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· Gastrointestinal Cancer Treatment and Cellular Senescence

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Advanced gastrointestinal (GI) cancers treated with chemotherapy and radiation exhibit disappointingly low 5–30% complete response rates. The majority of tumors are limited to only partial responses, and surgery continues to be the mainstay treatment for most GI cancers despite poor overall survival rates. For example, chemoradiotherapy for advanced rectal cancer often results in detectable tumor volume reduction following early treatment, but is often succeeded by tumor progression despite additional therapy.

Cellular senescence has long been described for primary tissues grown under culture conditions. This "aging"-associated physiological arrest has been shown to limit the replicative lifespan of cells in response to gradual erosion of the telomere. Replicative cellular senescence can also result from oncogenic signals. For example, *ras*-induced senescence has been increasingly recognized as a tumor suppression mechanism in carcinogenesis and accounts for the proliferative arrest observed in many benign tumors. Malignant tumors are characterized by their ability to bypass replicative senescence, but can be induced into a state of cell cycle arrest following multimodal therapy, termed **therapy-induced senescence.**

Mounting evidence now suggests that therapy-induced senescence is a prominent solid tumor response to therapy, and it most reasonably accounts for early provisional treatment responses by prolonged cell cycle arrest. However, certain senescent cancer cells are capable of escaping senescence and resuming cell division, leading to eventual tumor progression. Therapy-induced senescence is predicted to be a telomere-independent process since telomere erosion is not expected to occur given the lack of cell doubling.

Surprisingly, we discovered that massive telomere loss does indeed occur in senescent cancer cells following chemotherapy. Furthermore, we have also found that senescent cells that escape replicative arrest are able to partially recover their telomere loss. Based upon these observations, we propose the hypothesis that **modulation of telomerase activity regulates escape from therapy-induced senescence in colorectal cancer.** Therapy-induced cellular senescence is a novel paradigm of cancer therapy response that has recently been validated *in vivo* through work done by our laboratories and others. We aim to define the role of telomerase in regulating therapy-induced senescence and senescence escape in colorectal cancer.

This project is a key component of an ongoing effort to elucidate molecular mechanisms of therapy-induced senescence and identify markers that can reliably predict treatment response and reveal key checkpoints that could be targeted to block senescence escape and enhance clinical treatment responses in patients diagnosed with locally advanced or metastatic GI cancers.

Current Understanding of Cellular Senescence and Cancer

A requirement for the malignant transformation of tumor cells capable of infinite cell division is bypass of the physiological program of cellular senescence that limits the replicative lifespan of normal cells. In the lifespan of somatic cells, progressive loss of telomere length occurs with each successive cell division, reaching a critical shortening which has been shown to trigger a p53-mediated replicative arrest signal. Human diploid fibroblasts enter a state of replicative arrest after 60–80 population doublings, which has been termed *Hayflick's limit* or mortality stage 1 (M1).

The relatively slow onset of solid tumor responses to chemotherapy and the lack of a consistent correlation with apoptosis in numerous studies suggest that other pathways regulating cell death may predominate.

It has been well established that the replicative lifespan of these cells can be extended beyond this limit through inactivation of the p53 and other pathways. Immortalized cancer cells appear to bypass the M1 checkpoint through mutational inactivation or oncogenic viral targeting of these pathways. After bypassing M1 restriction, telomeres progressively shorten with each successive cell division until a critical second restriction point is encountered, termed M2.

How, then, do tumor cells keep dividing? Encountering this barrier provokes either of two mechanisms to avoid reaching a critical threshold of telomere loss that will result in cell death. One mechanism tumor cells utilize to preserve telomere length is over-expression of telomerase which catalyzes telomere repair, and the other involves activation of an alternative telomere-lengthening mechanism. Both of these mechanisms enable tumor cells to bypass the M2 restriction and thereby regain capacity for unlimited cell division and immortalization.

Despite having bypassed both M1 and M2 stages, cancer cells can still undergo terminal growth arrest in response to anti-cancer drugs or ionizing radiation. This telomere-independent response, termed therapy-induced senescence, is believed to overlap with the physiologic cellular senescence program. Senescent cells in replicative arrest are characterized by morphologic alterations, including enlarged and flattened cell shape with increased cytoplasmic granularity, nuclear polyploidy, and characteristic expression of the senescence marker, b-galactosidase (SA-b-gal; Figure 1). We have shown that therapy-induced senescence can be reliably induced in various tumor cell lines following exposure to a variety of chemotherapeutic agents, which suggests that therapy-induced senescence represents a primordial cellular stress response of epithelial cancer cells to anti-cancer drugs.

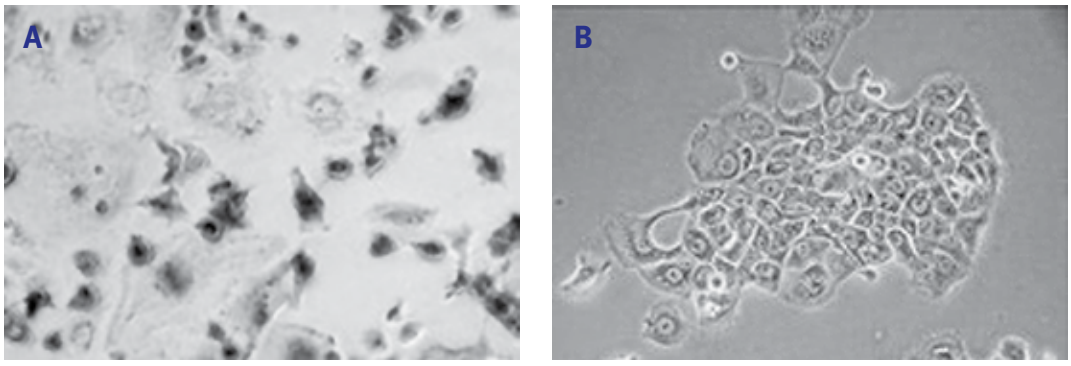

Figure 1. Light microscopy of blue-appearing tumor cells in therapy-induced senescence stained with X-gal. (A) H1299 lung carcinoma cells. (B) Bx-PC3 pancreatic carcinoma cells.

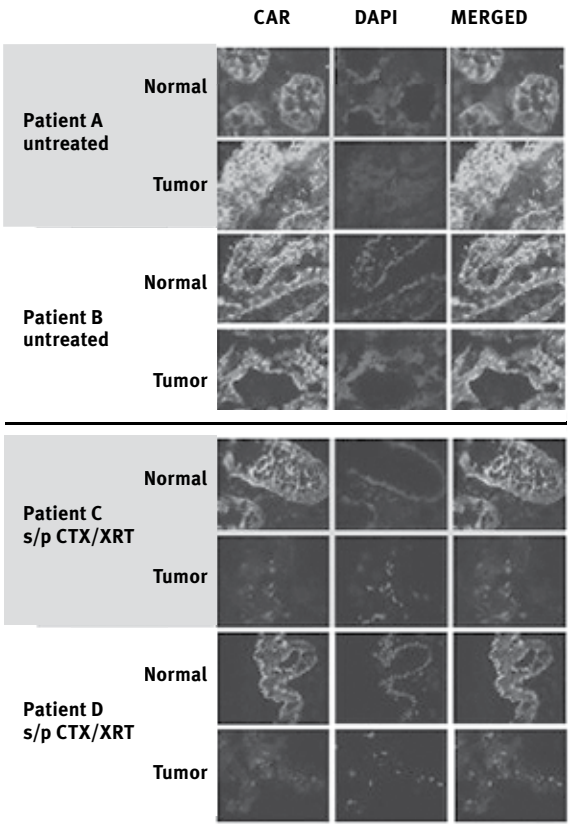

Figure 2. **CAR expression and senescence in colorectal cancer. Human tumor and adjacent normal tissue specimens were obtained from colorectal cancer patients undergoing curative resection treated with or without preoperative chemoradiotherapy. Freshly frozen specimens were stained with CAR primary antibody followed with FITC-conjugated secondary antibody or with DAPI. Immunofluorescence micrographs (40x) of CAR, DAPI and merged images are shown.**

Therapy-induced Senescence Occurs *In Vivo* and Represents Tumor Response to Treatment

The demonstration of therapy-induced senescence *in vivo* has been reliably shown in xenograft and transgenic murine models. Evidence of senescence has also been shown in a reported outside study of post-treatment tumor samples obtained from breast cancer patients treated with a neoadjuvant multi-agent chemotherapy. SA-b-gal expression, a reliable marker for senescence, was found in 41% of tumors resected from patients treated with chemotherapy, but in only 10% of untreated patients. Similar findings have been published in our laboratories showing evidence of chemotherapy-induced senescence in human colorectal cancer patients treated at the VA. Using coxsackie-adenovirus receptor (CAR) as a novel biomarker for senescence, we have shown that senescence response can be demonstrated in rectal cancer patients treated with neoadjuvant chemoradiation (Figure 2). Tumor specific down-regulation of CAR expression is observed in treated tumors compared to normal adjacent mucosa and a control DAPI nuclear stain.

Clinical Response of Solid Tumors to Multimodality Therapy is Best Described by Therapy-induced Senescence

The anti-tumor effects of chemotherapy have been commonly attributed to two forms of programmed cell death, apoptosis and autophagy. For most solid gastrointestinal cancers, however, these mechanisms cannot account for the modest (20–40%) disease response to chemotherapy observed weeks to months after treatment. Even in patients demonstrating near-complete responses to chemotherapy and/or radiotherapy, any remaining residual viable tumor cells will regain proliferative capacity resulting in cancer recurrence. For example, chemoradiotherapy used to treat locally advanced rectal cancer patients frequently produces detectable tumor volume reduction that is later overcome by tumor progression despite ongoing therapy.

The relatively slow onset of solid tumor responses to chemotherapy and the lack of a consistent correlation with apoptosis in numerous studies suggest that other pathways regulating cell death may predominate. Moreover, similarities in observed response rates, regardless of the particular chemotherapeutic agent applied to specific cancers, suggest that chemotherapy drugs may mediate their effect through non-specific drug/target mechanisms. The therapy-induced senescence model closely parallels the clinical observations of gastrointestinal malignancies treated with chemotherapy. Given that most solid tumors recur following therapy, it follows that some cancer cells undergo cell cycle arrest as a result of senescence *in vivo* and retain the ability to escape senescence in order to reproliferate, resulting in cancer progression.

Cdc2/Cdk1 Regulates Therapy-induced Senescence and Escape from Senescence

The cyclin-dependent kinase Cdc2/Cdk1 is a key control point that determines senescence phenotype. In order to examine key aspects of therapy-induced senescence and senescence escape, we have used the p53-null, p16-deficient NCI-H1299 carcinoma line as a model. H1299 cells exposed to moderate doses of camptothecin were shown to arrest in G2/M and enter a senescent state. Allowing recovery time, occasional senescent cells (frequency of 1:100,000 cells) were able to "escape" cell cycle arrest and form proliferating or "escape" colonies.

Analysis of these escape colonies showed that Cdc2/Cdk1 was aberrantly over-expressed compared to low-level expression observed in senescent cells. Furthermore, these cells were found to be dependent upon Cdc2/Cdk1 kinase activity to sustain viability, such that blocking Cdc2/Cdk1 via a selective inhibitor or competitive siRNA translated into rapid cell death. Specific inhibition of Cdc2/Cdk1 was also found to effectively abrogate escape from therapy-induced senescence. These findings suggest that down-regulation of Cdc2/Cdk1 mediates induction of senescence, and that its aberrant over-expression is essential for escape from senescence.

The Cdc2/Cdk1 Effector Protein Survivin Inhibits Apoptosis and is an Important Determinant of Clinical Outcome

Survivin, a 16.5 kDal nuclear protein, is the smallest member of the human inhibitor of apoptosis protein (IAP) family. Survivin is expressed in a cell cycle-dependent manner, and levels are markedly increased during mitosis. Survivin protein is stabilized by undergoing phosphorylation mediated by Cdc2/Cdk1 kinase, and appears to play a crucial role in mitotic spindle association and inhibition of caspase-9-mediated apoptotic activity. Administration of the chemotherapy drug Taxol, a microtubule inhibitor agent, in HeLa cells activates a survival checkpoint by up-regulation of Cdc2/Cdk1, resulting in activation and accumulation of survivin.

Conversely, suppression of survivin activation with the Cdc2/Cdk1 kinase-inhibitor flavopiridol enhances adriamycin-induced apoptotic cell death. Survivin knockout has been shown to be embryonic lethal, and fibroblasts derived from these animals exhibit catastrophic defects in microtubules, centrosomes, spindle poles, and in mitotic spindle microtubule formation. These results collectively suggest a critical role for survivin in cellular mitosis. Survivin has been found to be over-expressed in many types of human cancers; has been associated with unfavorable clinical prognosis in cancers of the breast, esophagus, stomach, pancreas, and colon; and has been shown to correlate with therapy resistance in a variety of clinical settings.

Survivin Enhances Telomerase Activity

In normal human cells, telomeres or nucleoprotein complexes located at the chromosome ends progressively shorten by 50–200 bp with each successive cell division through the loss of terminal DNA sequences. Telomeres are maintained by telomerase, a ribonucleoprotein polymerase that contains hTERT, a catalytic subunit providing reverse transcriptase activity. hTERT expression mainly determines telomerase activity and is expressed at high levels in embryonic stem cells and germ cells, which decreases during differentiation and disappears in fully differentiated somatic cells.

However, through unknown mechanisms, hTERT is reactivated in 80–95% of cancer cells. When telomere shortening reaches a critical threshold, cells are either prompted to enter into protective cell cycle arrest (i.e., senescence) or undergo apoptotic cell death. In contrast, cancer cells possess the ability to maintain and preserve telomere length and undergo sustained proliferation. Survivin has been shown to upregulate telomerase activity by augmenting the expression of human telomerase reverse transcriptase (hTERT) by phosphorylation of Sp1 and c-myc proteins that enhance binding to hTERT promoter. These findings support the concept that survivin enables cancer cells to escape senescence by promoting telomerase activity.

Telomeres and Cancer Senescence

Telomeres stabilize chromosomes and may act as a "mitotic clock" that determines the maximum replicative capacity of somatic cells. In humans, the telomere terminus is composed of 4–15 kbp of the hexanucleotide repeat TTAGGG, followed by a single-strand nucleotide overhang that loops back upon itself , forming a "t-loop," which is associated with telomere DNA-binding factors that function to preserve telomere integrity. Additionally, complex nucleoprotein structures also serve to protect the telomere ends. Loss of the "t-loop" and terminal nucleoprotein complex, termed "uncapping," exposes the telomere to degradative shortening.

Our finding that HCT116 senescent colorectal cancer cells suffer massive telomere loss suggests that telomere integrity is rapidly compromised following camptothecin exposure by

a mechanism that is unrelated to replication-dependent telomere attrition. The mechanism of such massive telomere shortening has not been thoroughly examined. Since telomere dysfunction in these situations is likely related to disruption of end structures *in vivo*, we propose that telomere uncapping leads to exonucleolytic degradation of telomere DNA in therapy-induced senescence.

Senescent escape cells that are able to recover their telomere length may regain replicative capacity. Survivin has been shown to enhance telomerase activity via up-regulation of Sp1 and c-Myc mediated telomerase gene transcription in colon carcinoma cells, and telomerase up-regulation has been observed in senescence-resistant breast cancer cells treated with adriamycin. It follows that survivin has a protective function in senescent escape cells by inhibiting apoptosis and promoting escape by up-regulating hTERT and promoting telomere lengthening.

Current Laboratory Objectives

We have shown *in vitro* that cancer cells exposed to chemotherapy can enter a state of reversible replicative arrest (i.e., therapy-induced senescence) characterized by shortened telomeres and low levels of survivin protein. While the majority of cancer cells will transition to irreversible senescence, small numbers of senescent cancer cells can escape cell cycle arrest. Cancer cells that escape senescence and reenter the cell cycle are presumably a major contributor to cancer progression. Although independent observations have been made regarding the protective effect of senescence and telomerase on cancer cell survival and negative impact on clinical prognosis, the relationship between the two has yet to be established. Our preliminary studies suggest that telomerase expression modulates escape from senescence. The purpose of our current work is to ask: "Does telomerase regulate senescence status during colorectal cancer treatment?"

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