Original Study

Therapy-Induced Cellular Senescence Induces Epithelial-to-Mesenchymal Transition and Increases Invasiveness in Rectal Cancer

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Abstract

We evaluated the effects of the senescence-associated secretome (SAS) in vitro and in clinical samples from patients with rectal cancer who had undergone neoadjuvant chemoradiotherapy (CRT). The effects of the SASs on colorectal cancer cells translated into increased invasiveness and induction of epithelial-to-mesenchymal transition (EMT). In the clinical samples, senescence and EMT co-occurred within a fraction of cancer cell clusters. These results could have important implications in guiding treatment after CRT.

Introduction: DNA damaging agents and ionizing radiation used in the therapy of human cancers can induce senescence of cancer cells. Senescent cells exhibit a secretory phenotype (senescence-associated secretome [SAS]) that can affect cancer cell behavior and, eventually, clinical prognosis. We assessed the effects of the SAS on the induction of epithelial-to-mesenchymal transition (EMT) in vitro and in clinical samples from patients with rectal cancer who had undergone neoadjuvant chemoradiotherapy (CRT). **Materials and Methods:** Colorectal cancer cells (HCT 116) were induced into senescence by exposure to either 5-fluorouracil (5-FU) or doxorubicin. The senescent state was confirmed by staining for senescence-associated β-galactosidase (SA-β-Gal). The paracrine effects of SASs were assessed on proliferating HCT 116 cells. The quantified parameters were cell proliferation, invasive capacity, and induction of EMT. Senescence and EMT in clinical samples were assessed by the expression levels (reverse transcriptase-quantitative polymerase chain reaction) of genes related to senescence and EMT after laser-assisted microdissection of cancer cell clusters that stained either positive or negative for SA-β-Gal. **Results:** We have shown that cultured colon cancer cells induced into senescence by exposure to 5-FU exhibit a SAS capable of paracrine induction of EMT in colon and rectal cancer cell lines and increased cell invasion in vitro. Using laser-assisted microdissection, we found that in rectal cancer samples from patients treated with neoadjuvant CRT, tumor cell niches enriched for senescent cells bookmark regions of increased mRNA expression levels of EMT-related proteins (Slug, Snail, vimentin) compared with the nearby senescent-null tumor cell niches. **Conclusion:** We have provided, first-hand, strongly suggestive evidence that senescent cancer cells emerging in the context of neoadjuvant CRT for rectal cancer influenced the tumor microenvironment by promoting EMT by way of short-range interactions.

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Introduction

Distant relapse affects about 15% to 20% of patients diagnosed with locally advanced rectal cancer, despite all the therapeutic advances.[1,2] Whether diagnosed as locally advanced or at any stage in the presence of positive lymph nodes, the standard of care treatment for patients with rectal cancer has been neoadjuvant

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chemotherapy with the thymidylate synthase inhibitor 5-fluorouracil (5-FU) and concomitant radiotherapy (chemoradiotherapy [CRT]), followed by surgery.[3,4]

It has been previously described that chemotherapy, in addition to its cytotoxic action, can induce a cellular state of irreversible proliferative arrest because of severe DNA damage, termed "therapy-induced senescence" (TIS).[5,6] Initially considered to be a phenomenon typical of normal somatic cells that lost their ability to divide and, thereby, termed "replicative senescence," it is now known that senescence can also be a response mechanism triggered by several factors, including oncogenic mutations, oxidative stress, and DNA damaging agents.[7,8]

The effect of cellular senescence in the context of cancer is not completely understood. Cell senescence can play a direct role in tumor growth inhibition, because it is an important antiproliferative mechanism.[9] In lung and breast cancer, the detection of cell senescence after neoadjuvant chemotherapy correlated positively with the response to treatment.[10,11] Also, in colorectal cancer, patients with sporadic senescent cells detected before treatment had increased susceptibility to TIS and a better response to adjuvant chemotherapy.[12] However, evidence has shown that senescent cells can also exert deleterious effects on the tissue microenvironment.[13] The so-called senescence-associated secretory phenotype (SASP) of these cells, which includes the secretion of several pro-inflammatory cytokines, epithelial growth factors, and tissue remodeling enzymes, can induce a more aggressive phenotype in nonsenescent cells in a paracrine fashion.[14] Data from studies of breast, prostate, and pancreatic cancer showed that senescent fibroblasts promoted tumor growth and progression by increasing proliferation and invasion and inducing an epithelial-to-mesenchymal transition (EMT) in premalignant and malignant cells.[15-19] It was further shown that neoadjuvant chemotherapy-induced senescence observed in patients with malignant pleural mesothelioma or lung cancer was potentially associated with a poor outcome.[20,21] Finally, senescent human prostate and breast tumor cells also have a SASP, raising the question of broader effects of SASPs on tumor behavior.[17,22] In the present study, we assessed the effects of the SASP from chemotherapy-induced senescence on induction of EMT in vitro and whether the coupling between cancer cell senescence and EMT induction was recapitulated in clinical samples from patients with rectal cancer who had undergone neoadjuvant CRT.

Materials and Methods

Cell Lines and Human Tissue Specimens

The human colon carcinoma cell lines HCT 116 and SW48 and the human rectal cancer cell line SW837 were obtained from the American Type Culture Collection (CCL-247, CCL-235, and CCL-231, respectively). HCT 116 was cultured in McCoy's 5A modified medium (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (Life Technologies), 100 U/mL penicillin/streptomycin (Life Technologies), 2 mM L-glutamine (Life Technologies), and 1% nonessential amino acids (Life Technologies). SW48 and SW837 were cultured in Dulbecco's modified Eagle medium (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies) and 100 U/mL penicillin/streptomycin (Life Technologies). All cell lines were kept at 37°C in 5% carbon dioxide. Rectal cancer specimens (n = 19) were collected during standard of care surgery from patients with rectal cancer who had or had not (controls) undergone neoadjuvant CRT, included in OCT compound (Sakura Finetek, Alphen aan den Rijn, The Netherlands), snapshot frozen in liquid nitrogen within 30 minutes of collection, and preserved at −80°C. The ethics commission of the Hospital de Santa Maria, Centro Hospitalar Lisboa Norte (Lisbon, Portugal) approved the study, and all the patients provided written informed consent.

Induction of Cell Senescence

A total of 4.0×10^4 HCT 116 cells were seeded in 60-mm diameter plates and continuously exposed to 5.0 μM 5-FU (Accord Farmacêutica Ltd, São Paulo, Brazil) or 0.5 μM doxorubicin (Sigma-Aldrich, St Louis, MO), for 7 days or 4 hours, respectively. The media with and without (controls) drugs were replaced every 48 hours. After drug removal, the cells were incubated with fresh medium. Conditioned media were collected 72 hours after drug release (senescence-associated secretome [SAS] medium). Culture media conditioned by exponentially growing (nonsenescent) cells (non-SAS medium) were collected 72 hours after seeding. The conditioned media were stored at 4°C and used within 48 hours of storage.

Cellular Assays

The detection of senescence-associated β-galactosidase (SA-β-Gal) activity in HCT 116 cells and frozen tissues was performed using the Senescence Cells Histochemical Staining Kit (Sigma-Aldrich), according to the manufacturer's instructions, followed by counterstaining with nuclear fast red (Sigma-Aldrich) and visualized in a Leica DM2500 bright field microscope (Leica Microsystems, Hannover, Germany).

Cell proliferation was assessed using the alamarBlue assay and 5-bromo-2′-deoxyuridine (BrdU) incorporation. The alamarBlue assay (Life Technologies) was performed according to the manufacturer's instructions. To determine the effect of the conditioned media on cell proliferation, HCT 116, SW837, and SW48 cells were plated in 96-well plates (2.0×10^3, 1.0×10^4, and 1.0×10^4 cells/well, respectively) and incubated for 24 hours in the presence of SAS medium or non-SAS medium. To test for BrdU incorporation, the cells induced into senescence by 5-FU or untreated control cells were exposed to 10 μM of BrdU for 24 hours or 1 hour, respectively. The cells were then fixed in 3.7% paraformaldehyde (PFA) for 10 minutes at room temperature. DNA was subsequently depurinated for 30 minutes in 4.0 N HCl, followed by a neutralization step in PBS supplemented with Tris buffer (100 mM; pH 8) for another 30 minutes. The cells were incubated with anti-BrdU antibody (1:50; clone BMC 9318; Roche, Basel, Switzerland) for 1 hour at 37°C, followed by incubation with anti-mouse Cy3 antibody (1:200; Jackson ImmunoResearch, West Grove, PA). Cover slips were mounted in VECTASHIELD with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA), and visualized in a Zeiss Axiovert 200M inverted wide-field fluorescence microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany). Apoptosis was assessed using the Caspase-Glo 3/7 Assay (Promega, Madison, WI), according to the manufacturer's instructions.