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Senescence and the Pro-tumorigenic Stroma

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Abstract

Hayflick and Moorhead first described senescence in the late 1960's as a permanent growth arrest that primary cells underwent after a defined number of cellular divisions in culture. This observation gave rise to the hypothesis that cells contained an internal counting mechanism that limited cellular division and that this limit was an important barrier to cellular transformation. What began as an *in vitro* observation has led to an immense body of work that reaches into all fields of biology and is of particular interest in the areas of aging, tissue regeneration, and tumorigenesis. The initially simplistic view that senescence limits cellular division and contributes to aging while stymying tumorigenesis has now evolved into an important and complex biological process that has numerous caveats and often opposing effects on tumorigenesis. In this review, we limit our discussion to the complex role senescence plays in tumorigenesis. Throughout the review we attempt to draw many parallels to other systems including the role senescent cells play in the tumor microenvironment and their significant molecular and phenotypic similarities to cancer associated fibroblasts (CAFs).

Keywords

senescence; SASP; tumor microenvironment; CAF

I. CELLULAR SENESCENCE IN VITRO

Cellular senescence was first described *in vitro* with the observation that primary cells have a finite replicative lifespan. When cells reach the end of their replicative lifespan they are unable to reenter the cell cycle, yet remain metabolically active.[1] Investigation into the mechanisms governing this finite replicative lifespan revealed that telomeres, the nucleoprotein structures located at the ends of the chromosomes, controlled replicative lifespan. Indeed, senescence is induced when telomeres become dysfunctional through telomere loss that results from the "end replication problem."[2–5] The end replication problem results from the inability of the DNA replication machinery to complete replication of the most distal end of the telomere. Dysfunctional telomeres also arise upon abrogation of telomere-specific binding proteins or other DNA replication and repair proteins that contribute to ongoing telomere function.[6] The importance of the telomere binding proteins in this process is underscored by the finding that mutations in telomere-specific structural

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proteins is associated with human disease. For example, mutation in the telomere protein Tin2 is associated with dyskeratosis congenita (DKC) while mutations in the Werner protein leads to the premature aging syndrome “Werner syndrome” (WS), which is characterized by dysfunctional telomeres.[7–9] Importantly, WS cells enter senescence earlier than cells from age-matched controls and display telomere defects despite having reasonably normal telomere lengths. While telomere dysfunction can drive the activation of senescence, there are also nontelomere signals that trigger a phenotype that is indistinguishable from telomere-based senescence. When cells are exposed to these varied stresses, which range from exposure to high levels of ROS or DNA-damaging agents to the expression of cell-cycle inhibitors, tumor suppressors, and oncogenes, it is referred to collectively as stress induced premature senescence (SIPS).[10] SIPS will be discussed in more detail below.

II. CHARACTERISTICS OF SENESCENT CELLS

Growth arrest is the most readily observed characteristic of senescent cells. Senescent cells typically arrest with a G_1 DNA content and display an enlarged and flattened morphology.[11] The growth arrest is permanent and senescent cells do not respond to strong mitogenic stimuli.[12] Growth arrest in senescent cells is mediated through activation of the senescence effector proteins Rβ and p53 and results following upregulation of cell-cycle inhibitors, including p21 and p16.[13–15] In contrast to quiescent cells, senescent cells are generally resistant to apoptosis.[16]

III. INDUCERS OF SENESCENCE

Senescence was originally described as a limit to replicative cellular potential but as alluded to above, it is now well accepted that senescence can be induced by a wide variety of stimuli. Indeed, telomere erosion or loss of telomeric integrity that results in exposure of chromosome ends that are identified as DNA breaks also induces senescence. However, a large number of other cellular stresses similarly induce senescence. Oxidative stress that arises from mitochondrial dysfunction and subsequent accumulation of reactive oxygen species (ROS) induces cellular senescence.[17,18] Overexpression of oncogenes can also result in cellular senescence due to persistent DNA damage caused by over-replication of the genome and uncontrolled cellular division.[19,20] Other forms of DNA damage, including those induced by chemotherapy drugs and irradiation, also result in cellular senescence.[21–24] Indeed, persistent DNA damage is a reoccurring theme in senescence-inducing stimuli, and one marker of senescent cells is the appearance of large, unresolved DNA-damage foci.[25] Interestingly, several groups have recently shown that these persistent damage foci are localized to telomeres, regardless of whether the senescence-inducing stimulus was specific to telomeres or affected the whole genome.[26,27] How these foci form and what function they play in the maintenance of the senescent phenotype is unclear.

IV. MARKERS OF SENESCENT CELLS

In addition to expression of cell-cycle inhibitors including p16, p21, and p53 and the formation of persistent DNA-damage foci, several additional senescence markers have been observed. The most commonly used marker of senescence is senescence-associated β-galactosidase (SA-β-gal).[28] SA-β-gal is active at pH 6, distinguishing it from other cel-lular β-gal activities, which typically are optimal at higher pH.[28] Senescent cells also accumulate foci encompassing areas of heterochromatin, termed senescence-associate heterochromatin foci (SAHF).[25] SAHF are marked by heterochromaatin-associated histone modifications including histone 3 methylated on lysine 9 (H3K9me) and the heterochromatin-binding protein HP1.[25] Interestingly, SAHF form specifically at E2F target genes, where they are thought to inhibit transcription of these genes and subsequent cell-cycle progression.[25]

V. CELLULAR SENESCENCE IN VIVO

The accumulation of senescent cells within tissues is hypothesized to contribute to age-related diseases and degeneration, possibly through the depletion of stem cell populations or through alterations of the tissue architecture through an altered secretory profile. The putative importance of these cells in the degeneration of tissue was shown when they were selectively removed from mice. Indeed, removal of senescent cells in these mouse models abrogated the development of a wide variety of age-related phenotypes, including sarcopenia and loss of adipose tissue.[29] The presence of senescent cells in human tissues has been documented using the markers previously described in a variety of human tissues including kidney, prostate, skin, and liver.[28,30–34] Evidence that senescent cells accumulate in tissues with age comes from observational studies of primate and human tissue. Indeed, skin biopsies from human donors revealed that in the skin dermal layer, very few senescent fibroblasts (identified by SA-β gal staining) are present in donors under the age of 40, while readily detectable senescent fibroblasts were identified in donors over age 60.[28] Perhaps the most stunning data were derived from baboons where senescent cells were found to increase over the life span of individual animals.[35] Together these data clearly demonstrated an age-related increase in senescent cells in primates and humans and suggest that they contribute to a wide variety of pathologies.

VI. SENESCENT CELLS PROMOTE MANY STAGES OF TUMORIGENESIS

The description of a limited replicative lifespan in the 1960's provided the basis for the hypothesis that senescence was a potent tumor suppressive mechanism.[1] The argument that was proposed was that because cancer cells were immortal, senescence would be a hurdle that would need to be overcome if an incipient tumor cell was going to progress to a fully neoplastic cell. This original hypothesis was validated decades later by investigators examining human tissues and animal models. Indeed, senescent cells were found in premalignant melanocytic naevi in humans that arose as a result of a mutation in the BRAF gene ($BRAF^{E600}$) that created a constitutively active protein.[36] Importantly, progression to neoplastic disease was associated with loss of senescent cells. In addition, senescent cells were found in a mouse prostate model. In this model, analysis of premalignant lesions of the prostate generated by loss of the tumor suppressor *Pten*[37] demonstrated that the lesions harbored senescent cells. Importantly, senescent cells were lost upon progression to malignancy.[37,38] Furthermore, inactivation of senescence-inducing pathways, either through inactivation of p53 or through inactivation of the DNA-damage response (a critical driver of senescence induction) in the *Pten* model as well as others, results in a more rapid progression from premalignancy to malignancy and larger tumor size.[37,38] Finally, restoration of p53 activity in sarcomas results in senescence induction and regression of the tumor.[39]

In contrast to the tumor-inhibiting effects that senescence mediates in transformed cells, senescence of normal cells in the surrounding microenvironment is tumor-promoting. Senescent human prostate fibroblasts stimulate the growth of epithelial cell harboring mutations that create preneoplastic cells in co-culture experiments while they have no effect on normal checkpoint competent cells.[40] Furthermore, senescent human lung fibroblasts stimulate preneoplastic epithelial cell growth in xenograft experiments in both the mammary fat pad and subcutaneous skin.[41] Senescent fibroblasts also promote epithelial-to-mesenchymal transition and invasion in breast preneoplastic cells,[42] indicating the ability of senescent fibroblasts to promote not only the growth of preneoplastic cells, but also the progression from precancerous to cancerous lesions. Thus, it is important to place senescence into the tissue context where it arises. When senescence arises in an incipient tumor cell that has begun its journey toward neoplasia, it is a potent tumor suppressor

mechanism. However, when senescence occurs in surrounding cells it can stimulate tumorigenesis.

The above observations raise two critical questions. The first question is if senescent cells are detrimental as is the case in the microenvironment, why don't cells activate the apoptotic pathway rather than senescence? The second question that follows is how a single mechanism, senescence in this case, can have such opposing effects in tumorigenesis. The first effect, anti-tumor, is obvious. Cells that cannot divide cannot form a tumor. The second effect is less clear; how do senescent cells stimulate tumorigenesis? Work over the past decade has begun to shed light on this second question and is discussed below.

VII. THE SENESCENCE-ASSOCIATED SECRETORY PHENOTYPE

How do senescent cells within the microenvironment promote tumorigenesis? Work from Campisi and colleagues demonstrated that senescent cells promote tumorigenesis through the upregulation and secretion of a wide variety of pro-tumorigenic proteins into the microenvironment.[41,43] These proteins are collectively referred to as the senescence-associated secretory phenotype (SASP).[21] SASP is enriched in proteins involved in inflammation (e.g., interleukins, cytokines, and chemokines), alteration of the extracellular matrix (e.g., matrix metalloproteinases), and cell division (e.g., growth factors).[21,40,44]

The SASP's pro-tumorigenic nature has been demonstrated extensively both *in vitro* and *in vivo*. Senescent fibroblasts stimulate the invasiveness of human umbilical vascular endothelial cells (HUVECs) *in vitro* and increase vascularization of tumors in xenograft experiments through secretion of vascular endothelial growth factor (VEGF).[45] Osteopontin (OPN) expression level is elevated in senescent fibroblasts and is necessary for the stimulation of preneoplastic cell growth induced by senescent fibroblasts *in vivo*.[44] Downregulation of OPN in senescent mammary fibroblasts also inhibits the invasion and migration of associated epithelial cells *in vitro*.[46] Interleukins IL6 and IL8 promote breast cancer epithelial cell growth. Indeed, treatment of co-cultures of senescence cells and preneoplastic epithelial cells with neutralizing antibodies against IL6 and IL8 results in decreased growth promotion.[21] Furthermore, treatment of breast cancer epithelial cells with recombinant IL6 and IL8 is sufficient to promote growth.[21] Finally, matrix metalloproteinase 3 (MMP3) from senescent cells promotes branching and proliferation of breast epithelial cell organoids as well as the growth of breast cancer epithelial cells in xenograft experiments.[42–47] These results demonstrate that senescent cells promote the establishment of primary tumors through the expression of SASP factors.

Like senescent fibroblasts within the tumor microenvironment, cancer-associated fibroblasts (CAFs) promote every step of the transformation process by stimulating tumor growth, angiogenesis, invasion, and metastasis.[48–50] The tumor -promoting activity of CAFs is partially mediated through an altered expression profile that overlaps significantly with the SASP. Indeed, several groups have isolated CAFS via fluorescence-activated cell sorting from human squamous cell carcinoma and pancreatic ductal adenocarcinoma,[51] through treatment with granulin (which induces the CAF phenotype *in vitro*) or through laser microdissection of breast tumors.[52–55] These studies have demonstrated that the expression profile of CAFs is enriched in many of the same pro-inflammatory factors including IL6, IL8, and a variety of CXCLs that are present in the SASP. Thus it was not surprising to find that like senescent cells,[52,56] CAFs also express increased levels of MMP3 and OPN expression,[51] which can promote tumor cell growth. Given the phenotypic similarities and emerging molecular similarities between senescent cells and CAFs, we have argued that senescent cells are an operational subtype of CAF. Even the choice of the acronym underscores the similarities between these cell types. SASP is in fact, the senescence-

associated secretory phenotype and we suggest that SASP or a portion of SASP is characteristic of all tumor-promoting stromal cells.

VIII. A ROLE FOR SENESCENCE IN TUMOR PROGRESSION

As already highlighted above, senescent cells within the microenvironment and their functionally analogous cousins, CAFs, promote transformation by stimulating tumor growth, angiogenesis, and invasion. The broad actions of CAFs and senescent cells in tumor progression have been ascribed to the plethora of pro-tumorigenic factors that these cell types secrete. Of particular importance is how the appearance of these cells shapes the local tissue environments by not only directly stimulating indolent tumor cell growth and progression, but also by recruiting bone marrow derived cells and altering the functions of many cell types within the existing tissue. There are many examples where this occurs by eliciting a local response such as by stimulating angiogenesis as well as systemic responses such as the recruitment of bone marrow derived cells. The current challenge to the field is to discern which changes are critical to tumor progression and to determine how cell autonomous mutations within incipient tumor cells influence stromal changes.

CAFs clearly impact tumor progression and this raises the possibility that senescent cells, through their secretory phenotype, also impact progression. Indeed, senescent cells can promote epithelial-to-mesenchymal transition (EMT),[21] a crucial step in tumor cell metastasis. It has been shown that treatment of human breast cancer cell lines with conditioned media from senescent fibroblasts resulted in decreased expression of cytokeratin and E-cadherin, hallmarks of EMT.[21] This promotion of EMT by senescent cells was mediated by MMP3.[42] Similarly, CAFs enhance the epithelial-mesenchymal transition (EMT) through the secretion of MMPs.[50]

When tumor cells leave a primary site, the mechanisms that drive selection of a metastatic site are complex. Interestingly, recent work has shown that prior to the arrival of metastatic cells at a distal site, numerous changes occur that prepare the site for growth of the tumor cells. Work from various groups has shown that systemic changes elicited by tumor cells within the primary site can initiate changes at the distal site.[57] However, an outstanding question is how the microenvironment at these distal sites is changed and what cell types are responsible for these changes. It is reasonable to hypothesize that the appearance of a reactive stromal compartment through the accumulation of CAFs, immune cells, or even senescent cells in tissues distant to the primary site condition the area for eventual establishment of metastases. If CAFs or senescent cells were present, expression of SASP factors could break down the extracellular matrix to allow for easier invasion and promote growth of the newly arrived tumor cells. Alternatively, tumors could bring CAFs with them from a primary site, and once in the metastatic site, facilitate tumor cell colonization and growth as was recently shown.[58] In this study the presence of CAFs with metastasizing tumor cells increased the likelihood of successful colonization at the distal site.[58] These results indicate that pro-tumorigenic changes induced by CAFs within the microenvironment have the potential to precondition and increase the likelihood of the establishment of metastases. Because senescent cells accumulate in tissue with age,[41] these observations raise the possibility that senescent cells also condition the premetastatic niche.

Finally, recent work has focused on identifying and elucidating the mechanisms that drive the emergence of cancer initiating cells or cancer stem cells (CSC), which appear to be responsible for forming distant metastasis.[7] While the identification of surface markers that precisely define CSCs has remained elusive, emerging data suggest that the tumor microenvironment can influence their prevalence.[59,60] Intriguingly, chemokines secreted by senescent cells have been shown to select for CSCs.[59] IL6, one of the most highly expressed

SASP factors, can play an important role in regulating breast CSC self-renewal.[61] Similarly, analyses of breast CAFs recently revealed that expression of chemokine (C-C motif) ligand 2 (CCL2) can stimulate CSC properties including sphere-forming capacity and self-renewal.[60]

IX. SASP EXPRESSION IS SUBJECT TO COMPLEX REGULATION

Given the potent tumor-promoting nature of SASP, identifying the regulatory mechanisms that govern its expression will contribute to the development of stroma-targeting cancer therapies. Additionally, it is important to again note the similarities between the SASP and the expression profile of CAFs. The overlap in expression profiles between these two tumor-promoting cell types suggests that regulatory pathways elucidated in senescent cells will be directly applicable in CAFs.

As discussed previously, persistent activation of the DNA-damage response is an important inducer of senescence, and ATM activity is required for expression of a wide variety of SASP factors.[62] Indeed, shRNA directed depletion of ATM from cells inhibits expression of the vast majority of SASP factors in senescent cells.[62] However, not all SASP factors are dependent on ATM for their expression; ATM dependency is predominantly a characteristic of the inflammatory SASP factors.[62] The SASP is also transcriptionally regulated by NFκB and C/EBPβ.[63–65] Interestingly, NFκB activity in senescent cells is decreased in response to ATM depletion, suggesting that ATM and NFκB function within the same signaling pathway.[63] Similar to ATM depletion, inhibition of NFκB or C/EBPβ results in an inability of senescent cells to activate a subset of SASP factors.[63,65]

In addition to DNA-damage response signaling, mitogen-activated protein kinase (MAPK) signaling is also an important regulator of SASP factor activation.[63] MAPK p38 inhibition, either through expression of shRNA directed at p38α or through treatment with small molecule inhibitors of p38, results in decreased expression of SASP in response to senescence.[63] Similar to the effects of ATM depletion, inhibition of p38 activity reduces the activity of NFκB.[63] Furthermore, constitutive activation of the p38-signaling pathway results in SASP activation even in the absence of ATM. ATM, however, is not required for p38 activation in response to senescence, suggesting that p38 and ATM function in parallel pathways, both of which end in NFκB activation and SASP factor expression.[63] As with ATM and NFκB, p38 is predominantly involved in the activation of the inflammatory SASP components. How p38 is activated in response to senescence is not understood, but the slow kinetics of its activation suggests a noncanonical mechanism. As indicated previously, many of the SASP regulatory pathways elucidated thus far do not control the expression of all SASP factors, and p38 is no exception. There remains much to be uncovered regarding the regulation of noninflammatory SASP factors. Furthermore, no central SASP regulator capable of coordinating upregulation of this diverse array of pro-tumorigenic factors has been identified.

Finally, it is important to note that SASP does not require activation of senescence for expression. Cells defective in the p53 and pRβ pathways, both central regulators of the senescent phenotype, either singularly or in combination retain the ability to express SASP factors in response to stress and persistent DNA-damage signaling.[21,66] This uncoupling of SASP expression and the senescent phenotype is further demonstrated by cells induced to senesce through overexpression of the cell-cycle inhibitors p16 and p21.[67] Senescence induced by ectopic expression of p16 or p21 fails to activate the SASP despite these cells displaying the hallmark cell-cycle arrest and cellular morphologies of senescence.[67] Thus, senescence represents merely one way to achieve expression of SASP factors. This concept parallels closely with our suggestion that senescent fibroblasts are a subset of CAFs, and that

SASP is a characteristic of tumor-promoting stromal cells in general regardless of whether they are senescent. Therefore, further characterization of SASP regulatory pathways in senescent cells and CAFs is critical to our understanding of these mechanisms and to future therapeutic approaches. Indeed, NFκB, a central transcriptional regulator of SASP expression in senescent cells, is also the mediator of expression of pro-inflammatory factors expressed by CAFs.[51] This observation suggests that therapeutic targets identified in SASP factor expression will be applicable to a wide variety of cancer-promoting microenvironments.

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Abbreviations

SASP	senescence-associated secretory phenotype
CAF	cancer-associated fibroblast
SIPS	stress-induced premature senescence
DKC	dyskeratosis congenital
WS	Werner syndrome
SA-β-gal	senescence-associated β-galactosidase
SAHF	senescence-associate heterochromatin foci
HUVEC's	human umbilical vascular endothelial cells
VEGF	vascular endothelial growth factor
OPN	osteopontin
MMP3	metalloproteinase 3
EMT	epithelial-to-mesenchymal transition
CSC	cancer stem cells
C-C motif	chemokine
GCL2	ligand 2

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