Evasion of a Single-Step, Chemotherapy-Induced Senescence in Breast Cancer Cells: Implications for Treatment Response

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Abstract

Purpose: The purpose of this study is to define the mechanistic basis for recovery of proliferative capacity in breast tumor cells after chemotherapy. Here, we test the hypothesis that evasion of senescence confers resistance to chemotherapeutic drugs and ionizing radiation.

Experimental Design: MCF-7 cells were treated with a single, clinically relevant dose (0.75-1.0 μmol/L) of Adriamycin. Two weeks following induction of senescence, clonal outgrowths were expanded and characterized in terms of senescence-associated β-galactosidase activity, gene expression profiles (Affymetrix U95 probe sets, Affymetrix, Santa Clara, CA) with confirmatory Western analyses, and telomerase activity following a second drug treatment. Levels of intracellular Adriamycin, as well as cross-resistance to other therapeutic agents, were also determined to define the resistance phenotype.

Results: A senescence-resistant (SR) clone (clone 2) was identified that was largely refractory to both Adriamycin-induced and γ-irradiation–induced senescence. Clone 2 continued to proliferate and maintain high levels of telomerase activity following a second drug treatment, when treated parental cells expressed very low levels of telomerase and many positive cell cycle regulators. SR clone 2 also expressed substantially more cdc-2 than parental cells and undetectable levels of MDR1, showed an intact p53 checkpoint and only a modestly lower level of intracellular drug accumulation, while exhibiting cross-resistance to other topoisomerase inhibitors.

Conclusions: SR clone 2 is intrinsically resistant to DNA damage–induced senescence perhaps through an ability to prevent down-regulation of cdc-2. Telomerase is a marker of proliferative recovery for breast cancer cells after chemotherapy exposure. Evasion or escape from a single-step, drug-induced senescence may represent a unique and previously unrecognized drug-resistance phenotype.

Bypassing cellular senescence is essential for immortalization, which is a critical and possibly rate-limiting step of malignant transformation (1). Replicative senescence is a growth-arrested state of aged cells mediated, in part, by telomere shortening and by the recognition of shortened, uncapped telomeres as damaged DNA (2). Cells that escape this proliferative barrier continue to divide, resulting in further telomere shortening and the accrual of additional mutations until crisis when the vast majority of cells die (1). The rare cell that adopts a telomere maintenance mechanism, which in most cases involves activation of telomerase (3), becomes immortal.

Both in vivo (4, 5) and in vitro (6–9) data are accumulating to indicate that cancer cells retain their ability to senesce. In fact, senescence may be the predominant response of cells derived from solid tumors following a wide variety of DNA-damaging agents including several conventional anticancer chemotherapeutics and γ-irradiation. However, unlike replicative senescence, this growth-arrested state is associated with more rapid kinetics (hence, the term “accelerated senescence” or stasis) and telomere dysfunction without overall net telomere shortening (6, 9).

We have recently reported that MCF-7 breast cancer cells undergo telomere length–independent senescence following a single, clinically relevant dose of Adriamycin (6). This senescence response is dependent on functional p53 and proceeded by a high frequency of telomere-specific chromosomal abnormalities. Compelling data have been generated from in vitro culture systems (6–9), transgenic murine models (5), and analyzing breast tumor specimens (4) to indicate that accelerated senescence is a physiologic mechanism of DNA damage response occurring in cancer therapy. This, coupled with the tendency for breast cancers to respond only transiently to standard chemotherapeutic regimens (10, 11), prompted us to use our MCF-7 model system to study mechanisms of proliferative recovery. Here, we extensively characterize a clonal population of MCF-7
Materials and Methods

Materials. RPMI 1640 and trypsin-EDTA (0.5% trypsin, 5.3 mmol/L EDTA) were obtained from Life Technologies, Inc. (Rockville, MD); t-glutamine, penicillin/streptomycin (10,000 units of penicillin per milliliter and 10 mg/mL streptomycin), and fetal bovine serum (FBS) were purchased from Whittaker Bioproducts (Walkersville, MD). Adriamycin was obtained from Sigma Chemical Company (St. Louis, MO), reconstituted in molecular biology grade water, and stored as aliquots at −20°C until dilution in culture media for treatments. Camptothecin, VM-26, and taxol were also purchased from Sigma Chemical, aliquoted, and stored at −20°C as recommended by the manufacturer.

Cell culture and treatment regimens. The MCF-7 breast tumor cell line was obtained from the National Cancer Institute Frederick Cancer Research Facility. The isogenic cell line, MCF-7/hTERT, was established by stable retroviral infection as previously described (6). Cells were maintained as monolayer cultures in RPMI 1640 supplemented with glutamine (0.292 mg/mL), penicillin/streptomycin (0.5 mL/100 mL media), 10% FBS. Cells were cultured at 37°C in 5% CO2 and 100% humidity. Twenty-four hours after plating, MCF-7 or MCF-7/hTERT were exposed to 0.75 or 1.0 humidity. Twenty-four hours after plating, MCF-7 or MCF-7/hTERT were

Results

We have previously reported that acute Adriamycin exposure causes a widespread p53-dependent, senescence arrest in MCF-7 breast cancer cells, which is characterized by length-independent
Evasion of Chemotherapy-Induced Senescence

Evasion of chemotherapy-induced senescence is a common occurrence in cancer cells. When breast cancer cells are exposed to Adriamycin, they often enter senescence, a state of irreversible growth arrest. However, some cells are able to evade this senescence and continue to grow. This phenomenon is not well understood but is believed to contribute to the development of chemoresistance.

In a study aimed at understanding the mechanisms behind evasion of senescence, researchers treated MCF-7 cells with Adriamycin and then isolated clonal populations that had either undergone senescence or evaded it. The clonal populations were then compared to understand the nature of this evasion.

Western data showed that p53 protein accumulates in MCF-7 cells following DNA damage, a characteristic of the senescent state. In contrast, SR clone 2 was largely refractory to the senescence response. This suggests that the evasion of senescence is unlikely due to differences in the levels of drug uptake and/or efflux via a MDR-1-independent mechanism.

Microarray analysis identified a number of genes that were differentially expressed in untreated versus Adriamycin-treated MCF-7 cells. The genes identified included those involved in DNA damage response, telomere maintenance, and cell cycle regulation. Among these, p21WAF-1 and BTG2 were upregulated in senescent cells, indicating that they are key regulators of the senescence response.

To test whether cells that evaded Adriamycin-induced senescence showed resistance to other chemotherapeutic agents, SR clone 2 was exposed to VM-26 (teniposide), camptothecin, and taxol. However, no differential effect was observed for taxol, a potent inducer of apoptosis in breast cancer cells.

The study demonstrates the importance of understanding the mechanisms behind evasion of senescence, as this process is crucial for the development of chemoresistance in cancer cells. Further research is needed to identify the specific genes and pathways involved in this process.

Fig. 1. Clonal outgrowth is characterized by evasion of Adriamycin-induced senescence. A, whereas MCF-7 parental cells undergo widespread senescence 72 hours after Adriamycin exposure as assessed by senescence-associated β-galactosidase staining (middle), SR clone 2 predominantly consists of cells refractory to senescence (right). Untreated (MCF-7 unt) MCF-7 cells show little or no staining for senescence-associated β-galactosidase. B, percent of β-galactosidase- positive cells was compared in parental (MCF-7) versus SR clone 2 (SR cl.2) cells 3 days after drug treatment; columns, mean from three representative fields of 100 cells; bars, SD.

5 Unpublished data.
both of which also exhibited cross-resistance to VM-26 and camptothecin, but not taxol (data not shown). Collectively, these data clearly show that SR clones are cross-resistant to DNA-damaging agents, a phenotype that is at least in part due to evasion of senescence.

Our microarray data (Table 1), coupled with confirmatory Western blots (cyclin B1 and cdc-2; Fig. 2) and/or OpArrays from Operon Technologies (Alameda, CA; cyclins B1 and E, and PCNA; data not shown), indicate that the expression levels of several positive cell cycle regulators are dramatically reduced in Adriamycin-treated parental cells, whereas expression levels are less severely diminished in treated SR clone 2 cells. Whereas the cyclin A transcript was undetectable by Affymetrix analysis in all cell lines tested, the protein was significantly reduced in parental MCF-7 cells 24 and 48 hours after drug exposure and much less diminished in Adriamycin-treated SR clone 2 cells (Fig. 2A). In contrast to the sustained down-regulation of cdc-2 protein in MCF-7 cells beginning 3 days after drug treatment (Fig. 2A and B), SR clone 2 continues to express high levels of cdc-2 mRNA (Table 1) and protein (Fig. 2B). The tendency for SR clone 2 cells to continue expressing positive cell cycle regulators after drug treatment, together with the presence of occasional, random SR clone 2 cells positively staining for β-galactosidase and exhibiting morphologic hallmarks of senescence (Fig. 1A), suggests that a minority subpopulation of SR clone 2 senesces whereas most have proliferative potential.

We were surprised that the constitutive RNA levels of the cell cycle regulators shown in Table 1 were expressed 4- to 7-fold higher in SR clone 2 relative to parental MCF-7, whereas the p53-related effectors were expressed at seemingly lower levels in SR clone 2 cells (column 4 of Table 1). However, when Western blotting was done to directly compare the basal protein levels for all of the genes listed in Table 1, this trend was not confirmed. In fact, the constitutive levels of cyclins A and B1 seemed slightly higher in parental MCF-7 cells compared with SR clone 2, whereas the differences in the basal levels of the other proteins were unremarkable (Fig. 2A; data not shown).

| Table 1. Expression profiles of selected genes after Adriamycin exposure |
|-----------------------------|-----------------|-----------------|-----------------|-----------------|
| Gene (accession no.) | Parental + AdR vs unt | SR cl2 + AdR vs unt | SR cl2 + AdR vs parental + AdR | SR cl2 unt vs parental unt |
| Cyclin B1 (M25753) | 8.4* Down† | 2.8 Down | 2.5 Up† | 7.1 Up |
| Cyclin B2 (AF091433) | 11.8 Down | NC | 6.0 Up | 7.5 Up |
| PCNA (J05614) | 3.9 Down | 2.1 Down | 2.1 Up | 4.1 Up |
| cdc-2 (M68520) | 10.9 Down | NC | 4.3 Up | 7.4 Up |
| p53-Related | | | | |
| p21Waf-1 (U03106) | 13.5 Up | 5.2 Up | NC | 10.0 Down |
| BTG2 (U72649) | 7.6 Up | 4.1 Up | NC | 6.6 Down |
| GADD45A (M60974) | 4.8 Up | NC | NC | 3.2 Down |
| Bax-α (L22473) | 3.0 Up | 5.5 Up | 2.2 Down | 12.0 Down |
| p53 (X02469) | NC | NC | NC | NC |

Abbreviations: NC, no change >2-fold in either direction; AdR, Adriamycin; SR cl2, SR clone 2; unt, untreated.
*Values represent fold change between the cultures listed in the column header.
†Direction of the fold change in the first culture listed in the column header relative to the second.
We have previously reported that concomitant with Adriamycin-induced senescence is a reduction of telomerase activity (6), likely reflecting a nonproliferative culture. Here the levels of telomerase activity were assayed by telomeric repeat amplification protocol beyond the initial senescence arrest into the recovery phase. As shown in Fig. 5, telomerase activity closely parallels the kinetics of repopulation of MCF-7 cells, as an increase in activity is observed at days 6 and 9 as recovered cells take over the population. Moreover, SR clone 2 continuously expresses high levels of telomerase despite a second drug treatment, suggesting that there is only a transient arrest and no substantial senescence in SR clone 2 cells after treatment. Taken together, these data show that telomerase is a marker of proliferative recovery in breast cancer cells after chemotherapy exposure.

Whereas a main cellular function of telomerase is to circumvent the "end replication problem" owing to the directional limitations of DNA polymerase (23, 24), telomerase may possess other biological functions (25–27). Here, we test whether one such role may be to facilitate proliferative recovery following a chemotherapeutic regimen associated with telomere-specific damage. Using MCF-7 cells stably expressing hTERT (MCF-7/hTERT; ref. 6), we compared its frequency of recovery to parental MCF-7 cells and found there is no significant difference following a 2-hour treatment with either 0.75 or 1.0 μmol/L Adriamycin (data not shown).

**Discussion**

Cellular senescence is becoming recognized as a common in vivo and in vitro response of cancer cells to DNA damage caused by many chemotherapeutic regimens. In fact, cultures of human cancer cells derived from solid tumors tend to undergo senescence arrest following exposure to standard chemotherapeutic agents, such as Adriamycin (15, 16), SN-38 (7), or VM-26.5 te Poele et al. (4) found that 41% of specimens from breast cancer patients who received adjuvant chemotherapy were positively stained for senescence-associated β-galactosidase, whereas only 10% of the specimens from patients who underwent surgery without chemotherapy were positive for senescence-associated β-galactosidase, which shows that chemotherapy induces senescence in vivo as well. Data generated from transgenic murine models indicate that tumor response to the chemotherapeutic agent, cyclophosphamide, positively

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Fig. 3. Comparison of the steady-state levels of Adriamycin (AdR) in MCF-7 versus SR clone 2. Intracellular drug accumulation was spectrophotometrically measured as described in Materials and Methods. Columns, mean from six independent experiments; bars, SD.

Fig. 4. SR clone 2 cells exhibit cross-resistance to other chemotherapeutic drugs and γ-irradiation, indicating intrinsic resistance to DNA damage–induced senescence. A, relative viability of parental and SR clone 2 cells 72 hours after drug exposure (0.75 μmol/L Adriamycin, 10 μmol/L camptothecin, 5 μmol/L VM-26, or 5 μmol/L Taxol for 2 hours). B, comparison of percent β-galactosidase positivity in parental versus SR clone 2, 7 days after exposure to 10 Gy γ-irradiation. Columns, mean from three representative fields of 100 cells; bars, SD.

Fig. 5. Telomerase is a marker for proliferative recovery. Telomerase activity was measured over time by telomeric repeat amplification protocol as described in Materials and Methods. IC, internal control, used to normalize sample-to-sample variation; Q, quantitation of telomerase activity, taken as a ratio of IC to the telomerase products (ladder).
correlated to the amount of senescence response (5), indicating that senescence is indeed a relevant factor in determining treatment outcome.

A persistent clinical challenge in the management of cancer is tumor recurrence. For most somatic malignancies, chemotherapy without concurrent radiation produces a 20% to 40% response rate with >95% of these cases being partial remissions (typically being defined as at least a 50% reduction in tumor volume; refs. 28, 29). In the present study, we show evasion of a single-step, Adriamycin-induced senescence as a novel mechanism by which breast cancer cells might recur and/or persist after chemotherapy. Our characterization of an early senescence arrest of MCF-7 cells followed by proliferative recovery correlates well with the sequence of chemotherapy treatment for breast cancers, although recognizably with a much more protracted kinetics of recurrence in vivo. Here, senescent-resistant cells are extensively characterized and found to be unique from the vast majority of Adriamycin-resistant MCF-7 cells previously described (18–20), which persists following chronic, high-dose exposure of Adriamycin owing to a strong selective pressure for efficient drug efflux via either amplification or transcriptional up-regulation of MDR-1. An analysis of the transcriptional profiles of such MDR-1 over-expressing MCF-7 cells has been previously reported (30). However, this former study focused on much earlier time points (i.e., hours rather than days after drug exposure), therefore making it difficult to compare data generated from the two studies.

SR clones arose following a single, clinically relevant dose of Adriamycin and have undetectable MDR-1 transcript and its protein product glycoprotein 120. Several lines of evidence indicate that evasion of senescence by SR clone 2 is not due to pharmacokinetic resistance, but rather an intrinsic resistance to DNA damage–induced senescence. Although SR clone 2 accumulates only ~65% of the steady-state level of Adriamycin as parental cells, sufficient drug is present intracellularly to cause DNA damage, as shown by the significant accumulation of p53 and induction of p21WAF1, as well as a comparable up-regulation of the p53-mediated DNA damage response gene, BTG2 (21), to that observed in Adriamycin-treated parental cells. The induction of additional downstream targets of p53 (i.e., GADD45 and bax-), after Adriamycin exposure further supports the presence of functional p53 in SR clone 2. Our finding that the vast majority of cells in SR clone 2 failed to grow arrest following treatment with another topoisomerase II inhibitor (VM-26) and a topoisomerase I inhibitor (camptothecin) suggests that evasion of senescence is not limited to Adriamycin specifically or to the class of type II topoisomerase inhibitors. However, our most compelling data to indicate that evasion of senescence is due to an intrinsic defect in DNA damage–induced senescence is that SR clone 2 was also refractory to senescence induced by γ-irradiation, which, like topoisomerase inhibitors (and not taxol), causes DNA damage predominantly in the form of double-strand breaks (31–33). Despite an inability to undergo senescence, SR clone 2 cells accumulated similar levels of p53 and p21WAF1 as parental MCF-7 cells 4 and 24 hours following irradiation (data not shown).

Based on the frequency of SR clone 2 recovery (1:27,000 with an initial seeding of 100,000 cells) and the typical size of the clonal outgrowths (<300 cells after 2–3 weeks), it seems reasonable to suggest that evasion of senescence is a relatively frequent event that involves an initial, transient growth arrest followed by re-entry into the cell cycle. The fact that we were able to identify two additional clones in a screen of 16 that evaded growth inhibition following acute exposure to senescence-inducing agents further shows that this is more than a rare, fortuitous event. Highly complementary to our data, Wang et al. (7) have recently reported a reversible senescence arrest in human glioblastoma cells following exposure to the topoisomerase I inhibitor, SN-38. Interestingly, this glioblastoma model system parallels the response of breast tumor cells to Adriamycin in additional ways. Specifically, in both systems, induction of accelerated senescence requires functional p53 and is independent of p16, whereas inactivation of p53 triggers apoptosis instead of senescence. A similar regrowth phenomenon has been described in tumor xenograft studies where irradiation or drug treatments produce what is commonly called "tumor growth delay" (34). Our comparative expression profiles and confirmatory Western blotting for positive cell cycle regulators are consistent with SR clone 2 (at least the majority of the population) proliferating after a second drug exposure. Further support for a proliferative state is that SR clone 2 cells continue to express high levels of telomerase activity after drug treatment. In contrast, parental cells, which are not dividing and, therefore have no need for a telomere maintenance mechanism, down-regulate telomerase. However, when recovered MCF-7 cells begin emerging (at around day 9), telomerase activity reappears, establishing telomerase as a marker of proliferative recovery for breast tumor cells after chemotherapy.

If SR clone 2 accumulates sufficient amounts of Adriamycin to incur DNA damage and to activate the p53 cell cycle check point, this begs the question "What is the defect or mechanism by which SR clone 2 overrides growth arrest following DNA damage?" One possibility that we plan to explore is that evasion of senescence may be due to a failure to down-regulate cdc-2 following acute Adriamycin exposure. When parental MCF-7 cells are beginning to undergo widespread senescence (day 3), there is a 90% reduction in the levels of cdc-2 mRNA coupled with a sustained (i.e., at least 12 days after Adriamycin treatment) down-regulation of cdc-2 protein. Previous studies have similarly reported that cdc-2 levels are down-regulated in senescent human and rodent fibroblasts (35), senescent colon cancer cells after Adriamycin exposure (16), and in H1299 cancer cells after γ-irradiation (36). In contrast, SR clone 2 continues to express high levels of cdc-2 after drug treatment. Maintaining high levels of cdc-2 could potentially allow SR clone 2 to bypass the G2-M checkpoint and to re-enter the cell cycle because inactivation of cdc-2 has been shown to be a requisite for p53-mediated G2-M arrest (37). Recognizing that elevated expression of cdc-2 has been associated with malignant breast lesions (38) and with resistance to therapy in non-Hodgkin’s lymphoma (39) makes this a particularly exciting area of further study.

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