reprint requests to:
Dr. M. Muller
10 Schaefer Terrace
Glenwood NSW
2768 Australia

E-mail: mmuller@med.usyd.edu.au

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