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Recent Advances in Cellular Senescence, Cancer and Aging

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Abstract How much do we know about the biology of aging from cell culture studies? Most normal somatic cells have a finite potential to divide due to a process termed cellular or replicative senescence. A growing body of evidence suggests that senescence evolved to protect higher eukaryotes, particularly mammals, from developing cancer. We now know that telomere shortening, due to the biochemistry of DNA replication, induces replicative senescence in human cells. However, in rodent cells, replicative senescence occurs despite very long telomeres. Recent findings suggest that replicative senescence is just the tip of the iceberg of a more general process termed cellular senescence. It appears that cellular senescence is a response to potentially oncogenic insults, including oxidative damage. In young organisms, growth arrest by cell senescence suppresses tumor development, but later in life, due to the accumulation of senescent cells which secret factors that can disrupt tissues during aging, cellular senescence promotes tumorigenesis. Therefore, antagonistic pleiotropy may explain in part, if not in whole, the apparently paradoxical effects of cellular sensescence, though this still remains an open question.

Keywords: replicative senescence, human fibroblasts, telomeres, antagonistic pleiotropy, oxidative damage, telomerase, reactive oxygen species

INTRODUCTION

Since the beginning of time, humans were intrigued by the question of how we age. The elucidation of the aging process has been the subject of much attention over many years. What controls aging? Can we control the pace of the aging process, or can we even prevent it? We are now beginning to understand the genetic and molecular basis of aging. Many theories of the prime causes of aging have been proposed. However, it is unlikely that any single theory can fully explain the complex aging process. Among several compelling aging theories [1-4] recently proposed, we will focus on what we know about cellular senescence, its relationship to oxidative stress, and also on what we can learn from studying cultured cells.

ORGANISMAL AGING

Basic biological processes such as the stress response and cell cycle control are strikingly well conserved from yeast to humans. Even different cell types (neurons, fibroblasts and epithelial cells) from different multicellular organisms are strikingly similar in their structure and functionality. Nonetheless, surprisingly, organisms can age at very different rates ranging from days to > 100 years. What does cellular senescence have to do with organismal aging? Hayflick noted that normal cells appeared aged when they exhausted their cell division potential in culture (replicative senescence). He therefore proposed that replicative senescence might also contribute to organismal aging. This speculative idea established the concept that cultured cells can shed light on the mechanisms of organismal aging and mortality. Correlative findings support this idea, but it remains to be critically tested.

REPLICATIVE SENESCENCE, CANCER AND AGING

Replicative senescence was first formally described by Hayflick and Moorhead in 1961 for human fibroblasts in culture [6]. Replicative senescence is the gradual decline in the capability to divide that is an intrinsic property of most normal somatic cell populations, unlike germ cells and certain stem cells [7]. Hayflick showed that normal fibroblasts initially grew well, but eventually reached a point at which all cells in the population were unable to divide further. Replicative senescence is limited to cells that have the potential to divide *in vivo*, and thus does not apply to post-mitotic cells. The number of population doublings (PD) at which a cell population reaches the end of finite division potential depends on the cell type, donor age, species, and geno-

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type [reviewed in 8,9]. What is the role of replicative senescence in mammals?

Tumor Suppression Imposed by Replicative Senescence

A finite replicative life span is a property of normal human cells, and most human tumor cells do not share this property [10,11]. A growing body of evidence suggest that replicative senescence, and the more general process of cellular senescence, is a powerful, albeit imperfect, tumor suppressive mechanism, limiting the proliferative potential of cells *in vivo* [9,11-15]. This evidence can be summarized as follows.

First, many tumors, naturally occurring and experimentally induced, very often contain immortal cells that have bypassed the limit to divide [12,16]. Therefore, most tumor cells have an extended replicative life span or are replicatively immortal. Of course, immortalization is not necessarily required for tumorigenesis (it may be required for metastasis though). Cells which are immortalized or have an extended replicative life span are at a highly increased risk for neoplastic transformation. This is because cells cannot accumulate the multiple mutations needed for tumorigenesis during their normally limited replicative life span. Many mutations are necessary for cells to acquire the properties of uncontrolled cell growth, invasion into the surrounding tissues, and metastasis. This shows that there is strong selection during tumorigenesis for mutations that permit cells to escape the constraints on proliferation that are imposed by replicative senescence.

Second, we now know that certain viral and cellular oncogenes transform cells primarily by allowing them to circumvent the senescence checkpoint, thereby conferring replicative immortality or extended replicative life span [Reviewed in 11].

Third, a number of regulatory proteins have been proposed to transduce senescence-inducing signals or mediate entrance of the cell into senescence. The INK4A locus is frequently found deleted or silenced in many human cancers and immortalized cell lines, inactivating both the $p16^{INK4A}$/pRb and the $p19^{ARF}$/p53 pathways through a single event. This suggests that inactivation of this locus is a crucial step in overcoming the cellular senescence checkpoint [17]. Consistent with this idea, the p53 and pRb tumor suppressor proteins are also critical for the ability of cells to sense the number of cell divisions they have completed, and enter and remain in the post-mitotic senescent state. The vast majority of tumor cells, if not all tumor cells, carry mutations that inactivate one or more component of the p53 and pRb pathways or both. However, it seems that the activation of either the $p16^{INK4A}$/pRb pathway or the $p19^{ARF}$/p53 pathway in cellular senescence depends on the cell type and species of origin. Mouse embryonic fibroblasts (MEFs) may rely primarily on the $p19^{ARF}$/p53 pathway [17], whereas human keratinocytes may rely primarily on both pathways, to execute the senescence checkpoint [18,19].

Next, there are several mouse models available in which germline inactivation of tumor suppressor genes gives rise to an organism in which cells cultured from these animals fail to undergo replicative senescence; at the very least, loss of p53 or p16 function very dramatically renders these animals cancer-prone, and they tend to die of cancer at an early age [11].

Factors Inducing the Senescent Phenotype

The senescent phenotype was first studied in cells that had undergone replicative senescence – that is, cells that expressed the senescent phenotype as a conesquence of completing a finite number of divisions. A variety of cell physiological stimuli can facilitate a cell to undergo senescence. It is now clear that telomere shortening is responsible for inducing the senescent phenotype that results from repeated rounds of cell division. Recently, however, it has become apparent that normal cells, following extensive passage in culture [6], exposure to oxidative damage [22,23], or activation of an oncogene [11,20,21], enter into irreversible growth arrest and express the hallmarks of a senescent-like phenotype. The fundamental features of the senescent phenotype are a large, flat morphology, resistance to stimuli that induce programmed cell death (apoptosis), senescence-associated (SA)-beta galactosidase enzymatic activity, and up-regulation of a variety of cell cycle inhibitory proteins. Although these various stimuli induce a similar senescent phenotype, they appear to activate distinct but not mutually exclusive biochemical effectors of senescence. For example, replicative senescence, after a finite number of divisions, is the cellular manifestation of progressive telomere shortening. However, there is no evidence that premature telomere shortening occurs in senescent cells induced by the RAS oncogene, supraphysiological mitogenic signaling by RAF and MEK, or overexpression of the growth regulatory E2F1 transcription factor. Taken together, these findings support the idea that the senescent response is induced by many stimuli that are potentially oncogenic, and is a fail-safe mechanism that protects normal cells from tumorigenic transformation.

Telomere Shortening and Replicative Senescence

How do cells sense the number of divisions they have completed, which can be quite large (>60-80 for human fetal fibroblasts)? What are the biochemical signals that provoke replicative senescence? It is now clear that the telomere shortening hypothesis [24] is the best explanation for the cell division "counting" mechanism.

Telomeres are the ends of linear chromosomes, and consist of a repetitive DNA sequence (TTAGGG in human and other vertebrates) and specialized proteins. Telomeres shorten during replicative senescence, both in culture [25] and *in vivo* [26] by about 48 ± 21 bp and 75 bp per PD, respectively. When one or more telomere becomes critically short, cells are triggered, by an as yet unknown mechanism, to enter a senescent state.

Because DNA replication is bi-directional, and DNA polymerases move unidirectionally and require a labile primer for initiation, each round of DNA replication leaves 50-200 3' bases at the telomere unreplicated. Olovnikov proposed that germ cells and tumor cells are able to protect their telomeric DNA from shortening by the expression of telomerase, the ribonucleic acid-containing enzyme that can add telomeric repeats to chromosome ends *de novo*. However, most somatic cells do not express this enzyme. Thus, the telomeres shorten progressively throughout the replicative life span of most somatic cells [24,25,27]. The average terminal telomeric restriction fragment (TRF) is 15-20 kb in the human germ line. Human cells proliferate until the average TRF reaches 5-7 kb, at which point they cease division with a senescent phenotype [24,25]. Eventually the shortened telomere may no longer be able to protect the ends of the chromosome, and the critically short chromosomal DNA end may release a senescence-inducing signal to the cell.

Several lines of evidence support a role for telomere shortening in replicative senescence. First, cultured cells display progressively shorter telomeres as the population reaches senescence. Second, cells cultured from old donors display both shorter replicative life spans and shorter telomeric DNA than those from young counterparts. Third, senescent cells have been used as a source of nuclei from which animals have been cloned, and the telomeres of these senescent cells are critically short. However, cells derived from the newly cloned animals are no longer senescent cells and sometimes show markedly lengthened telomeres. Introduction of telomerase activity into at least some normal human cells prevents telomere shortening as well as replicative senescence [28,29]. Thus, at least in human cells, telomere shortening is clearly an important regulator of replicative senescence.

NEW MEDIATORS: CELLULAR SENESCENCE, TUMOR SUPPRESSION AND AGING

In the past decade, it has become clear that cell proliferation and telomere shortening are not the only inducers of the replicative senescence (the irreversible growth arrest, resistance to apoptosis and altered cellular structure and function). There is now a growing body of evidence that additional stimuli can induce senescence, with little or no cell division or telomere shortening [reviewed in 11].

DNA Damage

Harman proposed that the continuous production of damaging oxygen free radicals as a by-product of mitochondrial oxidative metabolism would gradually result in excessive damage which would limit cellular and organismal life span [30]. Oxidative damage has long been suggested to be a causal factor of aging [31,32]. Cells or organisms accumulate oxidative damage to DNA, proteins and lipids over the lifetime [reviewed in 33]. Other types of DNA damage also induce normal mammalian cells to undergo a senescence arrest [34,35, reviewed in 36].

Oncogene Activation

The expression of certain oncogenes (for example, activated RAS, RAF or MEK), or supra-physiological mitogenic signals (for example, overexpression of E2F1), induce senescence, rather than apoptosis or transformation, in normal human and mouse fibroblasts [20,37, 38]. *ras*-induced cell senescence and replicative senescence share many phenotypic traits such as a large flat morphology, pH 6 beta-galactosidase activity, and high levels of the cyclin dependent kinase (CDK) inhibitors p21 and p16 [20]. Cells that have undergone *ras*-induced senescence may do so because RAS signals through reactive oxygen species (ROS). *ras*-induced senescence can be abrogated by culturing cells under hypoxic conditions or treatment of cells with antioxidants [39]. An important feature of oncogene or mitogen-induced senescence is that even in the presence of active telomerase, the cellular senescence machinery remains intact and can be activated by appropriate signals [40]. These findings support the idea that the senescence response is a fail-safe mechanism that prevents normal cells that experience potentially oncogenic insults.

Chromatin Remodeling

Genetic and pharmacological manipulations that open or decondense chromatin (loss of heterochromatin) also induce a senescent phenotype in human and mouse cells [41,42], and accelerate aging in yeast [43,44]. In mammalian cells, locus-specific heterochromatin may limit the expression of tumor suppressor genes.

THE TELOMERE/TELOMERASE HYPOTHESIS OF AGING

The current data on telomere length and telomerase expression strongly suggest that there is no basis for a general telomere and telomerase hypothesis of aging. Certainly, telomere length and telomerase may play a non-exclusive role in the aging of organisms that exhibit telomere-dependent replicative senescence. However, whether this is the case depends on whether the hypothesis that cellular senescence contributes to aging is correct. Moreover, while short dysfunctional telomeres clearly contribute to the atrophy or development of cancer in proliferative tissues [45,46,47, reviewed in 36], there is as yet no evidence that maintenance of telomere length or elongation of functional telomeres is instrumental in improving the health or longevity of complex multicellular organisms such as mammals.

Thus, the "telomere hypothesis of aging" is a misnomer. It is essentially an oversimplified statement of the cellular senescence hypothesis of aging, which of course remains to be critically validated.

OXIDATIVE DAMAGE AND SPECIES-SPECIFIC LIFE SPAN

Since neither telomere length nor the presence of telomerase predicts the replicative life span of cultured cells, much less the life span of organisms, then why does replicative senescence of cultured cells from different species correlate to the age of the species?

One possibility is that cells vary in their sensitivity to culture conditions, which may cause a telomere-independent damage arrest [48,49]. The most obvious culprit is oxygen – ambient oxygen concentrations (~20%) far exceed the physiological oxygen concentrations (2 to 5%) experienced by cells *in vivo*. Thus, cultured cells are exposed to oxidative insults. It has been shown that when cultured in 2-3% oxygen, human fibroblasts achieve 20-30% more population doublings (PD), compared to the population doublings achieved in ambient (20%) oxygen [23,50,51, reviewed in 36]. By contrast, we recently found that mouse fibroblast cultures achieve 2- to 3 fold greater population doublings when cultured in hypoxia (3% oxygen) (S. Parrinello and J Campisi, in preparation). These findings suggest that mouse cells are much more sensitive than human cells to oxidative stress damage, at least in culture. Moreover, they raise the possibility that species-specific differences in cellular replicative life span (under standard culture conditions) may reflect differences in the ability of cells to respond to oxidative stress damage, rather than telomere length-limited replicative potential. Thus, at least among the mammalian species studied thus far [52], the differences in replicative life span shown by the cell cultures may more accurately reflect species-specific differences in the ability of cells to proliferate under 20% oxygen conditions.

One of the few genetic manipulations that extended the life span of a mammal (mice) conferred oxidative stress resistance on fibroblasts derived from the animals. Inactivation of the p66/shc gene in the mouse germline extended the life span of mice by about 30% [53]. Embryo fibroblasts cultured from p66/shc-/- mice showed a corresponding increase in resistance to chemically induced oxidative stress. This magnitude of life span extension is similar to that achieved by caloric restriction. Thus, the replicative life span of cells, at least under standard culture conditions, may better reflect species-specific differences in the stress response, which, in turn, may predict organismal life span.

The data available so far suggest that the more general process of cellular senescence, which occurs in response to many stimuli, including oxidative stress, may provide a general reflection of organismal life span.

CONCLUSION

A great deal of progress has been made over the years in understanding how cellular senescence is induced and mediated at the molecular level mainly using cell culture systems. For years to come, this information will be instrumental in decoding the mechanisms that limit species life span.

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