HIGH SCHOOL EDITION
Journal of Student Research
Volume 13 Issue 4 (2024)

Exploring the Impact of Senescent Stromal Cells on Tumor Cell Stemness

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ABSTRACT

Aging is a great risk factor for cancer. Many types of cancer get more aggressive with aging. Cancer stem cells play a critical role during cancer progression and recurrence, which might enhance aging-associated cancer aggressiveness. At the cellular level, senescent cell accumulation is a main cause of aging. While senescence is traditionally considered a tumor-suppressive mechanism, recent studies suggest that senescent stromal cells may exert paradoxical effects on the stemness properties of adjacent tumor cells. This study delves into the interplay between tumor stromal cell senescence and the stemness in lung cancer cells. We investigated the impact of senescent MRC5 conditioned medium on tumor cell behavior. Initial experiments revealed that MRC5 stromal lung fibroblast induced a robust senescent state by H2O2 and carboplatin. Subsequent investigations using colony formation assays underscored an augmented clonogenic potential in cells cultured with senescent MRC5 conditioned medium, indicative of heightened tumor cell stemness. Expanding our analysis to drug resistance, we observed a pronounced resistance to carboplatin in tumor cells exposed to senescent MRC5 conditioned medium. To decipher the molecular underpinnings of these phenotypic changes, we quantified the expression of cancer stemness markers, revealing a specific pattern of upregulation. These findings collectively highlight the impact of senescent stromal cells on tumor cells stemness, indicating a distinctive molecular signature associated with the acquisition of stem-like properties. Understanding these complexities contributes to our knowledge of tumor heterogeneity and may inform novel therapeutic strategies.

Introduction

Cancer stem cells (CSCs) constitute a minute yet pivotal fraction of the cancer cell population, exerting significant influence on tumor initiation, progression, metastasis, and recurrence (Walcher et al., 2020). Unlike the majority of tumor cells, CSCs possess the unique ability to self-renew and generate diverse cell lineages within the tumor. This capacity for self-renewal is facilitated through asymmetric division, where CSCs give rise to both identical CSCs and differentiated progeny. The inherent plasticity of CSCs allows them to transition between stem cells and differentiated states, adapting to varying microenvironmental cues. Their heightened tumorigenicity, marked resistance to conventional therapies, and pivotal role in initiating and progressing tumors underscore the critical importance of understanding CSC biology (Bisht et al., 2022). The significance of studying cancer stem cells lies in their profound impact on tumor heterogeneity, therapeutic resistance, and tumor recurrence. CSCs contribute to the complex intratumoral landscape by generating diverse cell types within the tumor (Rich, 2016). Targeting these tumor stem cells holds considerable promise for enhancing therapeutic outcomes by tackling the root causes of tumor progression and resistance (Li et al., 2023). Moreover, as CSCs play a key role in tumor relapse post-treatment, investigating their biology becomes crucial for devising interventions to prevent recurrence and achieving sustained therapeutic success (Marzagalli et al., 2021).

A tumor represents a complex assembly of cells, where the intricate interplay between tumor cells and the surrounding normal stromal cells significantly influences the progression of the malignancy. The dynamic relationship between these two cellular components plays a pivotal role in shaping the tumor microenvironment (Quail & Joyce,

2013). One crucial aspect of this interaction involves the senescent state of tumor stromal cells, adding an additional layer of complexity to the modulation of tumor behavior.

The senescent status of stromal cells within the tumor microenvironment introduces a novel dimension to our understanding of tumor progression. As these stromal cells enter a state of irreversible cell cycle arrest, commonly known as senescence, they undergo alterations in their secretory profile, contributing to what is termed the Senescence-Associated Secretory Phenotype (SASP) (Huang et al., 2022). This senescent phenotype has the potential to exert profound effects on neighboring tumor cells. The communication between senescent stromal cells and tumor cells becomes a crucial factor that can either impede or promote tumor progression, depending on the specific molecular signals exchanged within this intricate cellular dialogue (Ye et al., 2023). Unraveling the nuances of this intercellular crosstalk holds promise for uncovering novel therapeutic targets and strategies aimed at manipulating the tumor microenvironment to impede or reverse tumor progression.

Cellular senescence, a natural process for non-tumor cells, serves as a mechanism to limit the proliferative potential of cells over time. Normally, senescent cells are efficiently recognized and eliminated by the immune system, contributing to the maintenance of tissue homeostasis. However, with aging, there is a well-documented tendency for the accumulation of senescent cells (Huang et al., 2022). The aging process is associated with a gradual decline in the immune system's efficiency, leading to a reduced capacity to clear senescent cells effectively. This age-related accumulation of senescent cells is believed to contribute to various aspects of aging-related tissue dysfunction and age-related diseases (Di Micco et al., 2021).

In the context of cancer patients undergoing treatment, both chemotherapy and radiotherapy are known to induce cell senescence as part of their mechanism of action. While these treatments aim to eliminate rapidly dividing tumor cells, they can also trigger senescence in normal, healthy cells (Bousset & Gil, 2022). The senescent state induced by chemotherapy and radiotherapy is considered a double-edged sword. On one hand, it may act as a protective mechanism by preventing damaged cells from further dividing and potentially becoming cancerous. On the other hand, the accumulation of senescent cells resulting from cancer treatments may contribute to therapy-associated side effects and long-term complications, as these cells can persist in tissues and contribute to inflammation and tissue dysfunction (Xiao et al., 2023).

In this paper, we delve into the influence of the senescent state of tumor stromal cells on the characteristics of tumor cells, with a particular focus on their stemness. To induce senescence in stromal cells, we employed different drugs on lung fibroblasts (MRC5). Subsequently, we exposed its corresponding tumor cells—lung adenocarcinoma (A549) to the conditioned medium derived from these senescent stromal cells. Our experimental outcomes were measured through diverse assays, encompassing the evaluation of tumor cell resistance to chemotherapeutics, visualization of colony formation on agarose plates, and the quantification of the expression of stemness marker proteins. By adopting this multifaceted approach, we aimed to comprehensively understand the effects of senescent tumor stromal cells on tumor cells, with a specific emphasis on the critical factor of stemness.

Methods

Cell Culture and Cell Treatment

MRC-5 human lung fibroblasts (CCL-171) and A549 lung adenocarcinoma cells were procured from the American Type Culture Collection (ATCC). MRC5 cells were cultured in Eagle's Minimum Essential Medium (EMEM, ATCC) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 1% penicillin-streptomycin (Gibco). The cell cultures were meticulously maintained in a controlled environment featuring a humidified atmosphere at 37°C and 5% CO2. As part of routine care, the growth medium was refreshed every other day, and the cultures were passaged using trypsin–EDTA (0.25%) when confluence levels ranged between 70% and 90%. The cell culture reagents primarily originated from Sigma Aldrich, Gillingham, UK, unless otherwise specified.

To induce cell senescence in MRC5 cells, the cells were treated with 20 μM H_2O_2, 50 μM carboplatin, or H_2O as a control for 3 days. Senescence was confirmed at this time point. Subsequently, the medium was replaced with normal medium without drugs for 1 day, and the conditioned medium was collected for the treatment of tumor cells. For tumor cell treatment, the conditioned medium was combined with fresh medium at a ratio of 2:1. Tumor cells were then cultured with this mixed medium for 3 days. This experimental design allowed for the investigation of the impact of senescent MRC5 cell-derived factors on tumor cell behavior.

Senescence β-Galactosidase Staining

In this experimental protocol, cells underwent a 5-day treatment regimen involving various fatty acids. Subsequently, an equivalent number of cells were distributed into separate wells of a 6-well plate, maintaining consistent treatment conditions for an additional 2 days. This period allowed the cells to reach an approximate confluence level of 80%. Following this incubation, the detection of senescent cells was conducted using the β-galactosidase staining assay, a well-established method for identifying cellular senescence. A commercial kit from Cell Signaling Technology was employed, following a standardized protocol. Essentially, the cells were fixed using a fixative solution (2% formaldehyde, 0.2% glutaraldehyde in PBS), followed by gentle washing with PBS. Subsequently, the cells were stained with a β-galactosidase staining solution containing the substrate (X-gal) and incubated at 37°C overnight, facilitating the emergence of blue-stained senescent cells. After staining, a careful PBS wash was conducted. The stained cells were examined under a microscope to ascertain the percentage of β-galactosidase-positive cells, which were then compared to the total cell population in different experimental groups. Notably, each treated group underwent three technical replicates, and the entire experimental procedure was conducted independently three times to ensure robustness and reproducibility.

Western Blotting

In this investigation, Western blotting was employed as a quantitative tool to evaluate both the cell senescence of non-tumor cells subjected to carboplatin and H_2O_2 treatments and the expression of cancer stemness markers in tumor cells cultured with conditioned medium for 3 days. Protein extraction was conducted using the RIPA buffer supplemented with protease inhibitors, and the protein content was quantified using the DC Protein Assay kit II (BioRad). Subsequently, 30 μg of total protein from each sample was separated via SDS-PAGE under reducing conditions. The resolved proteins were then transferred to a nitrocellulose membrane through electrophoresis.

The transferred membrane underwent blocking using TBS-based Odyssey Blocking buffer from LI-COR. Specific antibodies targeting key markers, including p16, p21, OCT4, NANOG, SOX2, KLF4, and c-MYC (1:2000, Cell Signaling Technology), were employed to probe for the proteins of interest. After an overnight incubation with primary antibodies at 4°C, the membranes were exposed to a donkey anti-rabbit Alexa Fluor 488 secondary antibody (A-21206—Thermo Fisher Scientific, 1:10,000) for 1 hour at room temperature. Signal detection was performed using the LI-COR Odyssey CLx Imaging System, and the blot intensities were assessed using the LI-COR Odyssey CLx imaging software.

Colony Formation Assay

A549 tumor cells were cultured with different conditioned medium for 5ays and then harvested and counted. 5000 tumor cells were seeded into each well on a 6-well plate. A semi-solid medium was created by mixing agarose and 2X complete medium at 1:1 ratio. Subsequently, the tumor cells were embedded in this semi-solid medium and allowed to solidify. The plates were then incubated for a period of 2-4 weeks at 37°C with 5% CO_2 to permit colony formation. After three weeks, the colonies were fixed using methanol, followed by staining with crystal violet for

enhanced visibility. The fixed and stained colonies were carefully examined under a microscope, and the number of the colonies was recorded. The colony number was normalized to the control group and results were presented as a percentage compared to the control.

Chemo-Resistant Assay

In the tumor cell drug resistance assay, tumor cells were initially cultured with conditioned medium derived from control, H_2O_2-treated, or carboplatin-treated MRC5 cells for a 4-day duration. Subsequently, the treated tumor cells were seeded into 96-well plates at a density of 2000 cells per well. Continuing with the same mixed medium, cells were exposed to varying concentrations of carboplatin and maintained in culture for an additional 4 days. Following this incubation period, the cells were fixed and stained with crystal violet to enable the quantification of cell mass.
To elaborate, the conditioned medium from non-tumor cells served as a modulator to simulate the microenvironmental influences on tumor cells. The choice of different conditions, including exposure to H_2O_2 or carboplatin, aimed to mimic cellular stress and induce senescent state, respectively. The subsequent seeding of treated tumor cells into 96-well plates allowed for a systematic evaluation of their response to carboplatin across a range of concentrations. This experimental design not only assessed drug resistance but also provided insights into how the cellular microenvironment might contribute to variations in tumor cell responses to chemotherapy. The fixation and staining process with crystal violet offered a reliable method for quantifying the cell mass, enabling a comprehensive analysis of drug resistance patterns in the context of different microenvironmental conditions.

Results

H_2O_2 and Carboplatin Induce Cell Senescence in Normal Stromal Cells

Both hydrogen peroxide (H_2O_2) and carboplatin have been recognized as potent triggers of cell senescence in normal stromal cells, with their action linked to the initiation of DNA damage and subsequent oxidative stress (Duan et al., 2005; Rutecki et al., 2024). In our experiment, fibroblasts were exposed to 20μM H_2O_2 and 50μM carboplatin to assess their impact on cellular senescence.

To evaluate the extent of cell senescence, we examined both cellular and molecular levels. Beta-galactosidase staining, a widely accepted method for measuring cell senescence, was performed following treatment with these senescence inducers (Valieva et al., 2022). It was evident that MRC5 cells treated with either carboplatin or H_2O_2 exhibited a significant increase in size compared to the control group, accompanied by numerous dark spots indicating larger, more prominent nuclei, characteristic of senescent cells. Additionally, a small proportion of nuclei were observed to be detached from cells in the H_2O_2 group, indicating aging-induced cell death. Moreover, a slight decrease in cell density was noted alongside increased cell size in the experimental groups, suggesting potential cell death. Notably, MRC5 cells in the carboplatin group displayed even larger size and lower density compared to the H_2O_2 group, indicating differential effects of these drugs in inducing cell senescence. Furthermore, a significant increase in positively stained cells was observed under the treatment of both H_2O_2 and carboplatin, consistent with changes in cell morphology (Figure 1A).

To delve into senescence at the molecular level and quantify these changes, total protein was extracted from the cells, and the expression levels of p16 and p21, both cell cycle regulators, were measured (Kumari & Jat, 2021). These proteins are known to exhibit higher expression in senescent cells where the cell cycle is inhibited, serving as senescence markers. The results showed darker bands for both p16 and p21 in cells treated with drugs compared to the control group, indicating elevated expression levels and confirming MRC5 cell senescence. Interestingly, p16 bands appeared darker and thicker in the carboplatin group, while p21 bands were slightly more prominent in the H_2O_2 group. This suggests that carboplatin may play a more significant role in facilitating p16 expression, while H_2O_2 may

aid in the expression of p21, supporting the hypothesis that these drugs induce cell senescence through different mechanisms (Figure 1B). In conclusion, both H_2O_2 and carboplatin were found to induce cell senescence in our model, affirming our decision to proceed with the study using senescent MRC5 cells.

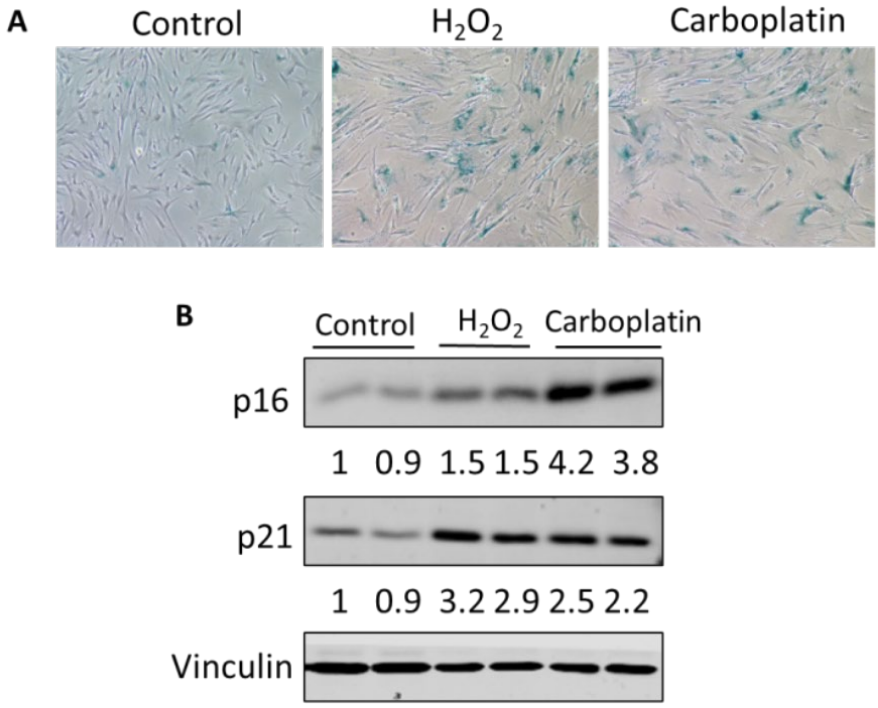

Figure 1. The induction of cell senescence in fibroblasts. A. MRC5 fibroblasts were exposed to 20 μM H_2O_2 or 50 μM carboplatin for 7 days. Beta-galactosidase staining was performed to identify senescent cells, which appeared blue. B. Total protein was collected from the fibroblasts after treatment, and the levels of p16 and p21 proteins were measured. The blot intensity was quantified using LI-COR and normalized to the control group.

The Senescent Stromal Cells Promotes Colony Formation of Tumor Cells

To assess the influence of stromal cells on tumor behavior, we collected conditioned medium from fibroblasts and utilized it to culture A549 tumor cells. Following a 5-day incubation period, an equal number of tumor cells from each group were seeded onto agarose plates to facilitate colony formation. These plates were then incubated for 3 weeks to allow for the development of visible colonies.

Upon visual inspection, plates conditioned with carboplatin and H_2O_2 exhibited a markedly higher number of colonies compared to the control plate, with the H_2O_2-conditioned plate displaying the greatest abundance of tumor cell colonies. To obtain more precise and quantitative results, plates were stained with crystal violet to enhance visibility, and the number of colonies on each plate was recorded. Colony numbers from the control group were normalized, and results were presented as percentages relative to the control group. Analysis of the data revealed that the number of colonies on the H_2O_2-conditioned plate more than doubled compared to the control group, while the relative colony number of the carboplatin-conditioned group was slightly less than double (Figure 2). These findings indicate that conditioned medium from either H_2O_2- or carboplatin-treated fibroblasts can effectively promote A549 colony formation. Given that only tumor cells with high stemness are capable of forming colonies under 3D culture conditions, colony formation serves as a marker of tumor stemness (Nakamura, 2023). Therefore, the increased number of

colonies suggests heightened tumor stemness. Consequently, it can be inferred that media from senescent MRC5 cells enhances A549 stemness, underscoring the potential role of senescent stromal cells in modulating tumor behavior.

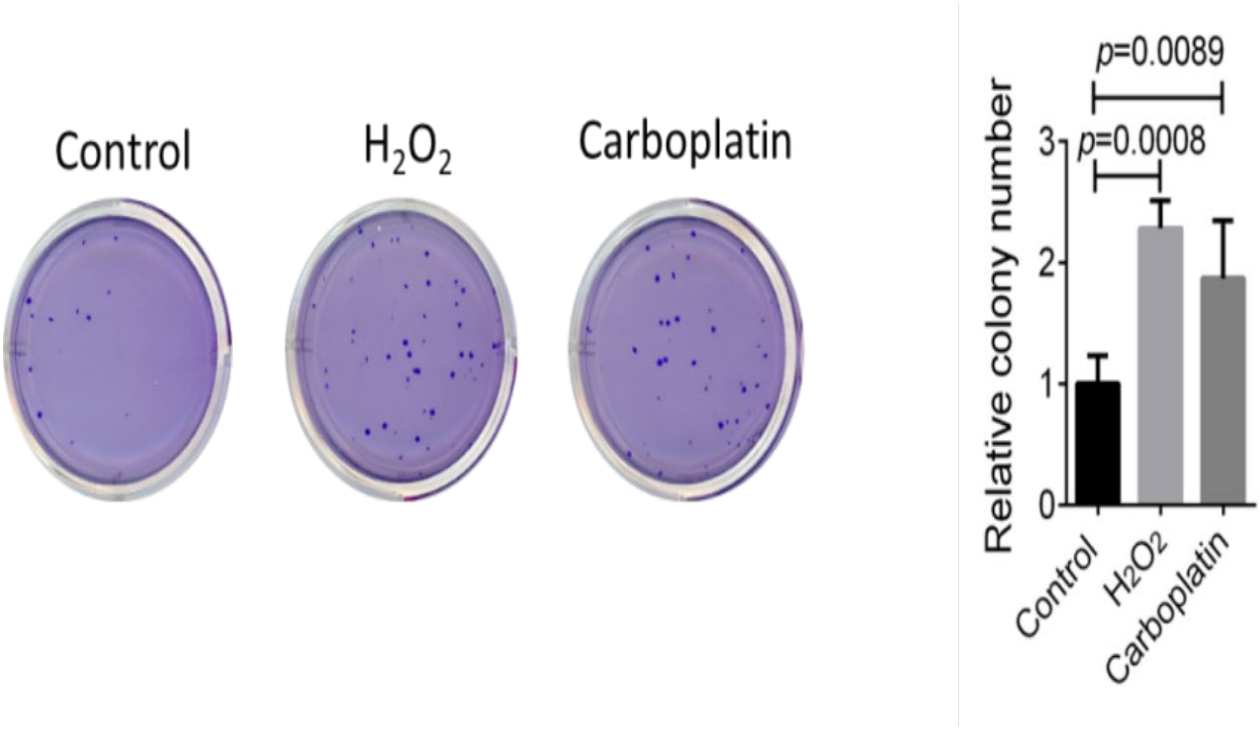

Figure 2. The effect of senescent fibroblasts on the colony formation ability of tumor cells. After treatment, A549 tumor cells were seeded in agarose medium and allowed to form visible colonies over approximately 3 weeks. The colonies were stained with crystal violet, and the number of colonies was counted and normalized to the control condition. Statistical analysis was performed using a two-tailed Student's t-test (n=3).

The Senescent Stromal Cells Promote Drug Resistance of Tumor Cells

Therapy resistance is a significant challenge in cancer treatment and a leading cause of mortality among patients. Interestingly, elderly cancer patients often exhibit increased susceptibility to developing therapy resistance compared to their younger counterparts (Lei et al., 2023). To investigate whether senescent stromal cells contribute to drug resistance, we pre-cultured tumor cells with different conditioned media from MRC-5 cells for 5 days. Subsequently, the tumor cells were seeded into a 96-well plate and cultured with conditioned media collected from three groups of normal stromal cells. To mimic the microenvironment during chemotherapy, tumor cells in each well were exposed to varying concentrations of carboplatin, a widely used clinical chemotherapeutic. After 4 days, the cells were fixed and stained with crystal violet to quantify cell biomass.

Our results revealed that regardless of the concentration of carboplatin (ranging from 0 μM to 150 μM), the biomass of tumor cells in conditioned groups consistently exceeded that of the control group. Moreover, as carboplatin concentration increased, the biomass of cells decreased, indicating a stronger inhibitory effect of carboplatin on tumor cell growth at higher concentrations within our tested range. Notably, among the three groups, tumor cells conditioned with carboplatin consistently exhibited the highest biomass. Given that a higher survival curve suggests increased resistance of tumor cells to carboplatin, our findings suggest that media from senescent stromal cells, specifically MRC5, promotes drug resistance in A549 cells (Figure 3). Further exploration into the unique properties of carboplatin that enhance resistance in carboplatin-conditioned tumor cells is warranted.

Tumor stem cells with heightened stemness are typically more resilient to chemotherapy drugs (Phi et al., 2018). Therefore, our results provide supporting evidence for our previous hypothesis that senescent stromal cells may enhance tumor stemness, contributing to increased drug resistance.

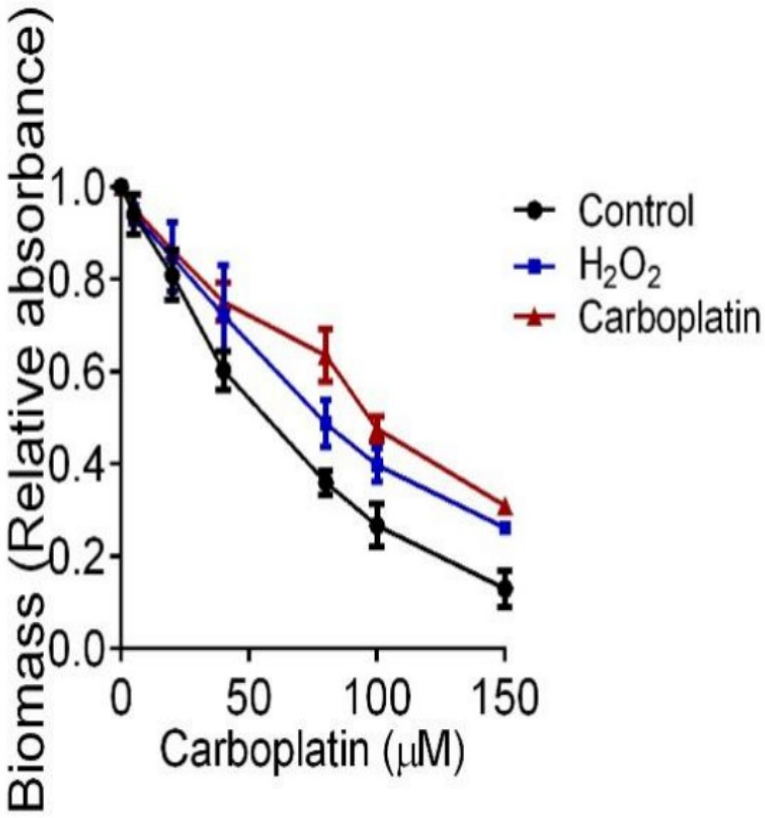

Figure 3. The effect of senescent fibroblasts on the drug resistance of tumor cells. A549 tumor cells were cultured with conditioned medium from fibroblasts for 5 days, followed by exposure to various concentrations of carboplatin for an additional 4 days. Cell survival was then measured and compared.

The Senescent Stromal Cells Enhance Tumor Stemness

Based on the aforementioned results suggesting enhanced tumor stemness by the senescent stromal cell conditioned medium, we aimed to further assess tumor stemness by examining the expression of stem cell markers. ALDH1A1, ALDH1A2, SOX2, OCT4, and NANOG are universally recognized markers for measuring stemness and identifying stem cells (Mohan et al., 2021). In our study, we observed changes in the expression of these protein markers after 3 days of cell culture. Relative to the control group, the expression levels of ALDH1A1, ALDH1A2, and SOX2 were all elevated in the H_2O_2 and carboplatin groups. However, no significant difference was noted in the expression of OCT4. Interestingly, under H_2O_2 treatment, we observed a slight decrease in NANOG expression, while no significant change was observed under carboplatin treatment (Figure 4).

Overall, the comparison of marker protein expression indicates that tumor cells treated with H_2O_2 and carboplatin conditioned media exhibit heightened stemness. This suggests that senescent cells, following drug treatment, secrete specific substances into the media, potentially enhancing the stemness of tumor cells. Consequently, this

heightened stemness may contribute to increased proliferation, metastasis, and drug resistance capabilities in tumor cells.

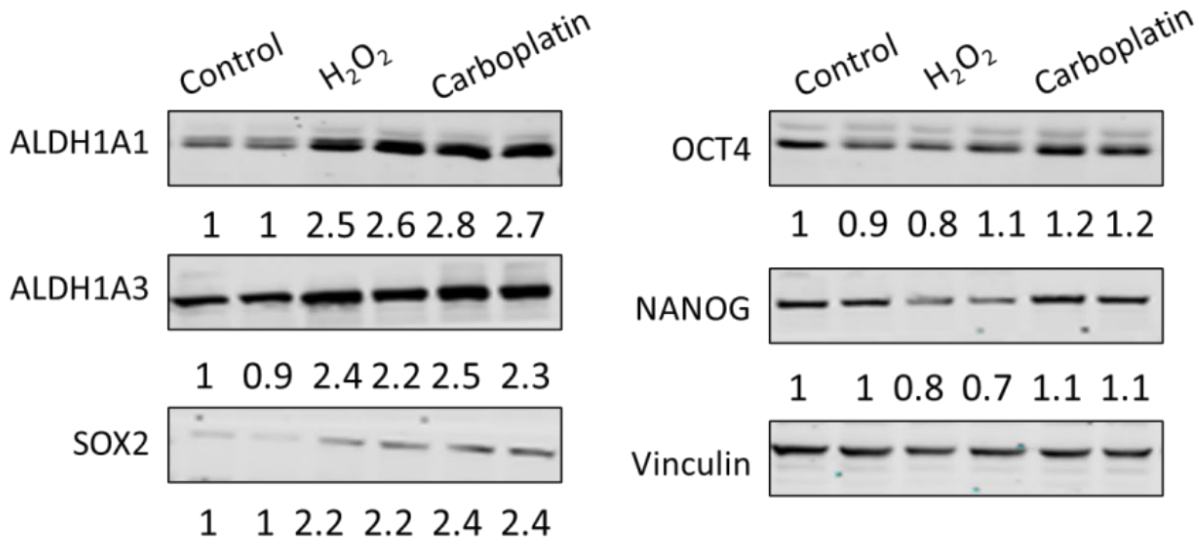

Figure 4. The effect of senescent fibroblasts on the tumor cell stemness. Tumor cells were cultured with conditioned medium from fibroblasts under different treatments for 4 days. The levels of stemness marker proteins were then measured, using vinculin as the reference protein. The intensity of the blots was quantified, normalized to vinculin, and subsequently normalized to the control group.

Discussion

In our study, we aimed to explore the effects of senescent stromal cells induced by two common agents, H_2O_2 and carboplatin, on tumor behavior. Through meticulous microscopic examination, we confirmed the efficacy of both treatments in inducing senescence in fibroblasts, a pivotal component of the tumor microenvironment. However, upon closer scrutiny, intriguing disparities emerged in the functions and effects of H_2O_2 and carboplatin.

Our findings revealed that both H_2O_2 and carboplatin significantly upregulated the expression of senescence-associated markers p16 and p21 in normal stromal cells. Notably, the carboplatin-conditioned group exhibited notably higher levels of p16 expression compared to the H_2O_2-conditioned group (Figure 1). This observation suggests a differential influence of carboplatin on p16 expression during cell senescence, hinting at distinct molecular pathways underlying the senescence-inducing properties of these agents.

Shifting our focus to tumor behavior, our investigation into colony formation by tumor cells unveiled intriguing insights. We observed that H_2O_2-conditioned media resulted in the highest number of colonies, indicative of its unique ability to promote A549 tumor progression (Figure 2). Furthermore, our experiment assessing drug resistance uncovered compelling results: the carboplatin-conditioned group exhibited the greatest resistance to clinical chemotherapeutics across all concentrations (Figure 3). This underscores the potential role of senescent stromal cells in enhancing drug resistance, thereby necessitating careful consideration in the development of therapeutic strategies.

In light of these findings, our study also delved into the impact of senescent fibroblasts on tumor stemness. Our results indicated that senescent fibroblasts increased the stemness of tumor cells, potentially through the secretion of senescence-associated secretory phenotype (SASP) molecules. Importantly, variations in the expression of stemness markers OCT4 and NANOG were observed under senescent fibroblast conditioned medium, underscoring the

complexity of the tumor-stroma interaction (Figure 4). This highlights the need for further investigation into the optimal treatment conditions and duration to better understand the underlying mechanisms driving tumor stemness in the context of senescent stromal cells.

Reflection on our findings prompts consideration for potential improvements and future research directions. Extending the research period and diversifying sample types would allow for the collection of more comprehensive data on stromal cell senescence and tumor cell behavior. Moreover, this study is more focused on the phenotypes, and delving into protein and molecular levels will enhance our understanding of underlying mechanisms.

Based on this research, deeper insights were provided to clinical medicine as well. Our findings demonstrated that senescent stromal cells positively impact tumor cell stemness, raising awareness among clinical professionals that older patients with increasing amounts of senescent stromal cells require additional care and interventions compared to younger cancer patients. Therefore, clinical therapy guidelines should be updated for elderly cancer patients. For instance, drugs targeting the removal of senescent cells, such as senolytics, can be employed for older cancer patients (Malayaperumal et al., 2023). This combination is expected to enhance the efficiency of cancer therapy and improve drug resistance. Furthermore, chemotherapy removes tumor cells by inducing cell senescence but also induces senescence in tumor stromal cells. Our results highlight the rationale and necessity of targeting senescent cells in cancer therapy. While this research is significant in providing information and ideas for treating cancers in an aging population, successfully translating these ideas into practice requires further exploration and understanding.

Acknowledgments

I would like to thank my advisor for the valuable insight provided to me on this topic.

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