



SASPECTS of Radiation Induced Senescence

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Abstract

Cellular senescence is a complex process of irreversible growth arrest which contributes to several physiological and pathological conditions. A variety of intrinsic or extrinsic stress signals, including those induced by Radiation Therapy (RT) also in combination with chemotherapeutic drugs, are able to cause stress-induced premature senescence in cancer cells (known as Therapy-Induced Senescence, TIS). Although TIS may inhibit tumor growth following RT, a number of outstanding issues about long-term tumor control and recurrence still remain unclear. The aim of this review is to describe the principal aspects of radiation induced senescence, the molecular pathways involved and the Senescence-Associated Secretory Phenotype (SASP). Finally, we report some therapeutic applications with the use of targeted molecules in TIS approaches.

Introduction

Cellular senescence represents a complex mechanism of irreversible cell-cycle arrest that contributes to several physiological and pathological processes of aging. Although senescent cells have irreversibly lost their capacity for cell division, they remain viable and metabolically active, a phenotype known as a Replicative Senescence (RS), associated with telomere erosion and increased sensitivity to cellular injury. RS may act as a defense mechanism to limit the expansion of older cells containing potentially dangerous mutations [1-4].

On the other hand, Stress-Induced Premature Senescence (SIPS) represents the other type of permanent growth arrest which rapidly occurs in response to a variety of intrinsic or extrinsic stress signals, including DNA damage due to Ionizing Radiation (IR), cytotoxic DNA damaging agents, oxidative stress and oncogenic activation. This process is also known as premature or accelerated senescence [5-7]. The RAS oncogene promotes premature senescence in addition to the accumulation of p53 or p16 [8]. Loss of tumor suppressors also leads to cellular senescence, both *in vitro* and *in vivo* [9-11]. In line with this evidence, it has been suggested that SIPS functions as a barrier to tumor growth and recurrence. Indeed, senescence is now recognized as an important tumor suppressive mechanism which prevents cancer progression *in vivo* [6,12]. Recent data suggest that senescence may play a significant role in the primary mechanism underlying loss of the self-renewal capacity in IR- or drug-treated cancer cells. Molecular mechanisms of radiation-induced cellular injury are influenced by several factors, such as radiation dosage and dose rate, cell type, and growth rate [12,13]. In contrast to this, much evidence suggests that senescence is also associated with the disruption of the tissue microenvironment and the development of a pro-oncogenic environment, principally via the secretion of senescence associated pro-inflammatory factors [14,15]. Several studies indicate that radiation therapy (RT) and chemotherapeutic drugs induce SIPS in cancer cells, also defined as therapy-induced senescence (TIS) [5-6,16]. Despite the tumor-suppressive potential of cellular senescence, senescent cancer cells secrete a characteristic profile of cytokines, growth factors and proteases, collectively termed the Senescence-Associated Secretory Phenotype (SASP). SASP influences tissue microenvironment and stimulates tumorigenesis, angiogenesis, Epithelial-to-Mesenchymal Transition (EMT) and metastasis *in vitro* and *in vivo*. Conversely, the anti-cancer function of SASP contributes to tumor cell clearance by the immune system [17-19].

Cells undergoing SIPS are morphologically indistinguishable from replicative senescent cells and exhibit many morphological changes, being large, flat, vacuolized and occasionally multinucleated.

In particular, when grown in culture, senescent cells display a specific and typical morphology with the macroscopic alterations of plasma membrane, nucleus and cytoskeleton, changes in cell-cell interactions, showing the so-called “fried egg” like appearance. The most widely used assay to test senescence is the histochemical detection of β -galactosidase activity, which is defined as

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senescence-associated β -galactosidase (SA- β -Gal) [20].

A collection of markers are generally known to define senescence both in cultured cells and in tissues. Other senescent features include, altered gene expression with increased expression of proteins such as p16, ARF, p53, p21, p15, p19, p38, p27 and hypophosphorylated RB [12,21].

Although TIS may indeed contribute to the early tumor growth inhibition following RT, a number of outstanding issues about the long-term perspectives of tumor control and recurrence still remain unclear. The aim of this review is to describe the principal aspects of radiation induced senescence, the molecular pathways involved and the SASP. Finally, we describe some therapeutic applications with the use of targeted molecules in TIS approaches.

Molecular Pathways Involved in the Radiation-Induced Senescence

Several studies provided important insights into the molecular pathways that lead to cellular senescence [3,22,23]. Significantly, it is known that the combined activity of two central proteins in cellular senescence, p53 and pRb, could determine whether cells undergo senescence or a cell death pathway [24,25]. Both of these tumor-suppressor proteins are activated in response to telomere shortening and various stresses including IR, which is the first step towards senescence [26,27]. After radiation exposure the initiating event in the cell, within minutes involves the recognition of DNA damage and the activation of the DNA Damage Response (DDR) pathway through the activation of many factors acting downstream of signaling cascades. The key mediators in this process include the Ser/Thr protein kinases Ataxia Telangiectasia Mutated (ATM) and Rad3-related (ATR), which phosphorylate important sensors and effectors of the DDR such as H2AX, 53BP1 and p53 [28-31]. This event induces the up-regulation of cyclin dependent kinase inhibitor p21 (CDKN1A), which in-turn inhibits the action of CDK2 kinase activity with the consequent blockage of the cell cycle [21,30]. p21 is a multifunctional protein which plays a key role in the p53 signaling pathway by activating transient cell cycle checkpoints. It also represents a senescence driver, because it positively regulates senescence genes and negatively regulates mitosis and apoptosis genes/proteins also through the inhibition of the Proliferating Cell Nuclear Antigen (PCNA) [16,32]. The upregulation of p21 during RS or SIPS via both p53-dependent and -independent mechanism is associated with telomere shortening and DNA damage. Overexpression of p21 can induce a senescence-like cell-cycle arrest, whereas depletion of p21 can delay senescence-associated arrest [15]. p21 also activates pRb through the inhibition of cyclin E/CDK2 [21]. During the activation of DDR, different cell cycle regulatory genes such as p38, p27 KIP1, p15 INK4B, pRb and Chk2 are able to trigger accelerated senescence. Due to their strong expression, p21, p16 and p53 are generally defined as molecular markers of accelerated senescence [6,33].

Regarding the tumor suppressor protein p53, also known as the genome guardian, it is activated in response to DNA Double Strand Breaks (DSB) following IR exposure by ATM/ATR kinase pathway to induce senescence p21-mediated [34-36]. An important target of p53 is p21, which antagonizes the activity of several cyclin-CDK complexes and can arrest cells in both G1 and G2 phases of the cell cycle. Indeed, many studies report that the p53/p21 pathway plays a crucial role in the regulation of cell cycle arrest and senescence [32,37].

On the other hand, when p53 is inactivated, as in cancer cells, the accumulation of DNA damage leads to the induction of senescence through p16 activity ROS-mediated, which plays an important role in the start and the maintenance of cellular senescence [38,39]. p16, a member of the INK4A family of CDK inhibitors, might play a tumor-suppressor role as p53 does. The former was first found to be increased in aged mammalian tissues and senescing fibroblasts and later was reported to be increased in oncogene-induced premature senescence [40]. p16 exerts its main effect on the cell cycle during the G1/S transition, by inhibiting the kinase activities of the cyclin D-dependent kinases CDK4 and CDK6, therefore prevents pRb phosphorylation [41].

The inactivation of pRb is often required to avoid senescence in human cells, showing that pRb is crucially involved in senescence pathways [42]. Cell-cycle progression is primarily regulated by CDK-mediated phosphorylation of pRb. Phosphorylated pRb releases the transcription factor E2F, allowing it to activate the transcription of genes for DNA synthesis and cell-cycle progression. So, the inactive pRb, in a hyperphosphorylated form, is required to enable entry into the cell cycle. In a senescent state, pRb protein is found in the active form, hypophosphorylated, which binds E2F protein and thereby blocks transcriptional activation of its targets, most of which are effectors of cell-cycle progression [42-44]. pRb phosphorylation is prevented by inhibition of CDK activity by negative CDK regulators such as p21 and p16 [42].

However, how the activation status of p53 and pRb determines whether a cell goes towards senescence is a complex and incompletely understood process.

Now it is widely demonstrated that the p53-p21 pathway is primarily responsible for senescence induced by telomere shortening or DNA damage, whereas the p16-pRb pathway is partly responsible for mediating SIPS [15].

A sequential involvement of p21 and p16 proteins was suggested, where p21 plays a crucial role at the early stage of senescence and p16 in the maintenance of cell-cycle arrest in senescent cells [4,26,32]. In addition to p16, the INK4a/ARF/INK4b locus encodes two other proteins, ARF and p15, which are important inducers of cellular senescence [45] like p16, ARF is induced in cellular senescence by various stimuli, including IR. It sequesters the MDM2 protein, an E3 ubiquitin ligase for p53 which promotes p53 degradation. Therefore, ARF positively regulates p53 levels and thereby enhances its activity [46,47]. In general, the DNA damage response, apoptotic and senescence pathways share common molecular mediators through p53 and p21. Depending on the cell type, the extent of stress, the dose administered inactivating the p53-p21 or p16-pRb pathway, separately or together, are necessary to avert senescence. In addition, the post-translational modifications that p53 undergoes may be important for cell fate [21]. Although there has been an increasing knowledge at a molecular level about senescent induced pathways, however what drives a cell towards senescence or apoptosis remains not quite clear.

Radiation and Senescence Associated Secretory Phenotype (SASP)

Several stress stimuli, such as those caused by IR induced DNA damages, can trigger senescence through the deregulation of the genes able to define cell fate decision. More precisely, in senescent cells several factors involved in cell cycle progression, survival and/or

cell death, DNA repair and inflammation processes, are deregulated [48].

Cellular senescence process essentially causes irreversible replicative arrest, apoptosis resistance, and frequently acquisition of a pro-inflammatory, tissue-destructive Senescence-Associated Secretory Phenotype (SASP) [49]. The SASP entails secretion of cytokines, chemokines, miRNA, growth factors, matrix metalloproteinases and other pro-inflammatory mediators. All these molecules are able to attract immune cells causing cell dysfunction and senescence induction in neighboring healthy cells, promoting tumor progression, and contributes to other age-related diseases such as cancer [50].

As described by Coppè et al. [51] inflammatory cytokines and chemokines, such as IL-6, IL-8, GRO- α , MCP-1 and GM-CSF were considered core features of the SASP. However, the composition of the SASP appears to vary depending on the cell type from which senescent cells originated and on the induction mechanism [49]. Indeed, SASP is not a fixed phenotype as different cell types display unique quantitative or qualitative features [51]. In their work, Coppè et al. [51] identified a hallmark of cellular senescence, the SASP, which confers cell non-autonomous paracrine functions, markedly exacerbated by gain of oncogenic RAS or the loss of p53 function. Particularly, they assayed by antibody arrays, the following SASP signature: IL-6, IL-8, GRO, GRO- α , GM-CSF, uPAR, TIMP2, ICAM-1, ENA-78, Osteoprotegerin, MCP-1, IGFBP2, PIGF, sTNF RI, HGF, MIF, IL-7, MIP3a, GITR, sgp130, TRAIL-R3, IL-6R, CCL-28, Amphiregulin, HCC-4, VEGF, GCP-2, IL-1 R4/ST2, IL-1a, IL-1b, IL-11, FAS/TNFRSF6, Oncostatin M, I-TAC, Axl, GDNF, bFGF, Acrp30, ICAM-3, BTC, IGFBP-6. The authors displayed how the SASP phenotype is highly complex and developed slowly over several days and only after DNA damage of sufficient magnitude to induce senescence, such DNA damages IR-induced. This phenotype can promote cellular behaviors associated with malignancy and they suggested that cells that acquire mutations, including those that inactivate p53 and/or activate RAS functions can be particularly malignant owing to the paracrine activities of the SASP [51].

While the cellular response to DNA damage initiates within minutes, literature data report that under *in vitro* conditions after IR exposure, cells develop a full SASP phenotype at least 5 days after senescence induction and also that cell growth arrest occurs within 24 hours [18,52-54]. Moreover, Rodier et al. [52] assayed 120 cytokines and other molecules secreted by senescent human fibroblast IR treated. Among these, the authors focused their attention on 16 factors senescence-associated, that were significantly modulated by irradiation (IL-6, GRO, IL-8, IGFBP-2, ICAM-1, sgp130, TRAIL R3, Osteoprotegerin, TIMP-2, TNF-R3, MCP-1, IGFBP-3, IL-1 R1, uPAR, LIGHT, IL-15). In addition, in ATM depleted cells, a reduced secretion of most of these factors and in particular IL-6 (of 50-fold) and IL-8 (of 10-fold), were measured highlighting that the ATM signaling does not regulate the entire SASP, but is required for a subset of SASP components, including the above mentioned major inflammatory cytokines (Figure 1).

Indeed, among the SASP factors, IL-6 and IL-8 are of particular interest also because they are deregulated after radiation exposure, as described by several authors. These cytokines initiate inflammatory responses, such as those associated with normal healing in damaged tissues, as well as many age-related pathologies including cancer [53]. Also high dose irradiation, increased IL-6 and IL-8 secretion

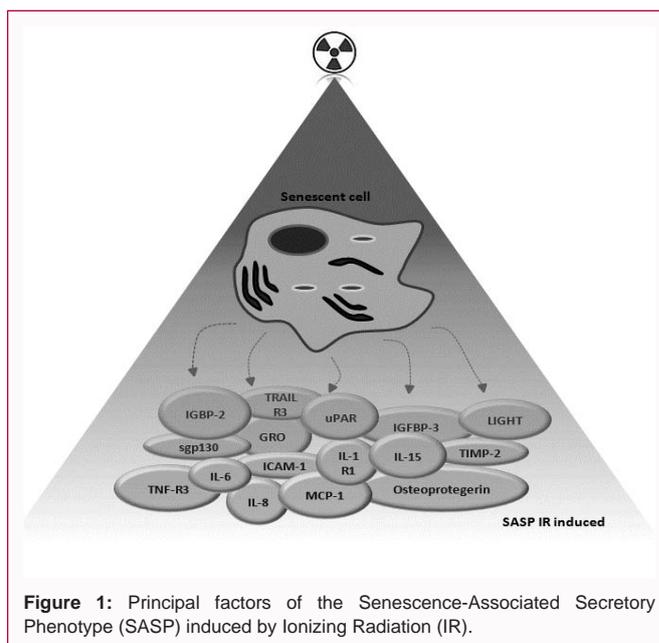


Figure 1: Principal factors of the Senescence-Associated Secretory Phenotype (SASP) induced by Ionizing Radiation (IR).

5 to 6 fold within 2-4 days post treatment. IL-6 is one of the most important pro-inflammatory cytokine and it has been reported to be increased in a variety of tumors, contributing to aggressive tumor growth and resistance to treatment [55]. IL-6 secretion has been shown to increase markedly after DNA damage and oncogene-induced senescence of mouse and human keratinocytes, melanocytes, monocytes, fibroblasts, and epithelial cells [51]. Interestingly, IL-6 was particularly important for the ability of senescent cells to promote cancer cell invasion. In addition, IL-8 was described [56] as a member of the CXC chemokines superfamily as well as a pro-angiogenic and a pro-inflammatory agent in a wide range of human malignancies. It resulted up-regulated in a dose dependent-manner, as described by Singh et al. [57] in human melanoma cells and by Meeren et al. [58] in endothelial cell lines, in tandem with IL-6 production.

On the other hand, IL-1 has often been described as a SASP molecule. In turn, its signaling pathway is upregulated by senescent cells and it represents the main key positive regulator of IL-6 and IL-8 expression by senescent human cells in culture. Moreover, IL-1 is so involved in breast cancer (BC) cell invasion acting as a chemo-attractant agent for MDA-MB-231 metastatic BC cells, induced by radiation [59]. Regarding this cytokine, Niu et al. [60] identified an autoregulatory feedback pathway involving IL1 α in induction of constitutive NF- κ B activation in pancreatic cancer cells. More precisely, this positive-feedback component mediated by IL-1 α , regulates its own synthesis in an autocrine receptor mediated, positive-feedback loop via NF- κ B transcription factor (TF), and intensifies IL-6 and IL-8 expression [60].

NF- κ B is known as the major inducer of SASP and a well-defined radiation-responsive transcription factor as also described recently by our group [61]. Its activity modulation increases cell sensitivity in several tumor cell lines and, also, NF- κ B down-regulation is probably required for p53-dependent apoptosis. NF- κ B is able to influence cell cycle regulation after irradiation and is supposed to be able to induce radioresistance by cell cycle regulation, alteration in apoptosis and changes in the ability to repair DNA damage. Disruption of NF- κ B aberrant survival signaling has recently become an important issue to study therapy of several chemoresistant/radioresistant cancers [62].

As described above, SASP molecules can induce senescence in many of the surrounding tumor cells in a paracrine manner. Indeed, SASP factors induce transformation processes in cells predisposed to proliferation [17]. In turn, literature data described the SASPs as able to cause both deleterious and beneficial effects in tissue, depending on the physiological context considered. More precisely, deleterious effects of senescent cells are mediated by “non-cell autonomous mechanisms” where the components of the secretory phenotype are also involved in tumor-promotion, involving invasion, angiogenesis, tumor growth, epithelial-mesenchymal transition activation and altered epithelial differentiation (through key SASPs factors such as IL-6, IL-8, MMP-3, etc.) [48,63]. Conversely, some anti-cancer functions of SASP molecules, were described as able to contribute to the cell clearance of the tumor. These beneficial effects of senescent cells are mediated by “cell-autonomous mechanisms” and could cause the growth at rest of senescent cells, activating the innate immune response, promoting tumor clearance and optimizing the repair of damaged tissues. Further investigations regarding this topic could clarify SASP controversial behaviors also in order to evaluate their role in targeted anticancer therapies in tandem with RT schedules.

Recently, we highlighted for the first time the cytokine profile secreted in a conditioned medium by the human MCF10A mammary epithelial cell line, MCF7 and MDA-MB-231 BC cell lines after single high radiation doses (9 Gy and 23 Gy). Our study has revealed that high IR doses may modify immunological factor secretion in time-dependent and cell line phenotype-dependent manners. Among the factors assayed, a discrete number of SASP proteins were included. In summary, we observed a senescent phenotype only in MCF7 and MDA-MB-231 BC cells following high dose irradiation [61,64,65]. The amount of SASP molecules secreted by the breast cell lines used in our experiment did not correlate with the induction of the SASP phenotype observed. Indeed, despite the low levels of IL-6, IL-8 and MCP-1, the MCF7 cells displayed a senescent phenotype. In contrast, MCF10A cells IR-treated, in which senescence processes were not observed, are described as able to secrete significantly higher levels of these cytokines compared with untreated control cells. Thus, we hypothesize that, despite the high amounts of SASP molecules that could be released in the tumor microenvironment, additional mechanisms are probably needed to induce the senescent process. These data are in line with those described by other researchers [66].

Overall, literature data indicate that the SASP is not an inevitable consequence of a senescence growth arrest and it is not tied to the senescence cell features as well as the SA-beta gal expression, but is a consequence of severe DNA damage (such those induced by IR) and not a permanent cell cycle arrest per se. In addition, no unique SASP signature could be proposed considering that its composition appears to vary depending on the cell type and on senescence signal induction [49]. Further works are needed in order to clarify this issue and the role of SASP molecules after IR exposure in specific cancer types.

Therapy-Induced Senescence and Radiation in Cancer Care

Cancer therapy has traditionally relied on cytotoxic treatment strategies with the assumption that complete cellular destruction of tumors optimizes the potential for patient survival. However, cancers often develop resistance to treatment and recur or progress to advanced primary and metastatic tumors. An alternative strategy is the induction of cytostasis, which permanently disables the proliferative capacity of cells without inducing cancer cell death.

A promising approach to induction of cytostasis in tumor cells is Therapy-Induced Senescence (TIS) also in combination with RT [67]. Indeed, literature data report that multimodality therapy approaches could be used in clinical practice with successful results. These are based on the principle that stand-alone, chemo or radio-therapeutic regimen are generally unable to control neoplastic lesions, whereas combining therapeutic agents with dissimilar action mechanisms potentially results in synergistic anti-neoplastic effects, as recently described by several authors [48,68,69]. Along this line, observations that some tumor cells can be forced into senescence by agents used in the management of human cancers are of clinical interest. Moreover many cancer cells possess intact, but silenced, signaling pathways that can be manipulated to stimulate senescence. This ability of tumor cells to undergo senescence in response to stress and damage has been noted with both radiation and chemotherapeutic drugs [5,67].

Considering the amount of drug or dose of radiation required to induce senescence is much lower than that necessary to kill cells, TIS could be able to enhance the efficacy of anticancer therapy, and at the same time could represent an effective way to treat cancer while lessening the side effects [70].

As reported by Ewald et al. [67] some drugs induce senescence in cancer cell lines *in vitro* and in tumor models *in vivo* with an action tumor type specific. For example, Cyclophosphamide+Doxorubicin+5-Fluorouracil therapy cause DNA damage senescence process in human breast tumors. Otherwise, Mitoxantrone and VO-OHPic drugs, causing DNA damage and PTEN signaling activation respectively, activate senescence in prostate xenograft tumors both *in vitro* and *in vivo* models.

As a therapeutic goal, TIS may provide an effective means to induce a persistent growth inhibitory response in both early- and late-stage cancers limiting toxicity. Moreover, small molecules targeting cellular responses to DNA damage have long been considered an attractive strategy to improve the effectiveness of genotoxic cancer therapy. For example, PARP inhibitors may have a significant impact in inducing senescence and have been proposed as a novel mechanism for sensitization to radiation [71]. As known, an early event in the Double Strand Breaks (DSB) response induced

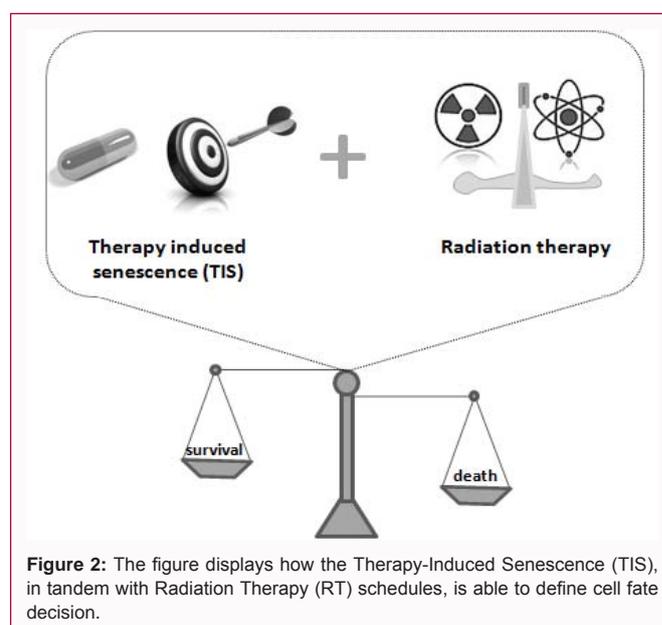


Figure 2: The figure displays how the Therapy-Induced Senescence (TIS), in tandem with Radiation Therapy (RT) schedules, is able to define cell fate decision.

by IR exposure, is the rapid recruitment and activation of PARP1, resulting in polymerization of poly (ADP ribose) (PAR) onto PARP1 itself, histones and other proteins at DSBs. Coincident with PARP1 recruitment, ATM-dependent phosphorylation of histone H2AX to form γ H2AX at DSBs promotes further chromatin modifications and assembly of proteins at specific sites, named IRIF (Ionizing Radiation-Induced Foci), involved in DNA damage signaling and DNA repair [72-74].

To block DSB repair in BC cells and tumors, Efimova et al. [71] targeted poly (ADP-ribose) polymerase with ABT-888 (veliparib), one of several PARP inhibitors currently used in clinical trials. More precisely, ABT-888 drug blocked IRIF induction and cell proliferation, driving the tumor cells towards accelerated senescence and suppressing tumor regrowth compared to IR alone. Moreover, the increase of senescence induction in BC cell, observed both *in vitro* and *in vivo*, allows the authors to propose this PARP inhibitor as a novel therapeutic strategy.

As discussed earlier, small molecule approaches are currently being developed to target DNA repair systems in an effort to enhance chemotherapy or radiation treatments also inducing senescence process. Some of these factors might modulate the activities of DNA helicases involved in the DNA damage response to radiation treatment, and have been recently proposed as prospective targets for anti-cancer therapy. In turn, Gupta and Brosh [75] suggest that the reduction of the WRN helicase by RNA interference therapy may lead cancer cells to senesce or undergo apoptosis. Several biological evidences, including results from RNA interference studies suggest that WRN protects cells from oxidative and other types of DNA damage. Innovative approaches to selectively introducing DNA damaging chemotherapy drugs or radiation to tumor tissues without adversely affecting normal cells is an important topic of investigation that needs to be clarified.

Other therapeutic approaches, currently under consideration, highlight the value of p53 as a target for pro-senescence therapy, showing that restoring p53 function, promotes tumor regression and tumor clearance via a senescence-inducing mechanism [76]. In this sense, novel p53-targeting compounds developed to date, include Ellipticine and PRIMA-1 (which restore wild-type activity to p53 mutants) and nutlins (which inhibit the binding of p53 to MDM2) [77,78]. For example, Luo et al. [79] demonstrate that IR-induced senescence in NSCLC cells was associated with p53 activation and increased p21 expression. In addition they observed that Nutlin-3a (a small molecule inhibitor of the MDM2/p53 interaction) enhances IR-induced tumor cell killing and senescence. Collectively, these findings suggest that pharmacological activation of the p53-p21 pathway can sensitize cancer cells to RT by promoting IR-induced senescence in irradiated cancer cells.

In summary, cancer therapy to date has focused on complete eradication of treatment-related complications. Regarding this topic, TIS approaches may be improved and employed in chronic tumors with the aim to allow patient survival and to maintain better quality of life.

Conclusion

Several stress stimuli, such as those caused by IR induced DNA damages, can trigger senescence through the deregulation of the genes able to define cell fate decision (Figure 2). Increasing data support an approach that incorporates the induction of senescence

in cancer therapy also in tandem with RT, a topic that needs to be explored further. Here, we have provided an overview of molecular pathways and SASP involved in therapy-induced senescence (TIS). This information represents an useful tool in therapeutically targeted intervention in the TIS contest.

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