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Original article

Therapy Induced Senescence Promote Expression of Death Receptors in Breast Cancer Cells

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Abstract

Chemotherapeutic agents that cause DNA damage also induce cellular senescence known as therapy-induced senescence (TIS). Cells undergoing senescence may exert detrimental effects by promoting tumor progression in healthy cells or supporting metastases in cancer cells due to "senesence-associated secretory phenotype" (SASP), involving secretion of chemokines, cytokines, metalloproteinases, and growth factors. Death receptors belong to the tumor necrosis factor receptor superfamily and implicated in induction of apoptosis via activation of extrinsic pathway. The most recognized death receptors are FAS (CD95), TNFR1 and TRAIL-R1 / 2 (DR4-DR5) etc. and capable of directly inducing apoptosis in the cell. In this study we aimed to investigate the expression of cell death receptors in response to TIS of breast cancer cells for their potential use in elimination of senescent cells.

Doxorubicin and etoposide were used to induce senescence selectively in MCF7 breast cancer cell line. Senescence induction was confirmed by β-galactosidase staining and cell cycle analysis. Activations of p53, p21, and γ-H2AX and expression levels of cell death receptors (FAS (CD95), TNFR1-2 and DR5 were tested by western blot analysis. Apoptosis was measured by Annexin V/7AAD analysis.

Here, we show that chemotherapy agents etoposide and doxorubicin induced senescence by arresting MCF-12A and MCF-7 cells in G1 and G2/M phases of cell cycle, respectively. Induction of senescence is confirmed by SA-β-gal staining and by activation of γ-H2AX, p53 and p21 proteins. Neither etoposide nor doxorubicin induced significant apoptosis in MCF12A or MCF-7 cells. Importantly, TIS increased the protein levels of TNFR1, TNFR2 and DR5 receptors selectively in MCF-7 cells but not in MCF-12A cells. These data suggest that chemotherapy agents induce senescence increased the expression of death receptors in breast cancer cell line MCF-7 thus provide a basis for further investigation of death receptor mediated targeting of senescent cells as potential therapeutic strategy.

Keywords: Therapy Induced Senescence, Etoposide, Doxorubicin, TNFR1, DR5, Breast Cancer.

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INTRODUCTION

Senescence was first identified *in vitro* in primary diploid fibroblasts as a result of replicative exhaustion and telomere shortening leading to the inhibition of cell division by arrest of the cell cycle. Subsequently, this phenomenon is called as "replicative senescence" (hayflick) (Hayflick 1965). Currently cellular senescence is widely recognized as an anti-proliferative state of cells which also serves as a tumor suppressor barrier can be induced by internal and external stimulus such as telomere shortening, DNA damage, oxidative stress, radiation exposure and activated oncogenes or inactivated tumor suppressor genes (Fridlyanskaya et al. 2015).

Chemotherapeutic agents that cause DNA damage (i.e. doxorubicin, cisplatin, bleomycin, camptothecin, etoposide and) also induce cellular senescence known as therapy-induced senescence (TIS) (Schosserer et al. 2017). Induction of senescence is achieved and maintained by DNA damage response signaling through activation of tumor suppressors Rb and p53. Accordingly, growth arrest is achieved in part at the G1 or G2 / M phase of the cell cycle via increased expression of specific cyclin-dependent kinase inhibitors (CDKIs), including p16 (Ink4a) p21 (Waf1/Cip1). Typically, senescent cells have a flattened and enlarged morphology, remain viable and metabolically active, but stop cell growth permanently (Schosserer et al. 2017). Unlike cells that undergo apoptosis or mitotic catastrophe in response to cytotoxic drugs, senescent cells can persist in this state indefinitely. Increased senescence-associated β-galactosidase activity is another hallmark of the senescent cells (Schosserer et al. 2017, Ewald et al. 2010, Hernandez-Segura et al. 2018, Wyld et al. 2020). Cells undergoing senescence also develop "senescence-associated secretory phenotype" (SASP), involving secretion of chemokines, cytokines, metalloproteinases, and growth factors (Itahana et al. 2007, Lopes-Paciencia et al. 2019). Recent reports suggest that senescent cells might have deleterious effects in the microenvironment due to the pro-inflammatory factors in SASP and even promote tumor progression in healthy cells or support immune evasion or metastases of cancer cells (Lopes-Paciencia et al. 2019, Ohtani 2019). Thus, anti-cancer therapies based on the induction of senescence in tumor cells must be taken into careful consideration and potential strategies aiming to eliminate senescent-like cancer cells are required.

Death receptors belong to the tumor necrosis factor receptor superfamily and implicated in induction of apoptosis via activation of extrinsic pathway. In brief, this process begins with binding of the ligand to the receptor and activates caspases 8 and 10 via the adapter proteins, resulting in a proteolytic cascade and cell death (Debatin and Krammer 2004). The most recognized death receptors FAS (CD95), TNFR1 and TRAIL-R1 / 2 (DR4-DR5) etc. are capable of directly inducing apoptosis in the cell (Elrod and Sun 2008, Ukrainskaya et al. 2017). Therefore, strategies aiming to increase expression or activation of death receptors are of great importance for cancer therapies. Previous studies suggest that chemotherapy agents may increase expression of death receptors in various cancel cells

(Ukrainskaya et al. 2017). Here, we aim to investigate the expression levels of cell death receptors in therapy-induced senescent breast cancer cells for their potential use in elimination of senescent cells.

MATERIALS and METHODS

Cell culture

MCF-7 breast cancer cells and the epithelial cell line MCF-12A cells were cultured in RPMI Medium (Gibco, USA), containing 10% fetal bovine serum (Gibco, USA) and %1 penicillin/streptomycin (Gibco, USA).

Senescence-Associated β-Galactosidase activity assay

MCF-7 and MCF-12A cells were seeded at 5.10^5 cells/per well in 12 well plates. MCF-7 and MCF-12A cells were treated with 50 μM etoposide (Eto) and 0.1 μM doxorubicine (Dox) for 72 hours (h) at 37 °C in a humidified atmosphere in a CO_2 incubator. Subsequently cells were tested for SA-β-gal activity as previously described (13). In brief, cells were washed with PBS, fixed with 0.5% glutaraldehyde (PBS [pH 7.2]) and washed in PBS (pH 7.2) supplemented with 1 mM $MgCl_2$. Cells were stained in X-gal solution (1 mg/ml X-gal [Merk, Darmstadt, Germany]), 0.12 mM $K_3Fe[CN]_6$, 0.12mM $K_4Fe[CN]_6$, 1 mM $MgCl_2$ in PBS at pH 6.0) overnight at 37ºC. Subsequently, all stained cells were photographed with, inverted bright field microscope (Zeiss, Germany), Axio Cam 305 color digital camera system and software ZEN Blue edition Version 2.1.

Cell Cycle Analysis

MCF-7 and MCF-12A cells were seeded at 2.10^5 cells/per well in 48 well plates and treated with indicated chemicals (Eto and Dox) for indicated time points at 37°C in a humidified atmosphere in a CO_2 incubator. After completing the incubation period, the cell-cycle distribution was measured using a Muse Cell Cycle Assay Kit (Millipore Austin, TX, USA) according to the manufacturer's instructions. Harvested cells were washed with PBS for two times and fixed in 70% ethanol overnight. Next day, cells were, washed with PBS and dissolved in 200 μl of Muse cell cycle reagent, and analyzed by Muse Cell Analyzer (Millipore, Austin, TX, USA).

Apoptosis Analysis (Annexin V/7AAD Assay)

MCF-7 and MCF-12A cells were seeded at 10^5 cells/per well in 48 well plates and treated with indicated chemicals (Eto and Dox) for indicated time points at 37°C in a humidified atmosphere in a CO2 incubator. Determination of apoptosis profile was achieved by using Muse® Annexin V & Dead Cell Assay according to the manufacturer's instructions. Quantitative analysis of apoptotic and necrotic cells was done by Muse Cell Analyzer (Millipore, Austin, TX, USA). The percentages of apoptosis and necrosis were calculated.

Protein Isolation and Western Blot Analysis

MCF-7 and MCF-12A cells treated with indicated chemicals (Eto and Dox) for indicated time points at 37 °C in a humidified atmosphere in a CO2 incubator. Cells were harvested and lysed in RIPA Buffer (0,5M TrisHCl, 5M NaCl, 0.5M EDTA, 0,1g SDS and 0,1 g $C_{24}H_{39}NaO_4$,) supplemented with protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany), and 1 mM Na_3VO_4 before use Western blot protocol was followed as described previously (Kilic-Eren et al. 2013). Primary antibodies used; rabbit monoclonal cell death receptors antibodies FAS, DR5, TNFR1 and TNR2, mouse monoclonal anti-p53, mouse anti-p21 monoclonal antibody and γH2AX antibody were from (1: 1000; Cell Signalling, Danvers, MA, USA), monoclonal mouse anti-GAPDH (1:10000; ProteinTech, Rosemont, IL, USA), Horseradish peroxidase-coupled secondary antibodies; anti-mouse IgG-HRP or anti-rabbit IgG-HRPwere (1:1500; Santa Cruz Biotechnology, CA, USA). The ECL solution was from. (Bio-Rad, Hercules, CA, USA). The membranes were visualized by ChemiDoc-ItR2 Imager (UVP, England) device at appropriate times.

RESULTS

MCF12-A and MCF-7 cells induce senescence in response to chemotherapy

In order to examine the expression of death receptors during TIS we used breast cancer cell line MCF-7 and non-tumorigenic (mammary gland epithelial) cell line MCF12-A as control. Cells were treated with DNA damaging chemotherapy agents etoposide (Eto, 100 μM) and doxorubicin (Doxo, 0,1 μM) which are well-known induce senescence rather than apoptosis when used at lower doses. After 72 h exposure to Eto and Doxo cells were stained for SA-β-gal acitivity. As shown in Fig 1, MCF-7 and MCF-12A (control) cells treated with Eto and Dox were positive for SA-β-Gal staining whereas there was no staining detected in controls or DMSO treated cells. (Figure 1).

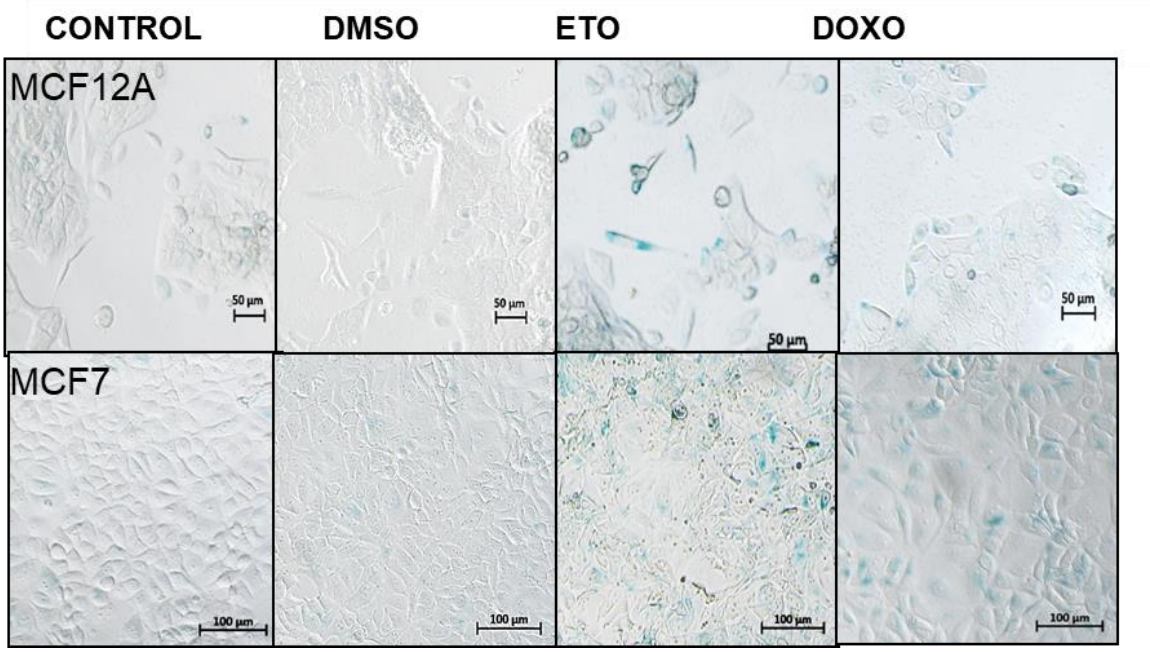

Figure 1. MCF7 and MCF-12A cells untreated (Controls), treated with DMSO, with 50 µM etoposide (ETO) and with 0,1 µM doxorubicin (DOXO), respectively were incubated for 72h. Induction of senescence was evaluated by SA-β-galactosidase activity assay and photographed with an inverted bright field microscope and digital camera system. Data shown are representative of three independent experiments.

Senescent cells are known to induce cell cycle arrest. Therefore, we also examined the cell cycle status of MCF-12 A and MCF-7 cells in response to Eto and Doxo treatment. As shown in Figure 2. 72 h Eto and Doxo treatment caused significant accumulation of cells in the G1/S and G2/M phases of the cell cycle in MCF-12 A and MCF-7 cells, respectively (Figure 2). Thus, lower doses of Eto and Doxo induced senescence both in MCF-12 A and MCF-7 cells.

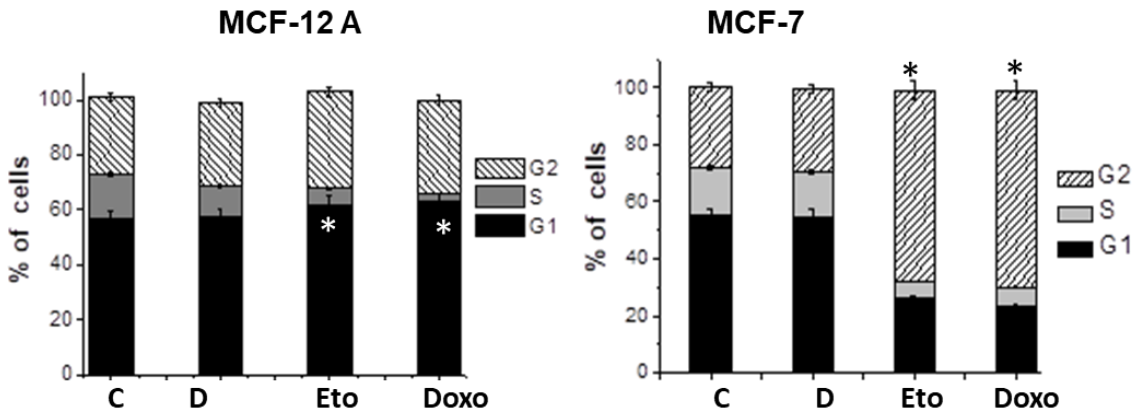

Figure 2. MCF7 and MCF-12A cells left untreated (C, control) and treated with DMSO (D), Doxo (0,1 µM), Eto (50 µM) were incubated for 72h and analyzed for cell cycle profile using Muse® Cell Analyzer. Data shown are the means of three independent experiments ± SD. * denotes p≤0,01 control vs eto, or control vs doxo.

MCF12-A and MCF-7 cells did not induce apoptosis in response to low dose treatment of chemotherapy

We also examined the Eto and Doxo treated MCF-12 A and MCF-7 cells for possible induction of apoptosis. After 72 h exposure to Eto and Doxo cells were stained for Annexin V/7AAD and analyzed by flow cytometry. As shown in Figure 3., Eto and Doxo treatment did not induce significant apoptosis compared to control or dmso treated cells (Figure 3A-B). Eto and Doxo induced 8,6 ± 0,8 % and 9,2 ± 0,5 % of apoptosis in MCF-12A cells, respectively (Figure 3). The amount of apoptosis in MCF 12A control or dmso treated cells were 9, 5 ± 0,5 % and 8 ± 1 %, respectively. Similar results were also obtained in MCF-7 cells where Eto and Doxo induced apoptosis 10,3 ± 0,7 % and 11,1 ± 0,4 %, respectively. The amount of apoptosis induced in control or DMSO treated MCF-7 cells were 10,5 ± 0,5% and 10,3 ± 0,7%, respectively. Our results demonstrate that there was no significant apoptosis induction in MCF12A or MCf-7 cells in response to treatments of Eto or Doxo compared to controls or DMSO treated cells (Figure 3A-B).

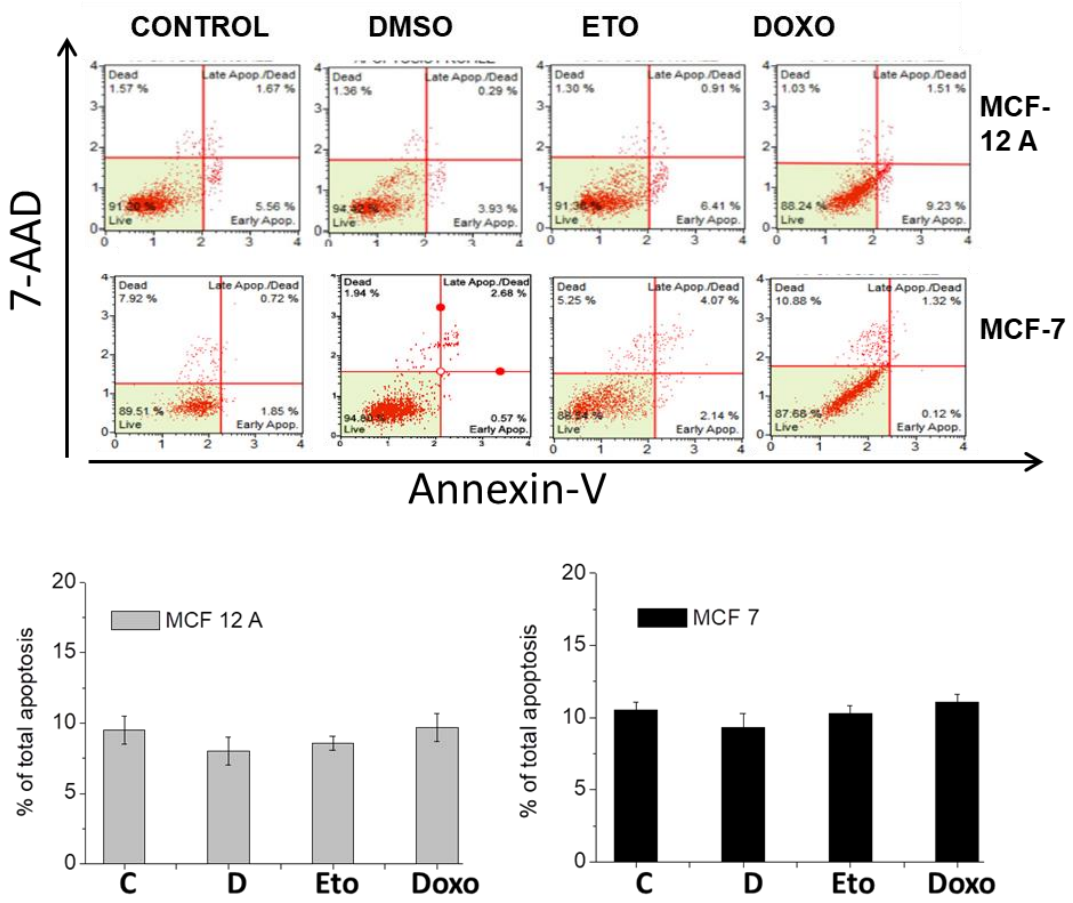

Figure 3. MCF7 and MCF-12A cells untreated (C, control) or treated with DMSO (D), Eto (50 µM), Doxo (0,1 µM) were incubated for 72h. Apoptosis profile was determined by Annexin V/7AAD assay using Muse® Cell Analyzer. Plotted data shown are the means of three independent experiments ± SD. Dot plot data shown are representative of three independent experiments.

Induction of senescence increased expression of cell death receptors selectively in MCF-7 cells

Next, we examined the expression levels of cell death receptors TNFR1 and 2, FAS, and DR5 in response to Eto and Doxo induced senescence in MCF-7and MCF-12A cells. Expressions of p53 and p21 as well as the surrogate marker of DNA damage γ-H2AX were also examined. Doxo and Eto increased protein levels of TNFR1, TNFR2 and DR5 receptors in MCF-7 cells but did not induce any alterations in MCF-12A cells (Figure 4). Interestingly, Eto and Doxo did not induce any change in FAS receptor expression in MCF-7 cells but there was a slight increase in FAS expression in MCF-12A cells. Furthermore, Eto and Doxo induced the expressions of p53 and p21, as well as γH2AX. These results suggest that Eto and Doxo induce senescence through activation of p53 and p21 and increase expression of TNFR1, 2 and DR5 receptors particularly in MCF-7 cells.

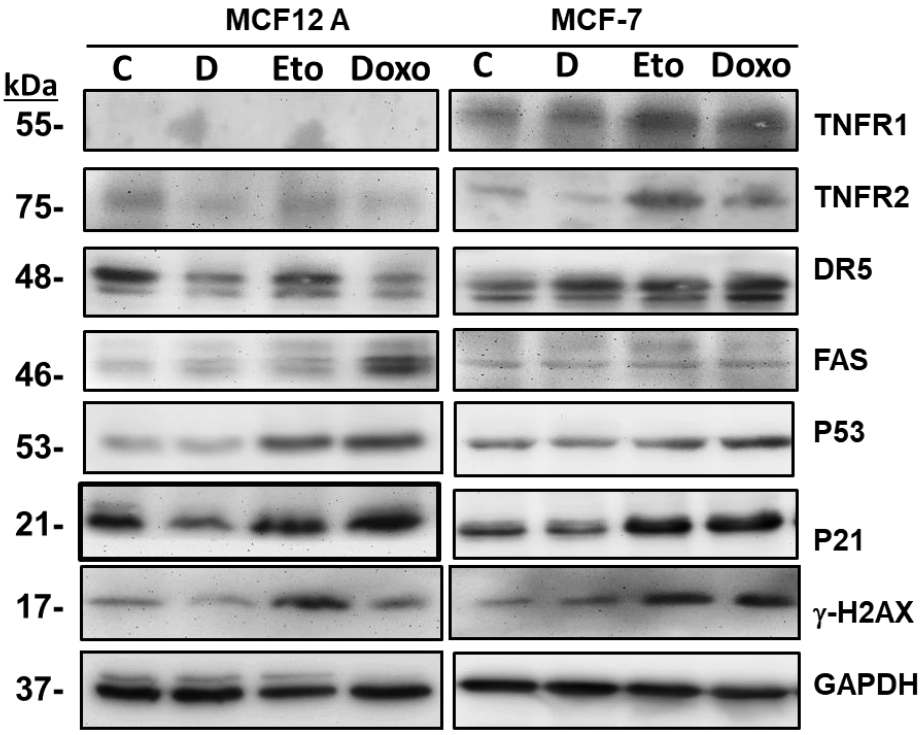

Figure 4. MCF7 and MCF-12A cells untreated (C, control) or treated with DMSO (D), Doxo (0,1 μM), Eto (50 μM) were incubated for 72h. Expression of TNFR1, TNFR2, DR5, FAS, γH2AX, p21 and p53 were assayed by western blotting. GAPDH was used as loading control.

DISCUSSION

Conventional anti-cancer therapies induce senescence in cancer cells both *in vitro* and *in vivo* (Fridlyanskaya et al. 2015, Schosserer et al. 2017). Accordingly, TIS has been identified in human tumor samples in response to radiation and genotoxic chemotherapy (Schosserer et al. 2017). TIS may be used

as an effective target as it can induce a persistent growth inhibitory response and a limited toxicity in early and/or late-stage tumors (Lee and Schmitt 2019, Dörr et al. 2013). It may also function as an alternate to disabled apoptotic response in cancer cells. TIS has been appreciated due to its beneficial effects, however, recent data suggest senescent cells may have some detrimental effects on bystander cells due to the activation of SASP (Dörr et al. 2013, Nardella et al. 2011, Watanabe et al. 2017). Although, much is unknown and consequences of induction of TIS may be context dependent and differ with the cancer type and chemotherapy agent used to induce senescence, strategies targeting senescent cells may be required in future. In this work, we evaluated the expression levels of death receptors in response to TIS in breast cancer cell line MCF-7 and transformed epithelial cell line MCF-12A. We showed that expressions of cell death receptors including TNFR1 and R2 and DR5 were increased selectively in MCF-7 cancer cells, in response to doxorubicin or etoposide induced senescence. We also showed that the induction of senescence was triggered by activation of DDR signaling and p53 and p21 activation.

We used two well-known DNA damaging chemotherapic agents, etoposide and doxorubicin at low doses to induce senescence. Our data on SA-β-gal activity confirm the induction of senescence in both cell lines. Additionally, we provide data on cell cycle analysis confirming, the G1/S and G/M cell cycle arrest in MCF-12A and MCF-7 cells, respectively, during induction of senescence. Our data showing that induction of p53, p21 as well as γH2AX (by western blotting) in both cell lines suggest that TIS involves DDR signaling and subsequent activation of p53 and p21. More importantly, our data provide evidence to TIS increased expression of cell death receptors TNFR1, 2 and DR5 in MCF-7 cells but not in MCF-12A cells. To exclude the possibility that expression of death receptors might have increased due to induction of apoptosis, we evaluated the ability of etoposide and doxorubicin to induce apoptosis when used for induction of senescence. Our data from Annexin V/7AAD measurements confirmed that low doses of etoposide or doxorubicin did not induce apoptosis in both cell lines. Hence, here we show that breast cancer cells display high expression of cell death receptors during TIS, which may be a potential target in senescent MCF-7 cancer cells.

Recent studies suggest that chemotherapy rarely induce pathological full response in p53wt human breast tumors which is associated with poor survival (McDermott et al. 2019). Further, the p53wt tumors have been found to induce senescence in response to chemotherapy with concomitant activation of SASP leading to a persistent cell population constituting residual disease that drives relapse and poor patient survival (Hassan et al. 2018). A previous report showing chemotherapy-induced senescent cells are capable of engulfing neighboring cells, explains how senescent cells remain persistent (Coppé et al. 2008, Tonnessen-Murray et al.2019). Data clearly suggest that remaining senescent cancer cells are associated with relapse and poor survival in breast cancer cells and warrants for development of further strategies targeting remaining senescent cells after chemotherapy. We believe that our data provide a

basis for further investigation of death receptor mediated targeting of senescent cells and may suggest a potential therapeutic strategy.

Conclusion

In conclusion, our study demonstrates that Etoposide and Doxorubicin induced senescent breast cancer cells selectively express death receptors including TNFR1, and 2, as well as DR5. Our data provide basis for investigation of selective activation of death receptors in breast cancer cells for elimination of therapy induced senescence which may contribute to develop potential novel therapeutical strategies in future.

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