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
cells arising following a single, clinically relevant dose of Adriamycin and propose escape from DNA damage–induced senescence as a novel mechanism by which breast cancer cells evade conventional chemotherapies.

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); 1-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), and 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 mol/L Adriamycin for 2 hours. In the case of parental cells, 16 days after drug treatment, foci of recovered cells were selectively trypsinized using cloning cylinders and then expanded for analysis. Recovered clonal populations (i.e., senescence resistance (SR) clone 2) were subsequently treated with 0.75 to 1.0 mol/L Adriamycin and analyzed (as described below). To test for senescence cross-resistant phenomena, SR clone 2 cells or parental cells as a reference were exposed to a single, 10 Gy dose of γ-irradiation or to a 2-hour treatment of camptothecin (10 mol/L), VM-26 (5 mol/L), or taxol (5 mol/L). Following all drug exposures, cultures were washed twice in PBS and then maintained in fresh media until analysis.

Growth assays. To determine the frequency of recovery for MCF-7 and MCF-7/hTERT, 100,000 cells were seeded in triplicate 100 mm2 culture dishes, exposed to Adriamycin (0.75 mol/L) for 2 hours, washed in PBS, and then maintained in culture media for 16 days with weekly media replacement. Dishes were fixed in 4% buffered formaldehyde and stained with 0.4% crystal violet in PBS. Clonogenic survival (i.e., frequency of recovery) was presented as mean number of colonies per dish ± SD. To determine the growth effects of acute Adriamycin exposure on MCF-7 and for cross-resistance studies, MCF-7 and SR clone 2 cells were seeded in triplicate wells at 8,000 cells per well of a 96-well cluster plate. At the indicated times after drug exposure, cell viability was assessed using a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This involved adding 100 μL of 2 μg/mL MTT (Sigma Chemical) per well, incubating in the dark for 3 hours, carefully removing the MTT, and then adding 100 μL DMSO per well. After gently rocking for 5 minutes, absorbance was measured at 490 nm and presented relative to untreated controls.

β-Galactosidase histochemical staining. Senescence-associated β-galactosidase histochemical staining was done as previously described (6, 12). The percentage of positively stained cells was determined by counting three random fields of at least 100 cells each. Images of representative fields were captured under 20× magnification.

Expression analysis using oligonucleotide (Affymetrix) microarrays. Total cellular RNA was isolated using TRIzol reagent (Life Technologies) from untreated MCF-7 and SR clone 2 cells and MCF-7 and SR clone 2 cells 3 days after acute (2 hours at 1.0 mol/L concentration) Adriamycin exposure. Forty micrograms of total RNA from each sample were used to generate double-stranded cDNA using a 24-mer oligo-dTMP primer with a T7 RNA polymerase promoter site added to the 3’ end (Superscript cDNA Synthesis System; Life Technologies). After second-strand synthesis, in vitro transcription was done using the Enzo BioArray High Yield RNA Transcript Labeling kit (Enzo Diagnostics, Farmingdale, NY) to produce biotin-labeled cRNA. Twenty micrograms of the cRNA product were fragmented at 94°C for 35 minutes into 35 to 200 bases in length. The fragmented cRNA was then added to a hybridization solution containing 100 mol/L MOPS, 1 mol/L Na+, and 20 mol/L EDTA in the presence of 0.01% Tween 20 to a final cRNA concentration of 0.05 mg/mL. Hybridization was done for 18 to 20 hours by incubating 200 μL of the hybridization mix to HG-U95Av2 containing 12,625 probe sets or HG-U133A containing >45,000 probe sets. Each microarray was washed and stained with streptavidin-phycocerythrin and scanned at a 6 μm resolution by the Agilent G2500A Technologies Gene Array scanner (Agilent Technologies, Palo Alto, CA) according to the GeneChip Expression Analysis Technical Manual procedures (Affymetrix). Data analysis from the scanned chips was done using the Microarray Suite 5.0 software (Affymetrix). Detailed protocols for data analysis of Affymetrix microarrays and extensive documentation of the sensitivity and quantitative aspects of the method have been described (13). Briefly, mismatch probes act as specificity controls that allow the direct subtraction of both background and cross-hybridization signals. To determine a particular RNA transcript abundance, the average of the difference representing perfect match-mismatch for each gene-specific probe family was calculated. This data was transferred to GeneSpring software (Silicon Genetics, Redwood City, CA) for additional analysis.

Western blot analysis. Cell cultures were lysed in 60 mol/L Tris (pH 6.8) containing 2% SDS and a cocktail of protease inhibitors (Sigma Chemical) at the indicated time points. Whole cell lysates were boiled for 5 minutes, briefly sonicated, and then centrifuged for 10 minutes at 10,000 × g at 4°C. Lysates from untreated cultures of MCF-7 and SR clone 2 cells served as a baseline for constitutive levels of expression of the various proteins. Protein concentrations were determined using a Lowry-based spectrophotometric assay (Bio-Rad, Hercules, CA), according to the manufacturer’s protocol. Ten to twenty micrograms of each sample were separated by SDS-PAGE and electrotransferred onto nitrocellulose membrane. A standard blotting procedure was done using monoclonal antibodies directed against p53 (1:5,000; Transduction Laboratories, Newington, NH), p21WAF1 (1:1,000; Transduction Laboratories), cyclin B1 (1:100; Novoceastra, Newcastle upon Tyne, United Kingdom), cyclin A (1:500, Novoceastra), cdc-2 (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), proliferating cell nuclear antigen (PCNA, 1:1,000; Santa Cruz Biotechnology), GADD45 (1:1,000, Santa Cruz Biotechnology), and bax-α (1:500; Santa Cruz Biotechnology), followed by peroxidase-conjugated anti-mouse IgG (1:10,000; Amer sham). To control for protein loading, all membranes were subsequently probed with a β-actin antibody (1:500, Sigma Chemical).

Cellular accumulation of Adriamycin. Parental and SR clone 2 MCF-7 cells were seeded at a density of 1.3 × 106 cells per milliliter. Twenty-four hours later, cells were treated in monolayer culture with 10 μmol/L Adriamycin for 3 hours at 37°C. Cells were then washed twice with ice-cold PBS, harvested by trypsinization, and pelleted by centrifugation at 4°C. Cell pellets were resuspended in 0.3 N HCl and sonicated for 10 pulses at 200 W-s. Following centrifugation at 1,000 × g for 30 minutes at 4°C, supernatants were removed and assayed spectrophotometrically using an excitation wavelength of 470 nm (14). Six independent experiments were conducted with values being presented as mean ± SD.

Telomeric repeat amplification protocol. Telomerase activity was measured using the TRAPEze kit (Intergen, Burlington, MA) as previously described (6, 15).

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...
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telomere dysfunction (6). Here, we find that following early senescence arrest, MCF-7 cells recover at a rate of ~1 in 27,000 following the treatment of 100,000 cells with 1 μmol/L Adriamycin for 2 hours. To define the mechanism(s) of this regrowth, we have isolated clonal populations that emerge 2 to 3 weeks after drug exposure.

One such clone evaded drug-induced senescence following a second, 2-hour Adriamycin treatment, hence the name SR clone 2. As we have previously shown (6), MCF-7 cells treated with Adriamycin widely express senescence-associated β-galactosidase activity and exhibit morphologic features indicative of senescence. In contrast, SR clone 2 was largely refractory to the senescence response (Fig. 1). To understand the nature of this senescence resistance phenotype, microarray analysis was done 3 days after drug treatment, a time when widespread senescence is consistently beginning to be observed (6). Fluorescent cDNA probes were prepared from total RNA derived from Adriamycin-treated parental and SR clone 2 cell populations, as well as untreated MCF-7 and SR clone 2 cells, and used for differential hybridization with Affymetrix oligonucleotide arrays (chip set HG_U95AV2 or HG_U133A containing >12,000 or >45,000 genes, respectively). We have identified a number of genes that were differentially expressed in untreated versus Adriamycin-treated MCF-7 cells (Affymetrix data, Table 1; confirmatory Western data, Fig. 2). As previously reported in human colon cancer cells following acute Adriamycin exposure (16), several cell cycle regulators were markedly down-regulated, including cyclin B1, PCNA, and cdc-2. Because p53 protein accumulates in MCF-7 cells following DNA damage, it was not surprising to observe the up-regulation of a number of p53-related genes, most notably p21WAF-1 with its 13.5-fold increase in mRNA transcript, which was confirmed by Western blotting (Fig. 2A). There was no change in the status of p53 mRNA levels following Adriamycin treatment as expected for a protein that is regulated posttranslationally (17).

Using untreated parental MCF-7 and SR clone 2, respectively, as baselines, gene expression profiles of Adriamycin-treated MCF-7 and treated SR clone 2 cells were compared to obtain clues for the mechanism(s) underlying evasion of senescence (Table 1). SR clone 2, which arose after a single, clinically relevant dose of Adriamycin, has undetectable levels of MDR-1 mRNA by Affymetrix analysis and protein (immunohistochemical data not shown), clearly distinguishing this clonal population from the more classic Adriamycin-resistant MCF-7 cells, which have been exposed to chronic, high-dose drug exposure and overexpress MDR-1 (18–20). To test whether decreased drug uptake and/or an increase efflux via a MDR-1–independent mechanism causes evasion of senescence, we spectrophotometrically measured the relative amount of steady-state Adriamycin in SR cells compared with parental MCF-7 following a single dose exposure. As shown in Fig. 3, SR clone 2 cells accumulate less drug (~35% less) than parental MCF-7. However, evasion of senescence is unlikely due to this marginal decrease because another recovered MCF-7 clonal population accumulates a similar amount of Adriamycin as SR clone 2 (~21% less than parental cells), yet still senesces (data not shown). Because Adriamycin-treated SR clone 2 exhibited a dramatic accumulation of p53 protein (Fig. 2A), induction of p21WAF-1 (Fig. 2A), and up-regulation of the DNA damage response gene, BTG2 (data not shown; ref. 21), both downstream effectors of p53, this close is exposed to sufficient levels of drug to incur DNA damage and is capable of triggering a damage response. Our Affymetrix data indicated that there is no difference (Table 1) in the expression levels of a number of p53-responsive targets when comparing Adriamycin-treated SR clone 2 relative to Adriamycin-treated parental MCF-7, further demonstrating that this SR clone has an intact DNA damage check point. Because the expression level of p21WAF-1 protein seemed to be slightly lower in SR clone 2 relative to parental cells at the 72-hour time point (Fig. 2A), we evaluated the levels of p21WAF-1 up to 9 days after drug treatment to determine whether an inability to sustain high levels of this growth inhibitor associates with evasion of senescence. However, both cell lines sustained similar, high levels of p21WAF-1 (data not shown).

To test whether cells that evaded Adriamycin-induced senescence showed resistance to other chemotherapeutic agents, SR clone 2 was exposed to VM-26 (teniposide), camptotheacin, and taxol. As shown in Fig. 4A, this clone clearly exhibits cross-resistance to other chemotherapeutic agents that induce senescence (camptotheacin (7), and VM-26).5 However, no differential effect was observed for taxol, a potent inducer of apoptosis in breast cancer cells (22). Moreover, SR clone 2 cells evaded γ-irradiation–induced senescence (Fig. 4B), despite accumulation of p53 protein and induction of p21WAF-1 (data not shown). Of note, two additional SR clones have been identified following a single dose exposure to Adriamycin.

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**

<table>
<thead>
<tr>
<th>Gene (accession no.)</th>
<th>Parental + AdR vs unt</th>
<th>SR cl2 + AdR vs unt</th>
<th>SR cl2 + AdR vs parental + AdR</th>
<th>SR cl2 unt vs parental unt</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell cycle regulators</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin B1 (M25753)</td>
<td>8.4* Down†</td>
<td>2.8 Down</td>
<td>2.5 Up†</td>
<td>7.1 Up</td>
</tr>
<tr>
<td>Cyclin E2 (AF091433)</td>
<td>11.8 Down</td>
<td>NC</td>
<td>6.0 Up</td>
<td>7.5 Up</td>
</tr>
<tr>
<td>PCNA (U05614)</td>
<td>3.9 Down</td>
<td>2.1 Down</td>
<td>2.1 Up</td>
<td>4.1 Up</td>
</tr>
<tr>
<td>cdc-2 (M68520)</td>
<td>10.9 Down</td>
<td>NC</td>
<td>4.3 Up</td>
<td>7.4 Up</td>
</tr>
<tr>
<td><strong>p53-Related</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p21Waf-1† (U03106)</td>
<td>13.5 Up</td>
<td>5.2 Up</td>
<td>NC</td>
<td>10.0 Down</td>
</tr>
<tr>
<td>BTG2 (U72649)</td>
<td>7.6 Up</td>
<td>4.1 Up</td>
<td>NC</td>
<td>6.6 Down</td>
</tr>
<tr>
<td>GADD45A (M60974)</td>
<td>4.8 Up</td>
<td>NC</td>
<td>NC</td>
<td>3.2 Down</td>
</tr>
<tr>
<td>Bax-α (L22473)</td>
<td>3.0 Up</td>
<td>5.5 Up</td>
<td>2.2 Down</td>
<td>12.0 Down</td>
</tr>
<tr>
<td>p53 (X02469)</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
</tbody>
</table>

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.

![Fig. 2. A, differential expression of select gene products after Adriamycin treatment confirms microarray data. B, a time course study reveals prolonged cdc-2 down-regulation in MCF-7 cells and sustained cdc-2 expression in SR clone 2 after Adriamycin treatment. Cell lysates were prepared as described in Materials and Methods from MCF-7 and SR clone 2 at the indicated times after drug treatment. Untreated cultures were included as a baseline for constitutive expression levels, whereas β-actin served as a loading control.](https://www.aacrjournals.org/CancerTherapyClinical/2005/11/7/2640/4)
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 chemotherapeutic 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

![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.](image1)

![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.](image2)

![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).](image3)
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 sequelae 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 persist 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-α) 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 checkpoint, 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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