Tumor cell escape from therapy-induced senescence as a model of disease recurrence after dormancy

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Introduction

Tumor cells induced into a state of senescence upon exposure to cancer chemotherapeutic drugs or radiation can recover self-renewal capacity, i.e. undergo “proliferative recovery”. We postulate that senescence in residual tumor cells that have survived after the bulk of the tumor cell population has been eliminated by therapy may represent one form of tumor dormancy. Consequently, senescence may represent an avenue whereby tumor cells evade the direct cytotoxic impact of therapy by entering a prolonged state of growth arrest, whereupon rare proliferating tumor cells will re-emerge months or years after patients have been cured of the primary disease.

Escape from Therapy-Induced Senescence (TIS).

Proliferating cells that undergo successive duplications will eventually cease to divide as they enter a state of senescence. It has been established that senescent cells (primarily aging fibroblasts) can persist in an arrested state indefinitely, indicating that senescence represents a highly stable form of growth arrest. However, while tumor cells maintain the potential to undergo an accelerated (or premature) form of senescence in response to severe genotoxic stress, hormonal deprivation or cell cycle inhibition, the possibility remains that the arrest, while durable and prolonged, may not be permanent for all cells in the population.

Early studies from our laboratory demonstrated that clinically relevant concentrations of Adriamycin (doxorubicin) induce senescence in (p53 wt, $p16^{INK4a}$ null) MCF-7 breast tumor cells from which a small population of cells evades the durable growth arrest, potentially developing resistance to senescence-inducing therapies (1). Similarly, the Wu group at the University of Washington established that H1299 lung cancer cells (deficient in $p16^{INK4a}$ as well as null in p53) can evade senescence induced by camptothecin to recover proliferative capacity (2). Both studies established an association between recovery from Therapy-Induced Senescence (TIS) with the ability of tumor cell to express the cyclin-dependent kinase, cdc2. In the Wu et al study, the frequency of escape/recovery was 1 in 10^6 cells, suggesting that (*i*) the stability of the senescent growth arrest is the more predominant phenotype, and that (*ii*) escape of tumor cells from senescence is a relatively rare event. Escape from senescence has since been reported by a number of investigators including the Sikora laboratory (studies on the potential contribution of chemotherapy-induced senescent tumor cells to cancer relapse) and the Bernards group (seminal studies on the reversibility of senescence, immortalization and escape from Oncogene-Induced Senescence, OIS).

Despite the accumulation of data in support of the premise that some tumor cells expressing classical hallmarks of senescence may not be terminally arrested, investigators have generally been conservative in their conclusions, often using terminology such as "senescence-like" or "pseudo-senescence" in order to distinguish the tumor cells that recovered proliferative capacity from tumor cells that appeared to be in a permanently arrested state. One caveat to the conclusion that tumor cells can re-emerge from senescence or a senescence-like state is that studies have generally involved cells in mass culture, where the origin of the replicating cells could not be unequivocally attributed to the senescent population. In an effort to circumvent this limitation, we have recently used a flow cytometric approach to enrich and select for tumor cells induced into senescence by chemotherapy based on Senescence-Associated β-galactosidase (SA-β-gal) activity and cellular size (3). Using live cell imaging and interferometry (3) confirmed what has been suggested by an extensive body of literature over the past decade and a half, specifically that senescent cells can undergo spontaneous cell division.

Features of Cells that Escape from Senescence.

A number of characteristics that have frequently been associated with tumor cells that escape from therapy induced senescence include polyploidy, stemness and aggressiveness.

Polyploidy. Polyploidy, a common feature of senescent cells, is consistent with the potential to generate daughter cells, and was evident in the camptothecin-induced senescent H1299 cell population described by Wu et al (2). Approximately 40% of these polyploid senescent tumor cells were able to take up EdU several days after senescence induction, suggesting that the cells retained the capacity to undergo DNA replication (4). Large polyploid cells induced into senescence by camptothecin that were sorted based on nuclear content were observed to generate colonies 7 –

10 days after seeding, findings that were supported by live cell imaging of cells escaping from senescence. Several studies by the Rajaraman group that included time-lapse microscopy suggested that senescent tumor cells replicate by budding (or neosis) from the polyploid state. Multiple follow up studies by other groups support the contention that polyploidy is a prerequisite for cells to re-emerge from senescence (5).

Stemness and Aggressiveness. Sabisz and Sklandanowski determined that about 1% of cells undergoing therapy-induced senescence expressed markers of cancer cell stemness (CD34 and CD117). Similarly, Was et al. presented evidence of cells undergoing therapy-induced senescence exhibiting stem cell features, specifically CD24+ (about 1.5% of cells) and NANOG in the treated cell population (6). Other laboratories have provided evidence that multiple breast cancer cell lines (MCF-7, MDA MB231, and T47D) and primary tumors that escaped from TIS could be derived from the cancer stem cell population. A recent report by the Schmitt group also focused on the relationship(s) between senescence regulatory pathways and cell "stemness" (7). This work demonstrated that a single exposure of Eμ-Myc – Bcl2-overexpressing lymphoma cells to Adriamycin (0.05 μg ml^{-1}) resulted in a robust senescence induction (marked by over 80% SA-β-gal staining) and an accompanying increased expression of stem cell related genes as well as elevated activity of aldehyde dehydrogenase (ALDH) and ATP-binding cassette (ABC), both associated with stem cell function (7). Importantly, enhanced stemness was not detected in cells exposed to the same concentration of Adriamycin *but which failed to undergo senescence* due to the absence of Suv39h1, the enzyme responsible for the senescence-associated epigenetic signature, H3K9Me3. Using an inducible expression model for p53 and Suv39h1, the authors were able to deactivate these pro-senescence proteins and facilitate resumption of S phase activity after Adriamycin-induced senescence (marked by EdU staining and gradual decline in SA-β-gal

activity). These authors argued that "senescence is, in principle, a reversible condition, which becomes evident when essential senescence maintenance genes are no longer expressed". Cells that escaped TIS and acquired stem cell properties were also more aggressive, forming rapidly growing colonies *in vitro* and more malignant tumors when implanted *in vivo* (in this study, in immunocompetent mice). Moreover, studies performed in melanoma, breast, colon and neuroblastoma cells have shown that Adriamycin-induced senescence was accompanied by elevation of Wnt ligands associated with the Epithelial-Mesenchymal Transition (EMT) and migratory properties.

Senescence, Tumor Dormancy and Disease Recurrence.

It is not difficult to imagine that the majority of tumor cells exposed to cytotoxic therapies undergo cell death and generate a robust immune response, leaving small and undetectable subpopulations of residual dormant cells. While having a pivotal impact on the natural history of cancers, our understanding of the mechanisms of dormancy and how tumor cells escape from dormancy are, unfortunately, quite limited (8).

Although it has been suggested that dormant tumor cells are in a quiescent state, senescence rather than quiescence would be more likely to reflect tumor dormancy since quiescence is a short-lived process from which tumor cells escape once DNA has been repaired or favorable conditions for growth recovery have been restored. In contrast, senescent cells, by definition, do not respond to growth promoting stimuli. Furthermore, quiescent cells will not have undergone the morphological and genetic modifications associated with TIS. In this context, it is noteworthy that common cancer mutations involve key proteins associated with the regulation of senescence such as p53, $p16^{INK4a}$ and Rb, all of which are likely to be relevant to the escape from senescence. Finally, the aggressive

nature of recurrent disease is also reflective of the aggressive phenotypes that evolve following escape from senescence, as demonstrated recently by the Schmitt group in lymphoma models (7).

It can be further argued that mechanisms that facilitate the recovery of dormant cells would be more closely associated with senescence rather than quiescence. For example, dynamic alterations of the microenvironment and restoration of the blood supply, critical events contributing to the capacity of dormant tumor cells to recover, are heavily influenced by mediators such as matrix metalloproteinases and angiogenic promoters such as VEGF, both classic components of the senescence-associated secretory phenotype. Moreover, senescent cells not only interact with, but also modulate the immune system, thus possibly contributing to evasion of immunosurveillance, which is a necessary step for cancer recurrence.

While senescence could reflect one form of tumor dormancy, we do not presume that senescence is the *only* form; in fact, senescence may be *only one among a number of forms* of tumor dormancy, such as those represented by circulating tumor cells or cells in the perivascular niche. We postulate that a subpopulation of cancer cells that escape cell death following repeated cycles of cytotoxic therapy can undergo senescence and persist for weeks, months or years, and, under the appropriate conditions, ultimately contribute to disease recurrence. These dormant senescent cells generate an array of soluble and non-soluble molecules that gradually alter the surrounding tissue and slowly promote angiogenesis. Eventually, a few senescent cells that manage to escape immunosurveillance and undergo proliferative recovery would be competent to exploit the changes in their extracellular environment and the restored blood supply to re-initiate tumor formation.

Strategies to Eliminate Senescent Tumor Cells in Efforts to Delay or Prevent Disease Recurrence.

It is well accepted that the senescence associated secretory phenotype has tumor promoting properties, although the bulk of the scientific literature on this phenomenon relates to senescence induced in normal (fibroblast) cells. Furthermore, senescence, while not formally a form of resistance, may provide a mechanism for evasion of the cytotoxic impact of various therapies by allowing the prolonged survival of tumor cells with the inherent potential to re-emerge from the growth-arrested state and generate progeny that retain self-renewal properties. This premise is supported by recent work by the Campisi group that demonstrated that senescent cells contribute to cancer relapse (9). Consequently, if senescence is a form of tumor dormancy, then tumor cells that escape from senescence and survive (fortunately a rare event) will, in some cases, be the source of recurrent disease, and their elimination would provide a survival advantage for cancer patients.

Recent work, largely in the field of aging, but also in cancer, has identified senolytic agents, drugs whose cytotoxicity has a high degree of specificity against senescent cells. Among these are drugs such as navitoclax that suppress anti-apoptotic proteins, Hsp90 inhibitors and histone deacetylase inhibitors (10). It is suggested that these drugs could be used as "clearing" agents to eliminate residual senescent tumor cells surviving after chemotherapy or radiation, with the goal of delaying or ideally preventing disease recurrence. Drug efficacy could be maximized, and patient toxicity reduced by treatment with senolytics after the standard therapy has been completed.

Summary and Conclusions

It is important to note that we do not propose that senescence is actually reversible in the manner of a reversible chemical reaction. Instead, we propose that while the bulk of a "senescent" population is likely to be indefinitely arrested, there will be subpopulation(s) of cells capable of recovering self-renewal capacity, particularly in the context of TIS in tumor cells that inherently

harbor genetic derangements (3,7). The results of our own recent studies confirm that only a subpopulation of tumor cells is capable of escaping the growth arrest (3), which likely reflects the heterogeneity of the senescent phenotype that has been established by the Demaria group. Furthermore, we show that both lung cancer and breast cancer cells selected for senescence can form tumors when implanted in mice

Certain caveats to these findings must be acknowledged. Many of the senescence markers, such as the induction of p21, expression of the cytokines and chemokines associated with the SASP and even the classical SA-β-gal enzyme, are not exclusively specific to senescence. Furthermore, escape of tumor cells induced into senescence by chemotherapy or radiation in tumor bearing animals remains to be conclusively demonstrated.

Despite these reservations, the possibility that therapy-induced senescence results in the survival of a residual tumor cell population from which cells with self-renewal capacity can emerge suggests that senescent tumor cells may represent one form of cancer dormancy. Given the tumor promoting properties of the senescence-associated secretory phenotype coupled with the potential for regrowth and disease recurrence, efforts to eliminate this small but significant tumor population may represent a clinically relevant strategy for prolonging the life of cancer patients.

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