Replicative Senescence: The Human Fibroblast Comes of Age

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Human diploid fibroblasts undergo replicative senescence predominantly because of arrest at the G_1/S boundary of the cell cycle. Senescent arrest resembles a process of terminal differentiation that appears to involve repression of proliferation-promoting genes with reciprocal new expression of antiproliferative genes, although post-transcriptional factors may also be involved. Identification of participating genes and clarification of their mechanisms of action will help to elucidate the universal cellular decline of biological aging and an important obverse manifestation, the rare escape of cells from senescence leading to immortalization and oncogenesis.

IN THE THREE DECADES THAT HAVE ELAPSED SINCE HAYFLICK and Moorhead (*1*) described the limited replicative life-span of human diploid fibroblasts (HDF senescence), definition of this phenomenon has been considerably extended and refined (*2, 3*). Thus, biologic rather than chronologic age is the prime determinant of the replicative limit. Taken together with the direct relationship between the maximum life-span of diverse animal species and the replicative life-span of their cultured fibroblasts (*4*), the data suggest a critical connection between functional decline in vivo and HDF senescence in vitro.

Theories of Cellular Senescence

The current prevailing theory is that HDF senescence resembles a state of terminal differentiation (*5*). The alternative theory that senescence represents the consequence of errors introduced during synthesis of major macromolecules or the result of genetic damage (*6*) has been rendered unlikely by observations that senescent HDF maintain viability (*7*) and the absence of an increase in protein (*8*) and DNA (*9*) synthetic infidelity in senescent HDF derived from normal persons and those showing premature aging, such as progeria and Werner syndrome. Although substantial interclonal variation is observed in replicative capacity (*10*), this variation can almost entirely be ascribed to a previous history of asynchronous cell division and unequal partition of cellular components in mitosis (*11*). The number of population doublings for the mass culture of a given cell strain is reproducible within relatively narrow limits, and cells appear to count the number of population doublings to a critical limit before they stop dividing (*12*), much like the replicative extinction that occurs during differentiation of many diverse cell lineages. Indeed, Bayreuther *et al.* (*13*) have defined seven stages in the "maturation" of HDF, three mitotic and four post-mitotic, on the basis of morphology and protein profiles identified on two-dimensional gel chromatography, which bolsters the concept that HDF senescence is a process of quasidifferentiation.

The Phenotype of HDF Senescence

Senescent HDF are generally larger, less motile, and exhibit decreased saturation density compared to early-passage HDF (*14, 15*). Additionally, nuclear size and the content of RNA, protein, glycogen, lipids, and lysosomes are all increased, but the amount of DNA reflects the G_1 or $2N$ complement in the majority of cells; those with a $4N$ DNA content represent G_1 tetraploid cells or occasional cells arrested in G_2 (*16*). These increases in cell size and in macromolecular mass, with the exception of DNA, are strongly reminiscent of unbalanced growth in bacteria (*17*), in which cells preserve much of their anabolic capacities but lose the ability to respond to signals for DNA replication and cell division.

The presence of senescent cells in early-passage cultures (*10–12*) is reflected in a typical thymidine labeling index of 80 to 90% during serial passage, followed by a relatively gradual involution as more cells undergo senescence (*15*). The salient feature of senescent HDF populations is that only a small fraction of cells cycle, and those that do, spend more time at cycling, particularly in the G_1 phase (*14*). Indeed, the nuclear fluorescence pattern of serum-stimulated senescent cells stained with quinacrine resembles that of proliferating young cells in late G_1 (*18*).

Flow cytometric measurements (*19*) indicate major differences between senescent arrest and the quiescent state of early G_1 arrest (G_0) induced by low serum or high density. Senescent HDF blocked in G_1 are larger than cycling G_1 cells and senescent cells "exit" from the cell cycle with a lower nucleocytoplasmic ratio than cycling G_1/S cells. Therefore, the mechanisms of senescence may disrupt the molecular circuitry for integration of cell cycle progression, in addition to simply blocking the initiation of DNA synthesis.

Such concepts help to reconcile the predominant block in G_1 with more distal perturbations. These include the small but rising incidence during normal HDF senescence of multinucleated cells (*7*)

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and karyotypic changes including tetraploidy and endoreduplication, plus frank abnormalities such as chromosome and chromatid breaks and gaps, complex exchange figures, dicentrics, and chromosome fragments (*19*). Indeed, these aberrations suggest problems beyond initiation and completion of DNA synthesis, involving chromosome condensation, pairing, and segregation and also nuclear cleavage and cytokinesis.

Alternatively, karyotypic instability may be explained by "leaky" replicative arrest, leading to overreplication at origins of replication followed by genetic recombination (*20*). Yet another mechanism could involve replication-dependent losses of DNA methylation that have been observed to vary not only between HDF clones, but also between specific gene loci and genetic domains within a given clone (*21*). Such apparently random demethylation would lead to sporadic gene derepressions in senescent HDF possibly predisposing to karyotypic instability.

Some Molecular Aspects of Senescence

Thymidine triphosphate (TTP) concentrations are increased as if senescent HDF are preparing to initiate DNA synthesis (*16, 18*). Senescent cells fully express several representative early- and mid-G_1 genes such as c-*myc* and c-H-*ras*. That serum induction of two genes, thymidine kinase and histone H3, which are usually coupled to DNA synthesis, was observed in one study of senescent HDF (22) but not in others (*23, 24*) would indicate that the block may vary from late G_1 to early S.

One potent method exists to overcome the replicative block: infection of senescent HDF with SV40 virus leads to reinitiation of DNA synthesis (*25*). Since both viral and host DNA replication depend on factors encoded by the host cell, these observations strongly suggest that the replicative machinery for DNA synthesis in senescent HDF, although turned off, is intact.

Senescent HDF are profoundly attenuated in their proliferative responses to epidermal growth factor (EGF), insulin-like growth factor (IGF-1), and platelet-derived growth factor (PDGF), but the receptors for these growth factors are normal with respect to affinity and number of binding sites per unit surface area of these enlarged cells (*21*). Moreover, autophosphorylation of tyrosine residues was also found to be normal (albeit labile under some conditions) for EGF (*21*) and PDGF (*26*). The number of binding sites per cell of glucocorticoids, however, was somewhat reduced (*21*). It appears, therefore, that these growth factors, their specific receptors, and some of their initial ligand-mediated transductions, are not appreciably altered, and studies need to be performed on other second-messenger signaling pathways to develop mechanistic clues.

Insights from Yeast

An understanding of the mechanisms involved may be gained by examining regulation of proliferation in yeast (*27*). Several mutants have been characterized in *Saccharomyces cerevisiae* that possess defects at virtually every stage of the cell cycle. Most of these events appear to be ordered into a few dependent pathways, so that the late events in the cell cycle depend on the completion of early events. The existence of a control mechanism is suggested when chemicals or mutants can relieve a dependent relationship, that is, conditions that permit a late event to occur even when the earlier, normally prerequisite event is prevented. For example, mutations in the yeast *RAD9* gene (which appears to negatively regulate a function essential for mitosis) allow cells with unrepaired DNA damage to proceed through cell division to yield cells with chromosomal aberrations. In a parallel manner, HDF senescence may be viewed as arrest at a specific checkpoint (G_1/S), or in a dependent pathway (DNA replication). Specific mutations in a subset of cells, for example, with reduced capacity for DNA repair (*28*), would then enable relief of dependence and resumption of cycling at the expense of karyotypic abnormalities.

The Dominance of Senescence

In short-term cell hybrids, containing one old and one young HDF nucleus in a single cytoplasm (heterokaryons), initiation of DNA synthesis in the young, actively proliferating nucleus is inhibited after fusion with senescent HDF (*29*), but ongoing DNA synthesis is not (*30*). Treatment of senescent cells with blockers of protein synthesis before fusion abrogates the inhibition, which indicates that this process is probably mediated by proteins (*29*). In strong support of a dominant inhibitory protein, Lumpkin *et al.* (*31*) microinjected polyadenylated [poly(A)$^+$] RNA from senescent HDF into proliferation-competent HDF and were able to inhibit DNA synthesis. Although this inhibitory activity was sensitive to ribonuclease treatment and appeared to be present in high abundance, attempts to clone it from complementary DNA (cDNA) libraries have so far not succeeded (*32*).

In long-term synkaryons (proliferating cell hybrids that contain a single cell nucleus in a heterologous cytoplasm) obtained from fusion of normal cells with immortal cells, there is a limited division potential, and immortalization, when it does occur, appears to result from alterations in a small number of specific genes (*33*). These results, coupled with a report that the senescent phenotype is induced in immortalized hamster cells after introduction of the long arm of human chromosome 1 (*34*), strongly suggest that cellular senescence is dominant and that immortality results from recessive changes in growth inhibitory genes.

Wright *et al.* (*35*) have obtained strong evidence to support this notion. They transformed normal HDF with the gene for SV40 T antigen under the control of the steroid-inducible mouse mammary tumor virus promoter and nurtured cells through crisis until they were able to isolate a rare immortal cell line. Cell proliferation depended on the induction of T antigen by dexamethasone in both the precrisis life-span and after immortalization. On removing dexamethasone, immortal cells divided briefly, then arrested in G_1. The authors proposed a two-stage model for HDF senescence: mortality stage 1 (M1) causes arrest near the G_1/S interface and is bypassed or overridden by the property of T antigen to stimulate DNA synthesis. Although circumvention of M1 occurs in virtually 100% of HDF once there is sufficient T antigen expression, it is a very rare event spontaneously. Mortality stage 2 (M2) is a distinctly different mechanism that causes failure of cell replication during crisis. Inactivation of M2 is very rare because it would require two independent events (each relatively rare) at a diploid locus. Such inactivation would probably be mutational in HDF and presumably involve a loss of function because hybrids between immortal and normal cells are mortal. Accordingly, T antigen–immortalized HDF would possess an active, bypassed M1 mechanism and an inactivated M2 mechanism. In short, two successive lines of defense would have to be breached (with extremely low probability) to escape from senescence. This model explains why no authentic cases of spontaneously immortalizing HDF have been reported [see Harley and Goldstein (*15*)] and why HDF are exceedingly resistant to immortalization by chemical mutagens and carcinogens. The model also predicts that single oncogenes, and even various combinations of oncogenes, would be unlikely to immortalize HDF unless the independent M1 and M2 mechanisms were both bypassed. In this

context, readily immortalized rodent cells would lack the M2 mechanism, or they would have a greater facility to override it or inactivate it by epigenetic mechanisms, such as loss of DNA methylation (*20*), and then proceed to oncogenesis.

Possible Role of Tumor Suppressor Genes

The possibility exists that tumor suppressor genes (*36*), such as the retinoblastoma susceptibility gene (*RB1*), the p53 gene, or the Wilms' tumor susceptibility gene, may also be involved in senescence. A simplistic view would predict that one or more of these genes becomes constitutively expressed or overexpressed in senescent HDF, but this does not appear to be the case, at least at the level of *RB1* messenger RNA (mRNA) (*37, 38*). However, senescence may be associated with the absence of the phosphorylated forms of RB1 protein (*38*) that normally accumulate as cells emerge from the quiescent state and approach the G_1/S boundary (*39*). The RB1 protein can form complexes with specific domains of viral proteins, including the SV40 T antigen, the adenovirus E1A protein, and the E7 protein of human papilloma virus, each of which is capable of stimulating DNA synthesis and tumorigenesis (*39*). It was proposed, therefore, that unphosphorylated RB1 protein is not just a passive consequence of growth arrest but rather actively inhibits cell cycle progression. Moreover, inhibition is abrogated by phosphorylating RB1 protein or removing it from the functional pool (*39*). Accordingly, the presence of unphosphorylated RB1 protein would account for the failure of senescent HDF to enter S phase (*38*).

How then can one explain the dominance of senescence in cell fusion heterodikaryons? Perhaps the replicating cell lacks the capacity to phosphorylate the RB1 protein contributed by senescent cells, leaving enough of the unphosphorylated form to inhibit DNA synthesis in both nuclei. Alternatively, senescent HDF may contain inhibitors of RB1 protein phosphorylation or a specific phosphatase. The presence of another DNA synthesis inhibitor would also explain the results. In any case, these possibilities and the connections between RB1 and viral proteins can now be studied with regard to the proposed M1 and M2 mechanisms for HDF senescence.

Are Stimulatory Genes Shut Off?

Consonant with the normal regulation of cell division (*36, 40*), HDF senescence may be viewed not only as the new expression of inhibitory genes, but also the reciprocal extinction of stimulatory genes. Expression of the c-*fos* proto-oncogene appears to be an early and essential prerequisite for the initiation of DNA synthesis by serum-stimulated fibroblasts (*41*). Thus, Seshadri and Campisi (*24*) have observed that serum was unable to induce transcription of c-*fos* in senescent HDF, whereas serum-induced transcription of c-*myc*, c-H-*ras*, ornithine decarboxylase, and actin genes was only minimally reduced. But actin and c-*fos* transcription is often coordinately induced in proliferating cells, most likely because their gene promoter regions contain the same serum-response elements. Since c-*fos* was also not induced in senescent HDF by phorbol esters, EGF, or elevated cyclic adenosine monophosphate (cAMP), all of which induce c-*fos* transcription through sequences distinct from the serum-response element (*24*), it would appear that the c-*fos* gene in senescent HDF is under specific transcriptional repression. PDGF was able to induce normal c-*fos* expression in senescent HDF (*26*), which suggests that the response pathways for growth factors other than PDGF (such as EGF or IGF-1) are blocked in the whole-serum experiments (*24*). Studies of these factors in defined medium should resolve this question.

If c-*fos* repression were the primary defect in senescence, one would predict that introducing and overexpressing c-*fos* in senescent HDF should restore DNA replicative potential. Indeed, such results have been obtained, at least in transient assays that show up to a fivefold increase in the number of cells synthesizing DNA after c-*fos* gene transfection (*42*). To establish causality, it will be necessary to obtain stable transformants that overexpress c-*fos* and rescue cells from senescence.

Microinjection of c-H-*ras* DNA into senescent HDF, whether in the proto-oncogene or oncogene forms, cannot induce DNA synthesis (*43*). Moreover, this block in DNA synthesis persists even if the *ras* oncogene is coinjected with the adenovirus E1A gene. These data are consistent with the proposal of a primary role for c-*fos* repression in HDF senescence as well as for the postulates of the M1 and M2 mechanisms. They also underscore earlier observations that transfection of various oncogenes into normal HDF fails to endow them with tumorigenic potential, while in contrast, rodent cells [which may lack the M2 mechanism (*35*)] are readily immortalized after similar transfections (*36*). Senescence of HDF, therefore, is not only a state of replicative arrest, but also of anti-oncogenesis.

Attrition of Telomeres: An Alternative Senescence Mechanism

Harley *et al.* (*44*) has invoked a different mechanism of HDF senescence based on progressive erosion of telomeres, specialized structures at the ends of linear chromosomes. These authors observed that mean telomere length decreased 2 to 3 kilobase pairs (kbp) during serial passage in several strains of HDF. This decrement occurred progressively and averaged 50 bp per population doubling. The total amount of specific telomeric sequence also decreased, which suggests true attrition of this DNA and not merely rearrangement. Loss of telomeric DNA did not result from general degradation or deletion of repetitious DNA in preparations from old HDF because other repetitive, nontelomeric sequence elements were not altered in size or amount. Of great interest is the *EST-1* mutant in yeast, which harbors a defect in telomere elongation leading to a senescence phenotype. In other words, it shows no immediate loss of viability, but rather a slow progressive death of cells (*45*). Indeed, the prediction of a continual decrease in mean telomere length was borne out in both the HDF and yeast studies.

Human telomeres consist of repeats of the sequence TTAGGG, which is added by a nontemplate mechanism involving a multifunctional telomerase enzyme. This ribonucleoprotein complex has also been shown in lower eukaryotes to contain a reverse transcriptase and RNA template for synthesis of the repeat sequence (*46*). Whether the loss of telomeric sequences in HDF and yeast relates to incomplete replication, for example, because of one or more functional deficiencies of telomerase, the degradation of termini, or unequal recombination coupled to selection of cells with shorter telomeres remains to be seen. However, such a mechanism is not easily reconciled with the dominance of senescent HDF over young HDF in fusion hybrids, particularly in short-term heterokaryons. One could again invoke the concept of dependence and the *RAD9* gene example (*27*), such that complete loss of one or a few telomeres leads to the elaboration of a negative signal that prevents initiation of DNA synthesis, thereby mimicking the differentiated state. This idea, although speculative, would not only explain senescent replicative arrest but also the chromosomal aberrations observed in senescent HDF (*19*) that would specifically ensue after loss of telomeres (*44*).

Current Perspectives and Future Directions

Current work should reveal the precise mechanism for repression of c-*fos*. It may resolve whether unphosphorylated RB1 protein specifically represses c-*fos* transcription, or conversely, whether c-*fos* repression prevents RB1 phosphorylation, for example, because it fails to activate a kinase. Whether other known oncogenes and hitherto undescribed proliferation-promoting genes are also turned off must be determined, as well as whether this occurs reciprocally with the turning on of antiproliferative genes. Attempts to clone antiproliferative genes are currently under way in several laboratories and include the use of prematurely senescent mutant HDF. Although this research has led to the discovery of novel overexpressed genes in Werner HDF (*37*), antiproliferative effects of these genes have yet to be shown. Similarly, expression of statin, a 57-kD protein that has been associated with the nonproliferative states of senescence and quiescence (*47*), and pSEN, a highly abundant, senescence-induced cDNA (*48*), have now been shown, over part but not all of their sequences, to be identical to elongation factor 1 alpha, the protein synthesis factor. An inhibitory role of such gene products can readily be imagined at the level of translation, although neither statin nor pSEN has yet been shown to inhibit protein synthesis nor to display any other causal link to HDF senescence. Nonetheless, serum stimulation of senescent HDF fails to elicit the normal induction patterns for ornithine decarboxylase (*22, 23*) and calmodulin (*16, 49*), despite mRNA patterns similar to those of vigorously dividing early-passage cells. Reduced concentrations of such key regulatory proteins at critical times in the cell cycle suggest further mechanisms for the inhibition of DNA synthesis in HDF senescence and thus inculpate post-transcriptional defects in this process. Other possible examples range from the multiple determinants of the quality and quantity of mature mRNA to increased activity in specific pathways of protein degradation (*50*). In short, these and any other mechanisms that ultimately deplete the concentration of crucial proteins could result in proliferative arrest.

The role of extracellular matrix proteins in HDF senescence also needs to be clarified. Genes for fibronectin and various collagens, to name but two matrix components, are overexpressed at the mRNA level in Werner HDF (*37*), whereas senescent normal HDF accumulate more fibronectin in the extracellular matrix than young HDF (*51*). Moreover, the physical nature of fibronectin also seems to be altered in senescent HDF (*52*). Thus, excessive accumulation of extracellular proteins, either as normal or variant species, could serve to alter topographic relationships of cells to the substrate, to growth factors, to nutrients, and to each other. They could then act synergistically, as cofactors, along with extinction of positive factors and new expression of negative factors, acting intracellularly, to inhibit cell cycling (*37, 53*).

Conclusions

Although the mechanism of HDF senescence may at this juncture be viewed in analogy with terminal differentiation, research may reveal molecular details to be distinctive for each process. The predominant block at G_1/S and perturbations in other phases of the cell cycle will ultimately be understood once specific alterations in molecular circuitry involving transcriptional and post-transcriptional levels of regulation are delineated. In any case, current concepts must accommodate nature's directive that replicative senescence evolve as the dominant, universal cellular norm, with immortalization signifying the infinitesimally rare, random escape of a cell from senescence, an apparently early event in the multistep process of oncogenesis.

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Research Article

Enzymatic Coupling of Cholesterol Intermediates to a Mating Pheromone Precursor and to the Ras Protein

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The post-translational processing of the yeast a-mating pheromone precursor, Ras proteins, nuclear lamins, and some subunits of trimeric G proteins requires a set of complex modifications at their carboxyl termini. This processing includes three steps: prenylation of a cysteine residue, proteolytic processing, and carboxymethylation. In the yeast *Saccharomyces cerevisiae*, the product of the *DPR1-RAM1* gene participates in this type of processing. Through the use of an in vitro assay with peptide substrates modeled after a presumptive a-mating pheromone precursor, it was discovered that mutations in *DPR1-RAM1* cause a defect in the prenylation reaction. It was further shown that *DPR1-RAM1* encodes an essential and limiting component of a protein prenyltransferase. These studies also implied a fixed order of the three processing steps shared by prenylated proteins: prenylation, proteolysis, then carboxymethylation. Because the yeast protein prenyltransferase could also prenylate human H-ras p21 precursor, the human DPR1-RAM1 analogue may be a useful target for anticancer chemotherapy.

ISOPRENOIDS ARE A CLASS OF STRUCTURALLY RELATED LIPOphilic molecules that perform a wide variety of essential cellular functions. Isoprenoid lipids include such functionally diverse molecules as cholesterol, ubiquinone, dolichols, and chlorophyll, yet all isoprenoids are derived from a common precursor, mevalonic acid. Polyisoprenoid molecules are attached post-translationally to a small class of eukaryotic proteins, which includes nuclear lamins (*1*), trimeric G proteins (*2*), lipopeptide pheromones (*3*), and the Ras family of oncoproteins (*4–6*). The biological activities of several of these proteins require association with the inner surface of the plasma membrane, and this membrane localization is dependent on the post-translational attachment of the polyisoprenoid lipid residue to the COOH-terminus of the protein. This type of protein modification is referred to as protein prenylation. In the case of the *Saccharomyces cerevisiae* **a** mating pheromone (**a**-factor), prenylation is necessary for secretion and biological activity (*6*). Similarly, oncogenic Ras proteins require prenylation for both membrane association and transforming activity (*4–6*). Protein prenylation is a stable and irreversible protein modification that plays a critical role in directing the modified protein to the plasma membrane (*5*).

The mechanism of protein prenylation has recently become of interest because of both the wide range of proteins that undergo this modification and the ability of inhibitors of prenylation to suppress some phenotypes of oncogenic Ras proteins (*7*). Information on protein prenylation comes primarily from studies of the processing of yeast and human Ras proteins, nuclear lamins, and yeast **a**-factor. Analysis of the structure of the modified COOH-termini of Ras protein and **a**-factor revealed at least three chemical modifications that occur post-translationally. These include (i) attachment of an isoprene moiety to a cysteine residue near the COOH-terminus through a thioether linkage, (ii) proteolytic removal of the three amino acids distal to that cysteine, and (iii) formation of a methyl ester at the new COOH-terminus (*3–5, 8, 9*). Secreted **a**-factor and nuclear lamin B contain a farnesyl (C_{15}-lipid) group (*1, 3*), whereas the precise identity of the isoprene group attached to Ras proteins has not been fully resolved (*4, 5*). In some but not all Ras proteins, the COOH-terminus is further modified by the addition of a

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