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Review Article

Cellular Senescence as a Target in Cancer Control

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Somatic cells show a spontaneous decline in growth rate in continuous culture. This is not related to elapsed time but to an increasing number of population doublings, eventually terminating in a quiescent but viable state termed *replicative senescence*. These cells are commonly multinucleated and do not respond to mitogens or apoptotic stimuli. Cells displaying characteristics of senescent cells can also be observed in response to other stimuli, such as oncogenic stress, DNA damage, or cytotoxic drugs and have been reported to be found *in vivo*. Most tumors show unlimited replicative potential, leading to the hypothesis that cellular senescence is a natural antitumor program. Recent findings suggest that cellular senescence is a natural mechanism to prevent undesired oncogenic stress in somatic cells that has been lost in malignant tumors. Given that the ultimate goal of cancer research is to find the definitive cure for as many tumor types as possible, exploration of cellular senescence to drive towards antitumor therapies may decisively influence the outcome of new drugs. In the present paper, we will review the potential of cellular senescence to be used as target for anticancer therapy.

1. The Biology of Senescence

Over 40 years ago, Hayflick [1] established that human diploid fibroblasts show a spontaneous decline in growth rate in continuous culture related not to elapsed time but to an increasing number of population doublings, eventually terminating in a quiescent but viable state now known as *replicative senescence*. These cells show a flat, enlarged morphology with low pH β-gal activity, are commonly multinucleated, and are irresponsive to mitogens or apoptotic stimuli. Similar behaviour has since then been observed in a wide variety of normal cells, and it is now widely accepted [2] that normal human somatic cells have an intrinsically limited proliferative lifespan, even under ideal growth conditions. Moreover, the senescent phenotype is associated with a typical gene-expression profile [3–5]. Cells displaying characteristics of senescent cells, however, can be observed in response to other stimuli, such as oncogenic stress, DNA damage, or cytotoxic drugs [6].

Cells displaying senescent characteristics have not only been observed in cell culture but also in their maternal tissue environment. A number of reports have related reduced cellular lifespan with metabolic disease, stress sensitivity, progeria syndromes, and impaired healing, indicating that entry into cellular senescence may contribute to human disease. Indeed, it has been suggested that cellular senescence is in part responsible for the pathogenesis of a number of human diseases, such as atherosclerosis, osteoarthritis, muscular degeneration, ulcer formation, Alzheimer's dementia, diabetes, and immune exhaustion.

Most cancers contain cell populations that have escaped the normal limitations on proliferative potential. This capability, known as *immortality*, contrasts with the limited lifespan of normal somatic cells. It has therefore been proposed that cellular senescence is a major tumor suppressor mechanism that must be overcome during tumorigenesis [2].

The kinetics of replicative senescence do not show an abrupt arrest of the whole population, but a gradual decline in the proportion of dividing cells [7], the exact timing of which varies between both cell types and sister clones [8]. This behaviour is best explained as the result of (i) an intrinsic control mechanism linked to elapsed cell divisions—*the senescence clock*—which progressively desensitises the cell-cycle machinery to growth factor stimulation, together with

(ii) a stochastic component probably having the same (still unknown) basis as that observed in immortal cells under conditions of growth factor restriction. Stem cells can give rise to differentiated progeny and are capable of autorenewal. In some renewing tissues, stem cells undergo more than 1000 divisions in a lifetime with no morphological signs of senescence [8]. This indicates that at a certain point of lineage differentiation, cells activate the senescence clock that ultimately induces cell senescence through a series of effectors.

More recently, the finite number of divisions—referred to as the "*Hayflick limit*"—was attributed to the progressive shortening of chromosomal ends. *Telomere shortening* is considered to be the most probable molecular mechanism explaining the existence of such a senescence clock controlling replicative senescence [9, 10]. Eukaryotic cells cannot replicate the very ends of their chromosomes, the telomeres, resulting in shortening their lengths with every cell division until they reach a critical threshold, at which point cells stop replicating [11]. However, enforced replication despite short telomeres ends in high chromosomal instability and apoptosis, a process known as *crisis*. Many other mechanisms, however, have been also proposed (Table 1).

Senescent cells display molecular markers characteristics of cells bearing double-strand breaks. These markers include nuclear foci of phosphorylated histone H2AX and the localization at double-strand break sites of DNA-repair and DNA-damage checkpoint factors, such as 53BP1, MDC1, and NBS1 [12, 13]. Senescent cells also contain activated forms of the DNA-damage checkpoint kinases Chk1 and Chk2. These and other results suggest that telomere shortening initiates senescence trough a DNA-damage response. This will explain why other DNA-damaging stresses, such as culture shock, might initiate senescence without telomere involvement. The initiation of senescence triggers the generation and accumulation of distinct heterochromatic structures known as senescence associated heterochromatic foci (SAHF). The formation of SAHF coincides with the recruitment of heterochromatic proteins and the pRB tumor suppressor to E2F-responsive promoters. SAHF accumulation is associated with stable repression of E2F target genes and does not occur in reversibly arrested cells. SAHF formation and promoter repression depend on the integrity of the pRb pathway [14]. These results provide an explanation for the stability of the senescent state.

Consistent with a role in aging, senescent cells accumulate with age in many rodent and human tissues [15]. Moreover, they are found at sites of age-related pathology, including degenerative disorders such as osteoarthritis and atherosclerosis [15] and hyperproliferative lesions such as benign prostatic hyperplasia [16] and melanocytic naevi [17]. A limited number of cell culture and mouse xenograft studies support the idea that senescent cells secrete factors that can disrupt tissue structure and function and promote cancer progression [18–20]. Recent studies on the senescence-associated secretory phenotype (SASP) of human and mouse fibroblasts show that it is conserved across cell types and species and that specific secreted factors are strong candidates for stimulating malignant phenotypes in neighboring cells [21–23].

The idea that a biological process such as cellular senescence can be both beneficial (tumor suppressive) and deleterious (protumorigenic) is consistent with a major evolutionary theory of aging termed antagonistic pleiotropy [23]. The SASP may be the major reason for the deleterious side of the senescence response [24].

In addition to telomere dysfunction, cellular senescence can be elicited by other types of stress, including oncogene activation [25]. This phenomenon is not observed for oncogenic RAS exclusively; many—but not all—of its effectors, including activated mutants of RAF, MEK, and BRAF, were shown to cause senescence as well [26–29]. Some oncogenes, such as RAS, CDC6, cyclin E, and STAT5 which induce senescence also trigger a DNA-damage response (DDR), which is associated with DNA hyperreplication and appears to be causally involved in oncogene-induced senescence (OIS), *in vitro* [30–33]. During most of the last decade, OIS has been studied predominantly in cell culture systems, triggering a long debate as to whether or not OIS corresponds to a physiologically relevant phenomenon *in vivo*. In favour of OIS representing an *in vitro* phenomenon only is that artificial conditions, such as the use of bovine serum and plastic dishes, as well as the presence of supraphysiologic O2, generate a stress signal that at the very least contributes to triggering a cellular senescence response [34, 35]. However, conversely, senescence bypass screens have identified several genuine human oncogenes, including TBX2, BCL6, KLF4, hDRIL, BRF1, and PPP1CA [36]. Furthermore, virtually all human cancers lack functional p53/pRB pathways, two key senescence-signalling routes [37], and often carry mutations in sets of genes, which are known to collaborate *in vitro* in bypassing the senescence response.

2. Effector Pathways

Cellular senescence pathways are believed to have multiple layers of regulation, with additional redundancy built into these layers [38]. On the basis of the complementation studies, there are at least four senescence genes or pathways. There are, however, many more chromosomes that can induce senescence than there are senescence complementation groups. Furthermore, there are some immortal cell lines that have been assigned to multiple complementation groups [39]. This indicates that in any one immortal cell line, there are probably multiple senescence genes/pathways that are abrogated [40]. Many of the functional studies, where a putative senescence gene is overexpressed in cells, indicate that although multiple genes/pathways may be abrogated in a particular cell line, as little as one gene/pathway is required for repair and subsequent reversion to senescence.

Pathways known to regulate cellular senescence/immortalisation, including the p16INK4a/pRB pathway, the p19ARF/p53/p21CIP1/WAF1 pathway, and the PTEN/p27KIP1 pathway, are reviewed in [36, 41–44]. Other genes that have been shown to induce a senescence-like phenotype include PPP1A [45], SAHH [46, 47], Csn2, Arase and BRF1 [48], PGM [49], IGFBP3 and IGFBPrP1 [50], PAI-1 [51, 52], MKK3 [53], MKK6 [53, 54], Smurf2 [55], and HIC-5 [56]. All these genes have shown to be related to human

Table 1: Cellular clock driving senescence hypothesis.

Cellular clock	Cause	Molecular readout
Error-catastrophe theories		
Somatic mutation accumulation	Metabolism/oxygen free radicals	Altered protein function, DNA damage
Mitochondrial DNA mutation	Oxygen free radicals	Altered mitochondrial function
Posttranslational modification of proteins	Oxidation, glycosylation, acetylation, methylation, and so forth	Altered function of proteins
Altered proteolysis	Errors in proteolysis machinery	Accumulation non functional proteins
Deterministic theories		
Telomere shortening	no replication of the telomere ends	DNA damage, exposure ends of telomeres, Liberation regulatory proteins, and so forth
Changes in heterochromatin domains		changes in transcription
Changes in DNA methylation		changes in transcription
Codon restriction	Switching codon preferences in early development, restrict availability later In life	Altered protein synthesis
Terminal differentiation	Senescence is a form of terminal differentiation genetically controlled	

Several hypotheses for cellular clocks driving senescence have been proposed. Most of them lay into error-catastrophe theories, suggesting that senescence is a byproduct of cell living, and deterministic theories, suggesting a genetic program for cellular senescence. Some of the most representative theories are collected in this table.

tumorigenesis. However, all these genes and their pathways, as indicated earlier, can act in sequential steps conforming a well-regulated process.

Two major effector pathways have been directly related to senescence: the p14ARF/p53/p21 pathway and the INK4/CDK/pRb pathway [57] (Figure 1). The absence of p53 function induced by dominant negative mutants, specific p53 antisense mRNA, oligonucleotides, or viral oncoproteins (such as SV40 T antigen or HPV16 E6) is sufficient to substantially extend the lifespan of several cell types in culture [58]. Consistent with this, senescence is associated with a switch-on of the transactivation function of p53 in culture [59]. Coincident with telomere shortening, DNA-damage checkpoint activation, and associated genomic instability, p53 is also activated *in vivo*[60]. Deletion of p53 attenuated the cellular and organismal effects of telomere dysfunction, establishing a key role for p53 in the shortening response [60].

Other p53 regulatory proteins are also involved in senescence (Figure 1). MDM2 protein has p53 ubiquitin ligase activity and forms an autoregulatory loop with p53 [61]. Overexpression of MDM2 targets p53 for degradation and induces functional-p53 loss [62]. The product of another gene upregulated in senescence—p14ARF—can release p53 from inhibition by MDM2 and cause growth arrest in young fibroblasts [62]. Seeding mouse embryonic fibroblasts (MEFs) into culture induces the synthesis of ARF protein, which continues to accumulate until the cells enter senescence [63]. MEFs derived from ARF-disrupted mice [63] or wild-type fibroblasts expressing an efficient ARF antisense construct [64] are also efficiently immortalised. Concomitant with this observation, overexpression of MDM2 in naïve MEFs produces efficient immortalisation [64].

Activation of p53 induces the upregulation of the cyclin-dependent kinase (CDK) inhibitor p21WAF1, which has a direct inhibitory action on the cell-cycle machinery [37] and correlates well with the declining growth rate in senescing cultures. In mouse embryo fibroblasts, however, the absence of p21WAF1 does not overcome senescence [65, 66]. This suggests that at least one additional downstream effector is needed for p53-induced growth arrest in senescence. In contrast, a different behaviour is observed in human cells, where elimination of p21 by a double round of homologous recombination is sufficient to bypass senescence [67]. Other p53 effectors might be also involved, such as 14-3-3 and GADD45, which inhibit G2/M transition or downregulation of Myc [68] (Leal and Carnero, Unpublished results).

The retinoblastoma tumor suppressor pathway, pRb, has also been related to senescence (Figure 1). Overexpression of pRb, as well as some of the regulators of the pRb pathway such as CDK inhibitors, leads to growth arrest mimicking the senescent phenotype [26]. Moreover, inactivation of pRb by viral oncoproteins such as E7, SV40 large T antigen, and E1A leads to extension of lifespan [69–71]. Other members of the pocket protein family comprising pRb, p130, and p107 may also be involved. In MEFs, p130 levels decrease with population doublings and MEFs from triple pRb, p130, and p107 knockout mice are immortal [72]. Nevertheless, since a certain degree of complementation has been observed among the pocket protein family [72], it is difficult to assess the role of each protein in replicative senescence.

Given that p16INK4a functions to inhibit the inactivation of pRb by CDKs [73], a loss of functional p16INK4a may be expected to have similar consequences with the loss of functional pRb. Several types of human cells accumulate p16INK4a protein as they approach senescence [74]. Senescent fibroblasts may contain p16INK4a levels at least 40-fold

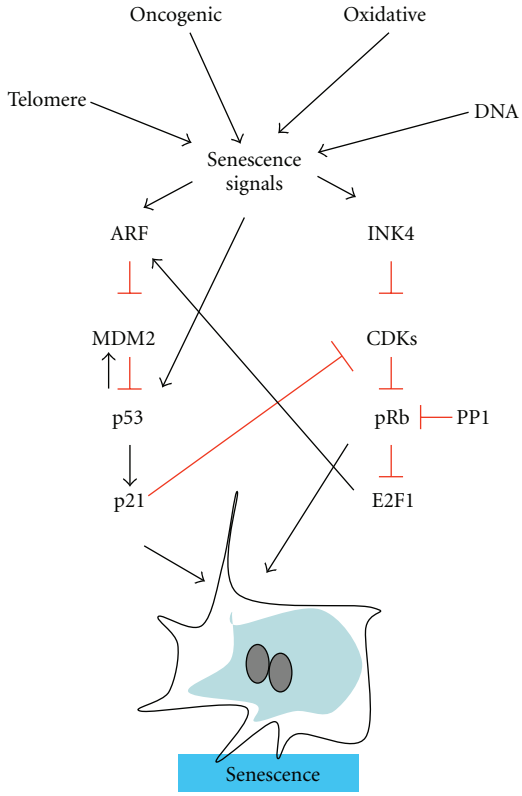

Figure 1: Scheme representing the senescence effector pathways crosstalk.

greater than early passage cells. The deletion of p16INK4a is common in immortalised tumor cell lines [75], and several nontumorigenic *in vitro* immortalised cell lines also lack functional p16INK4a protein. Expression of p16INK4a-specific antisense in naïve MEFs increases the probability of immortalisation of these cells [64]. In accordance with this observation, mice cells which are made nullizygous for p16INK4a by targeted deletion undergo immortalisation more readily than normal control cells [76, 77] although they show normal senescence kinetics. Knockout mice for p16INK4a proteins develop normally to adulthood and are fertile, indicating that the individual INK4 proteins are not essential for development. p16INK4a deficiency, however, results in a low susceptibility to spontaneous tumor development and increased tumor susceptibility under specific carcinogenic protocols [76, 77]. A crosstalk among the different pathways involved in senescence has been found. This crosstalk might ensure the correct functioning of the senescence program. Moreover, genes such as *myc* that are involved in all the pathways are able to bypass senescence in human primary cells. Myc can bypass CDK4/6 inhibition by activating CDK2-cyclinA/E complexes and inducing the Cdk-activating phosphatase Cdc25A [78]. Moreover, *myc* induces degradation of p27, thus influencing the inhibitory effects of PTEN. Finally, expression of myc induces telomerase activity by activating the transcription of the catalytic subunit [79]. The overall result is a single step immortalisation of human cells induced by myc gene amplification [80].

Over all steps, DNA methylation regulates expression of senescence genes, with the capability of controlling the process [44]. In human cancers, the silencing of tumour suppressor genes through aberrant DNA methylation of the CpG island(s) in promoters in these genes is a common epigenetic change [81]. There are an assortment of pathways from which genes have been shown to be hypermethylated in cancer cells, including DNA repair, cell-cycle control, invasion, and metastasis. The tumour suppressor genes BRCA1, p16INK4a, p15INK4b, p14ARF, p73, and APC are among those silenced by hypermethylation although the frequency of aberrant methylation is somewhat tumour-type specific. Recently, we found S-adenosylhomocysteine hydrolase (SAHH) [46], which has also been previously identified in an independent short hairpin RNA (shRNA) screening [82], the inactivation of which confers resistance to both p53- and p16(INK4)-induced proliferation arrest and senescence. SAHH catalyzes the hydrolysis of S-adenosylhomocysteine to adenosine and homocysteine. In eukaryotes, this is the major route for disposal of S-adenosylhomocysteine formed as a common product of each of the many S-adenosylmethionine-dependent methyltransferases, therefore, regulating the methylation processes. Interestingly, SAHH inactivation inhibits p53 transcriptional activity and impairs DNA-damage-induced transcription of p21(Cip1). SAHH messenger RNA (mRNA) was lost in 50% of tumour tissues from 206 patients with different kinds of tumours in comparison with normal tissue counterparts. Moreover, SAHH protein was also affected in some colon cancers [46, 47].

3. Clinical Implications

The implication of senescence as a barrier to tumorigenesis first comes from the realisation that a limited number of duplications necessarily reduces the possibility of tumor growth. However, the proliferative lifespan before reaching the Hayflick limit could be sufficient to generate a tumor mass greater than that required for lethality. This argument fails to take into account the existence of ongoing cell death and differentiation within a tumor and the occurrence of clonal selection driven by different senescence barriers or barriers unrelated to senescence. Finally, a clinically significant cancer can be composed of entirely mortal, presenescent cells if the cell of origin has a sufficient proliferative lifespan, and the tumor develops with few successive clonal expansion steps and/or with a low cell death rate. Even with these examples, however, senescence may of course still be a significant barrier to the recurrence of tumors from the small number of residual cells remaining after therapy.

As mentioned, several studies *in vivo* show that oncogene-induced senescence provides a bona-fide barrier to tumorigenesis. Michaloglou and coworkers [83] have shown that an oncogenic BRaf can induce senescence in fibroblasts and melanocytes and that human nevi display markers of senescence. Therefore, sustained exposure of melanocytes to aberrant mitotic stimuli provokes senescence after an initial proliferation burst. Collado and coworkers [84] identified senescent cells *in vivo* after generating new

senescence biomarkers from array studies. Using conditional Kras-val12 mice strains, they observed senescence markers to be predominant in premalignant lesions of the lung and pancreas, but not in those that have progressed to full-blown cancers. Direct evidence that hyperproliferative signals can trigger a program of permanent arrest *in vivo* have been provided in a transgenic model conditionally expressing E2F3 in the pituitary gland [85]. E2F3 induced hyperplasias that failed to progress because the cells became insensitive to further mitogenic signals. This insensitivity correlated with the appearance of senescence markers and a terminally arrested cellular state. Disruption of PTEN in mice also produces hyperplastic conditions analogous to prostatic intraepithelial neoplasia (a precancerous lesion in men). These lesions display senescence markers [86]. Loss of p53 prevents senescence in response to PTEN ablation and cooperates to produce invasive prostate carcinomas. These results are consistent with the notion that senescence actively limits malignant conversion.

In human fibroblasts in culture, the senescence program involves chromatin reorganisation involving H3 methylation at the Lys9 residue concomitant with the recruitment of heterochromatin proteins to some proliferation-related genes. Braig and coworkers [87] found that disruption of Suv39h1 methyltransferase, which methylates the Lys9 residue of H3, blocked ras-induced senescence and accelerated ras-induced lymphomagenesis in mice. Interestingly, Suv39h1-expressing tumors responded through senescence to chemotherapy; however, Suv39h1-null tumors did not show any senescent response but still maintained the apoptotic response. Treating ras transgenic mice with DNA-methyltransferase or histone deacetylase inhibitors, which mimic the effects of Suv39h1 disruption, accelerated ras-induced tumorigenesis.

The concept of cancer being a disease whereby cells have lost the ability to senesce leads to a critical evaluation of the benefits that can be achieved for cancer diagnosis, and therapy through the knowledge surrounding molecular pathways (both genetic and epigenetic in origin) that induce senescence. Until just a few years ago, it was accepted that tumor cells were no longer capable of senescence. Today, however, it is accepted that neoplastic cells can be forced to undergo senescence by genetic manipulations and by epigenetic factors, including anticancer drugs, radiation, and differentiating agents [26, 88]. However, although not fully studied *in vivo*, it has been shown that senescent cells might increase the oncogenic potential of tumor cells. Therefore, it will be necessary to understand the contribution of senescent stromal cells to tumors, before applying drug-induced senescence program to tumors.

Immortalising defects are recessive and can be blocked by imposing the process of senescence [89]. The first approach to inducing senescence to tumor cells was through somatic cell fusion. These studies identified four senescence-determining complementation groups. In recent years, it has been found that different tumoral cell lines show cellular growth arrest along with senescence markers after the genetic expression of tumor suppressor genes commonly involved in senescence, such as p53, p21, p16, pRb, or p21 [90]. Similarly, the restoration of cellular levels of p53 in a cell line conditionally immortalised by p53 antisense expression induces growth arrest with a senescent phenotype [91]. Adenovirus vectors carrying CKIs (p16INK4a, p15INK4b, p21cip1, and p27kip1) as vehicles for delivery and expression are a powerful approach to examining therapeutic applications both *in vitro* and *in vivo*, with promising results [92]. When a 16-amino acid transmembrane carrier segment derived from the *Drosophila* antenappedia protein was linked to the third ankyrin repeat of the p16INK4a protein and inserted into cells, Rb-dependent G_1 arrest was observed. In a breast-derived cell line, the chimera containing the antennapedia peptide and the carboxyl-terminal residue of p21waf1 had higher specificity for CDK4/cyclin D than for CDK2/cyclin E and arrested the cells in G_1 phase [93].

These observations indicate that tumor cells maintain at least some of the components of the cellular senescence program, including terminal growth arrest. It is now clear that depending upon the cell proliferation kinetics of the tissue of origin, tumor development can be initiated by genetic events, causing either a block in terminal differentiation or/and inappropriate activation of growth stimulatory signaling pathways. The net result in both cases is the generation of a cellular clone capable of infinite expansion if it is not constrained by physical barriers or lack of blood supply. Schmitt and collaborators [94] convincingly showed that in a lymphoid mouse tumor model, an intact senescence pathway appears to be pivotal to the efficacy of cyclophosphamide, and its disruption makes tumor cells highly refractory to the drug. On the other hand, as mentioned, Suv39h1-expressing tumors responded to chemotherapy by inducing senescence. However, Suv39h1-null tumors did not show any senescent response but still maintained the apoptotic response. Suv39h1-null tumors with altered apoptotic response do not respond to therapy.

These results suggest that drug efficacy and tumor formation are not fully independent processes. Until recently, tumor formation and the development of drug resistance were thought to be independent processes. Mutations in factors that regulate tumor-suppressive fail-safe mechanisms, such as apoptosis and senescence, allow transformation. Chemotherapeutic compounds activate a separate set of effector pathways that eliminate malignant clones. Mutations in factors that are involved in these separate pathways inhibit the effect of chemotherapy to induce the effector programs to eliminate the tumors. Consequently, defects in antineoplastic fail-safe programs, even if required to allow for tumor formation, do not interfere with the effector program initiated by therapeutic agents. Nevertheless, preclinical data have provided evidence that key regulators, such as p53, participate in tumor prevention and drug action and that tumor mutations acquired during tumor development also confer chemoresistance [95]. Therefore, the "joint model" [96] proposes a functional overlap between the fail-safe and therapeutic effector programs, such that some of the mutations that allowed transformation can also confer chemoresistance by disabling drug effector programs.

The *in vitro* observation that DNA-damaging agents not only promote apoptosis but also induce cellular senescence [97, 98] indicates that genes that control senescence might

also determine treatment outcome. Using a MYC-driven mouse lymphoma model, p53 and p16INK4A were recently shown to control drug-induced senescence *in vivo* [94]. Drug-treated lymphomas with apoptotic defects were forced into senescence, and tumors that resumed growth frequently displayed defects in either p53 or p16INK4A. Importantly, drug-induced senescence was shown to contribute to long-term host survival after cancer therapy, as mice bearing lymphomas that were unable to enter senescence in response to therapy had shorter survival times. Notably, drug-inducible senescence is not a phenomenon that is restricted to a mouse lymphoma model, as tissue specimens taken from human breast tumors after chemotherapy also displayed typical features of cellular senescence [98].

Depending on the initiating oncogene, transformation relies on fail-safe defects that disrupt either apoptosis or senescence. There are a number of reports that drug-inducible senescence could become detectable only after apoptosis has been disabled [99]. It is conceivable that senescence occurs with much slower kinetics, serving as a "backup" fail-safe program in case the first-line response is corrupted. This is supported by sequential disruption of apoptosis- and senescence-controlling genes during tumor formation and subsequent therapy reported in human cancers [100, 101].

4. Senescence-Based Therapy

Different chemical agents can induce cellular senescence epigenetically. Treatment of primary cells with H_2O_2 or butyrate provokes early senescence [102]. Similar results were obtained after treatment with high doses of radiation and other damaging agents [102]. Interestingly, the treatment of different tumor cell lines with different chemotherapeutic agents, radiation, or differentiating agents induces irreversible growth arrest, with enzymatic and morphologic changes resembling those occurring during replicative senescence. Moderate doses of doxorubicine induced a senescent phenotype in 11 out of 14 tumor cell lines analysed, independently of p53 status [103]. A similar effect has been observed in lines from human tumors treated with cisplatin [104], hydroxyurea [105], and bromodeoxyuridine [106]. In mammary carcinoma cell lines treated *in vitro* and *in vivo* with differentiating agents, terminal proliferative arrest with minimal toxicity for normal cells has been observed [107].

The propensity of tumor cells to undergo senescence in response to different kinds of damage induced by commonly used chemotherapeutic treatments was compared on cell lines from different tumor origins [66]. Under equitoxic doses, the strongest induction of a senescent phenotype was observed with DNA-interacting agents (doxorubicin, aphidicolin, and cisplatin), and the weakest effect was observed with microtubule-targeting drugs (Taxol and vincristine). A medium response was observed with ionising radiation, cytarabine, and etoposide. Induction of senescence by the drugs was dose-dependent and correlated with the growth arrest observed in the cultures [102, 105–107]. The drug-induced senescent phenotype in tumor cells was not associated with telomere shortening and was not prevented by the expression of telomerase [108].

Drug-induced senescent phenotypes have been confirmed *in vivo* ([94] and references therein). A study from Poele et al. [98] revealed the correlation between chemotherapeutic treatment in clinical cancer and the senescence response. In frozen samples from breast tumors treated by neoadjuvant chemotherapy (cyclophosphamide, doxorubicin, and 5-fluoracyl), senescent markers were detected in 41% of samples from treated tumors. Normal tissue was negative, suggesting that the chemotherapy-induced senescence was a specific response of tumor cells. Interestingly, senescence response was associated with wild-type p53 and the increased expression of p16. Similarly, in treatment-induced senescence, murine Eμ-myc lymphoma response required wild-type p53 and p16 [94].

The Chk2 kinase is a tumor suppressor and key component of the DNA damage checkpoint response that encompasses cell-cycle arrest, apoptosis, and DNA repair. It has also been shown to have a role in replicative senescence resulting from dysfunctional telomeres. Some of these functions are at least partially exerted through activation of the p53 transcription factor. High-level expression of Chk2 in cells with wild-type p53 led to arrested proliferation with senescent features [109]. These were accompanied by p21 induction, consistent with p53 activation. However, Chk2-dependent senescence and p21 transcriptional induction also occurred in p53-defective cells. Small interfering RNA-mediated knockdown of p21 in p53-defective cells expressing Chk2 resulted in a decrease in senescent cells. DNA-damage response is also induced by cytokines, such as interferons. Sustained treatment with interferon triggers a p53-dependent senescence program. Interferon-treated cells accumulated gamma-H2AX foci and phosphorylated forms of ATM and CHK2. The DNA-damage-signalling pathway was activated by an increase in reactive oxygen species (ROS) induced by interferon and was inhibited by the antioxidant N-acetyl cysteine. RNA interference against ATM inhibited p53 activity and senescence in response to betainterferon [110]. It seems that p53 activation is the primary response to DNA damage, but its absence does not preclude a response with a senescent phenotype.

Comparable to p53, which functions as a fail-safe mediator of DNA-damage response, the p16 inhibitor has been implicated in both response to DNA-damage and control of stress-induced senescence. Although the molecular mechanism used by p16 to control not only temporary but permanent cell-cycle arrest is unclear, p16 responds to DNA-damage in a delayed manner and appears to be indispensable for the maintenance of cellular senescence [94, 98]. A synthetic inhibitor of CDK4, possibly mimicking the role of p16, produced a DNA-damage-independent form of senescence in cells lacking p16 expression and inhibited the growth of tumors in mice. Use of siRNAs to inactivate the papilomavirus oncoproteins E6 and E7, which deregulate p53 and pRb, restored cellular senescence in cervical cancer cells. Introduction of E2 protein, a negative regulator of E6 and E7, induced senescence in almost all cervical carcinoma cells tested. The effect of E2 was not accompanied by

telomere shortening, nor was it prevented by telomerase expression. Induction of senescence by E2 was associated with p53 stabilisation and strong induction of p21, and it was prevented by using p21 antisenses [111].

Many observations indicate that p53, p21, and p16, which regulate cellular senescence, play an important role in treatment-induced senescence of tumor cells. Since these genes are commonly lost in human tumors, we can expect that most human tumors do not respond by undergoing senescence. However, this is not the case. Chemotherapeutic drugs induced senescence in p53- and p16-defective tumor cell lines [107]. *in vivo*, 20% of tumors undergoing senescence after treatment showed p53 mutations [98]. We have been able to induce senescence with several chemotherapeutic drugs in p53-null cells independently of p16 (Moneo and Carnero, unpublished). We have found that the induced senescence correlated with p53-independent p21 induction. Moreover, knockout of p53 or p21 in HCT116 cells decreased but did not abolish cellular senescence. Hence, p16, p53, and p21 might acts as positive regulators but are not absolutely required for this response. Other related tumor suppressors, such as p63 or p73, could be involved, and their role in drug-induced senescence should be explored.

Treatment with 6-anilino-5,8-quinoline quinone, a previously described inhibitor of guanylate cyclase, induced cellular senescence [112]. Microarray analysis revealed that this compound induced the Cdk inhibitor p21WAF1 in a p53-independent manner. Furthermore, p21, though not p53, was required for inhibition of proliferation by the drug. The lack of p53 involvement suggests that this compound acts independently of DNA-damage induction. Growth inhibition was also observed in malignant melanoma and breast cancer cell lines. Functional inactivation of the retinoblastoma tumor-suppressor protein converted 6-anilino-5,8quinolinequinone-induced growth arrest into apoptosis. Tumor cell senescence was also found to be induced by TGFb and by differentiating agents including retinoids. The induction of senescence has been analyzed in more detail with derivatives of vitamin A, which regulate cell growth and differentiation through their effects on gene expression [113].

A prominent feature of immortal cells is a resistance to oxidative stress. By contrast, primary cells undergo senescence when grown for extended periods in tissue culture or exposed to agents that increase production of reactive oxygen species. It has been also found that enhanced glycolysis enables primary mouse cells to avoid senescence by protecting them from oxidative damage, and that immortal ES cells have intrinsically high levels of glycolysis [49]. siRNA downregulation of PGM, an enzyme regulating glycolytic flux, triggers senescent phenotype recovery in tumor cells. Therefore, regulation of glycolysis and/or ROS production might be interesting approaches to the induction of senescence in tumors.

5. Telomerase Inhibitors

Restoration of the limited replicative potential in tumors as an anticancer therapy has been widely examined through the targeting of telomerase activity. Early studies indicated that telomerase activity is absent in somatic tissues and present in most cancers [114]. It was, therefore, reasonable to suggest that inhibition of telomerase activity, with a consequent shortening of telomeres and arrest of cell growth, might be an effective treatment of cancer.

Several different approaches to telomerase inhibition have been adopted to prevent the multiplication of neoplastic cells in culture. These have included treatment of the cells with the alkaloid berberine, transfection with an antisense vector for the human telomerase RNA component, introduction of a catalytically inactive, dominant-negative mutant of human telomerase reverse transcriptase, and low-level expression of a mutant-template telomerase RNA. All of the treatments inhibit the multiplication of neoplastic cells in culture, and those tested also inhibit tumor formation in mice. It should, however, be noted that the transfection of neoplastic cells with telomerase-inhibitory vectors was accomplished either in culture before their inoculation into mice or (in the case of the antisense RNA) through daily injections into the growing tumors for 7–14 days. No attempt was made to assess the long-term systemic injection of vectors into mice carrying the tumors, leaving the matter of effects on normal cell function yet to be investigated. Telomere shortening has been observed in the treated tumor cells and correlates with inhibition of their proliferation [115]. The expression of threshold levels of mutant-template telomerase RNA decreases cell viability despite the retention of endogenous wild-type telomerase RNA, wild-type telomerase activity, and unaltered stable telomere lengths.

One reported advantage of telomerase inhibition as a cancer chemotherapy was that it was not expected to induce cancer in normal cells, as telomerase activity is closely associated with advanced tumors [114]. Knockout of the gene for the RNA component of telomerase in mice does not, however, prevent either tumor formation or neoplastic transformation of cells cultured from such mice [116, 117]. The incidence of spontaneous malignancies is even higher than that of normal mice [117]. A similarly increased risk of cancer is found in individuals with the inherited syndrome dyskeratosis congenita (DKC) that is caused by a mutation in one of the components of telomerase, such that individuals with DKC are deficient for telomerase activity [118]. This increased incidence of cancer is presumably a result of end-to-end fusion of chromosomes destabilized by inadequate capping [119]. There is, therefore, the distinct possibility that systemically introduced inhibition of telomerase in cancer chemotherapy would increase the frequency of chromosome aberration and the risk of secondary cancers in normal tissue, particularly when p53 mutations already exist [120].

The situation became more complicated when it was found that telomerase activity is present in stem cells and dividing transit cells of renewing tissues, and even when cell division is induced in tissues conventionally regarded as quiescent. Thus, it seems likely that all tissues with cells able to divide have either ongoing or potential telomerase activity with a capacity for telomere maintenance during cell division.

Treatment of cancer by telomerase inhibition is still considered potentially valid for several reasons that might mitigate side effects on normal tissues [121]. One reason is that telomeres are longer in normal tissues than in most cancers, and treatment of tumors can be designed to end before telomere depletion in normal tissues [120]. However, further studies with this approach must be carried out to protect renewing tissues, such as intestine, epidermis, and hematopoietic tissue, in which stem cells and transit cells are constantly dividing at a high rate.

It is expected that telomerase inhibitors will be developed that have far fewer side effects than many of the cancer chemotherapeutic agents that are currently available. Individuals with DKC show features that include abnormalities of the skin and nails and eventual failure of proliferation in the bone marrow, which indicates that telomerase is required for normal proliferative capacity in these somatic tissues. Despite this telomerase deficiency, onset of pancytopaenia in these individuals does not occur until a median age of 10 years, which indicates that it might be relatively safe to administer telomerase inhibitors continuously for several years.

Telomerase inhibitors will not be useful, however, for the minority of tumors that use ALT. In addition, in telomerase-positive tumors it can be predicted that effective telomerase inhibitors will exert an extremely strong selection pressure for the emergence of resistant cells that use the ALT mechanism. Activation of ALT was not observed in cell-culture experiments in which telomerase-positive cell lines were treated with small-molecule inhibitors of telomerase or dominant-negative TERT mutants [122], indicating that it is not a high-frequency event. This might be a problem, however, in clinically significant tumors containing as many as 10^{12} cells. Development of ALT inhibitors may, therefore, be necessary. For tumors that use both telomere maintenance mechanisms, treatment might need to be initiated with a combination of telomerase and ALT inhibitors. Both telomerase and ALT must access the telomere, but how this might be achieved is at present unknown. A further possibility could be to identify molecular targets for simultaneous inhibition of both telomere maintenance mechanisms, since proteins involved in telomerase-based and ALT-mediated events may overlap.

6. Concluding Remarks

The concept of senescence as a barrier to tumorigenesis, either by natural replicative limits or as stress-induced senescence leads to a critical evaluation of the benefits that can be achieved for cancer diagnosis and therapy. It is accepted that neoplastic cells can be forced to undergo senescence by genetic manipulations and by epigenetic factors, including anticancer drugs, radiation, and differentiating agents. These senescent features can be imposed even in the absence of the two functional effector pathways, p53 and pRb. This lead to speculate the possible benefits of inducing an unspecific senescence program to stop tumor growth. This might be of value added to surgery or radiation; however, possible escape from a yet uncontrolled senescent phenotype and the unknown effect *in vivo* of senescent stromal cells might hamper these efforts. A more controlled induction of senescence through the knowledge of pathways involved and targeting specific targets might rend a less profitable but more valued effort. The use of tools such as oncolytic viruses driven by telomerase promoters might also work better than direct inhibition of the protein. However, it is too early and more research is needed in the basic understanding of the molecular mechanisms driving the senescence processes before embarking patients in such therapy.

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