Escape from Therapy-Induced Accelerated Cellular Senescence in p53-Null Lung Cancer Cells and in Human Lung Cancers

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Abstract

Accelerated cellular senescence (ACS) has been described for tumor cells treated with chemotherapy and radiation. Following exposure to genotoxins, tumor cells undergo terminal growth arrest and adopt morphologic and marker features suggestive of cellular senescence. ACS is elicited by a variety of chemotherapeutic agents in the p53-null, p16-deficient human non–small cell H1299 carcinoma cells. After 10 to 21 days, infrequent ACS cells (1 in 106) can bypass replicative arrest and reenter cell cycle. These cells express senescence markers and resemble the parental cells in their transcription profile. We show that these escaped H1299 cells overexpress the cyclin-dependent kinase Cdc2/Cdk1. The escape from ACS can be disrupted by Cdc2/Cdk1 kinase inhibitors or by knockdown of Cdc2/Cdk1 with smaller interfering RNA and can be promoted by expression of exogenous Cdc2/Cdk1. We also present evidence that ACS occurs in vivo in human lung cancer following induction chemotherapy. Viable tumors following chemotherapy also overexpress Cdc2/Cdk1. We propose that ACS is a mechanism of in vivo tumor response and that mechanisms aberrantly up-regulate Cdc2/Cdk1 promotes escape from the senescence pathway may be involved in a subset of tumors and likely accounts for tumor recurrence/progression. (Cancer Res 2005; 65(7): 2795-803)

Introduction

A requisite for immortalization of cancer cells is the bypass of physiologic programs of cellular senescence. Although the senescence program remains incompletely understood, the telomere paradigm suggests that cellular senescence is governed by telomere length, which progressively shortens in the replicative life span of somatic cells (1, 2). A pivotal element to this hypothesis is p53, postulated to mediate replicative arrest in response to a DNA damage signal produced by a sufficiently short telomere (3, 4). Through mechanisms both dependent and independent of pRb, p16INK4A has also been shown modulate this telomere length–dependent arrest of cell division (5). In human diploid fibroblasts, the replicative arrest occurs at 60 to 80 population doublings and has been termed mortality stage 1 (M1; ref. 6). Indeed, the replicative life span of normal human diploid cells can be extended beyond this limit by the inactivation of either the p53 or the p16-pRb pathway (7–9). Immortalized cancer cells seem to bypass the M1 restriction through mutational inactivation of these pathways or through specific targeting by viral oncoproteins (6, 10, 11). Beyond M1, as cells further divide and telomeres shorten, a second restriction point (M2 or crisis) is encountered, at which time aberrant expression of telomerase or alternative telomerase-independent mechanisms of telomere lengthening enables rare cells to traverse this restriction and become immortalized (12, 13). One of these two telomere maintenance mechanisms is believed to be operating in nearly all immortalized tumor cell lines.

In response to anticancer drugs, ionizing radiation, differentiating agents, oxidative stress, or the presence of selective oncogenic mutations, immortalized cancers cells can undergo terminal growth arrest despite having bypassed both M1 and M2 (14–16). This telomere-independent response, termed accelerated cellular senescence (ACS), is believed to overlap with the physiologic cellular senescence program and shares with it the replicative arrest phenotype. The phenotype includes morphologic alterations, such as enlarged and flattened shape with increased cytoplasmic granularity, presence of polyploidy, and expression of the pH-resistant, senescence-associated β-galactosidase (SA-β-gal; refs. 14, 17, 18). Both physiologic cellular senescence and ACS seem to be modulated by p53 effector pathways not restricted to p21Waf1/Cip1 and p16 through its regulation of pRb (19, 20). The conditional expression of p53, p16, or p21 alone in neoplastic cell lines results in irreversible growth arrest and senescence phenotype (21–25).

Recent investigations suggest that ACS occurs in vivo (26). The presence of SA-β-gal was reported to occur in 41% of specimens from breast cancer patients who received induction chemotherapy but in only 10% of specimens from patients who underwent surgery without chemotherapy. In transgenic murine models using Bcl-2-overexpressing lymphomas, tumor response to cyclophosphamide was shown to correlate to the amount of senescence response, and the response was reduced with the accumulation of either p53 or p16 mutations (27). These findings collectively suggest that ACS leading to terminal growth arrest is a physiologic mechanism of DNA damage response occurring in cancer therapy.

Given that p53 is mutated in 50% of all solid tumors (28) and that the response in solid tumors to chemotherapy is often transient, we postulated that p53-defective solid tumors arrested in senescence following chemotherapy can circumvent ACS and again replicate. We undertook this investigation to define p53-independent pathways that regulate ACS and mechanisms by which senescent cells may circumvent the ACS program and reenter replicative cell cycle. Here, we show that p53-null human lung carcinoma H1299 cells arrest in G2-M in response to chemotherapy using the ATM/ATR DNA damage response pathways. Following arrest, ~1 in 106 cells escape replicative arrest and reenter cell cycle. These cells express senescence markers and resemble the parental cells in their transcription profile. The H1299 cells that escape senescence do so by overexpressing the cyclin-dependent kinase Cdc2/Cdk1, and the senescence escape can be disrupted by Cdc2 inhibitors, which
results in cell death of senescence escape cells, and promoted by expression of exogenous Cdc2. Finally, we present preliminary evidence that senescence responses occur in lung cancer patients receiving neoadjuvant chemotherapy. Our results suggest that further understanding of senescence escape pathways may have significant influence on clinical outcome.

Materials and Methods

**Cell culture.** H1299 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (BioWhittaker, Walkersville, MD). Cells were treated with chemotheraphy at indicated concentrations at 15% to 30% cell density for 1 to 3 days, rinsed with PBS, and harvested or allowed to recover for the indicated time in full medium before harvest. Serum-deprived cells were plated at 1% cell density. On the next day, the cells were rinsed in PBS and a new medium containing 0.5% fetal bovine serum was added for 7 days. Caffeine and SB202190 were added 2 hours before addition of the camptothecin. Cells were harvested 24 or 48 hours later and fixed in paraformaldehyde. Senescence escape cells were directly treated with pharmacologic inhibitors or transfected with small interfering RNA (siRNA; SMARTpool, Upstate, Charlottevile, VA) using Effectene (Quagen, Valencia, CA) at concentrations indicated. Alamar blue reduction assay was carried out following the manufacturer's protocol (Biosource, Camarillo, CA).

**PKH2 staining, paraformaldehyde fixation, and cell cycle profiling.** Untreated H1299 and ACS cells following camptothecin were stained with PKH2 following Chang et al. (14) and recovered in growth medium for indicated times. Cells were then harvested, fixed in 1% paraformaldehyde, and analyzed on Becton Dickinson (San Diego, CA) FACScan flow cytometer. For cell cycle profiling, cells were fixed in paraformaldehyde, stored in 70% ethanol, and stained with propidium iodide (40 μg/mL) before fluorescence-activated cell sorting analysis.

**Quantitative in situ SA-β-gal.** In situ SA-β-gal staining was done on indicated cells after three PBS washes and fixation in 4% paraformaldehyde with 1.7 mg/mL of 5-bromo-4-chloro-3-indoly-β-D-galactoside at pH 6.0 (17). Tumor samples were freshly frozen within 30 minutes of surgery in Tissue-Tek cryopreservation medium, cryostat sectioned, and stained with hematoxylin.

**RNase protection assays and statistical analyses.** RNase protection assays (RPA) were done as per instructions for Riboprobe kits (PharMingen, San Diego, CA) using the following probe sets: hcC1-1, hcC1-2, hcC1-3, hjC1-2, hjStress-1, hjApO-1b, and hjApO-3. Five to 10 μg RNA were used per lane, and samples were separated on 6% Tris-borate EDTA/urea gels in 1× Tris-borate EDTA buffer. Dried gels were analyzed using the Cyclone scanner and Optilquant software (Pakard Bioscience, Billerica, MA). Probe signals were normalized using the internal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and L22 controls. Paired data were entered into an online statistical program provided by Department of Physics, College of Saint Benedict/Saint John’s University (Collegeville, MN; http://www.physics.csbsju.edu/stats/t-test.html).

**Western blot analysis.** Western analyses were done on β-actin normalized cellular proteins (40 μg) extracted with WE16 buffer [50 mMol/L Tris (pH 7.5), 250 mMol/L NaCl, 5 mMol/L EDTA, 0.1% SDS, 1% NP40, 1% deoxycholate, 20% glycerol] supplemented with protease (Complete Mini-EDTA free, Roche, Indianapolis, IN) and phosphatase inhibitors (P2, Sigma, St. Louis, MO) following the manufacturer’s recommended concentrations. The proteins were then separated by 10% SDS-PAGE and transferred onto Immobilon-P membranes (Millipore, Bedford, MA). Immunodetection of the proteins was carried out following the manufacturer’s suggested antibody concentrations and 1:10,000 for horseradish peroxidase–conjugated secondary anti-mouse or anti-rabbit antibodies (Sigma). Lysates were normalized on a Western blot using antibodies against β-actin antibody (E5;00; clone AC-15, Sigma) and then p16 (501, RB (IF8), chk1 (G-4), Cdc25 c (20), Cdc2/p34 (clone 17), cyclin B1 (H-433), survivin (D-8), cyclin D1 (A12), or phosphorylated Cdc2 (Tyr15, Cell Signaling Technology, Beverly, MA) or all other antibodies were from Santa Cruz Biotechnology, Santa Cruz, CA) following the manufacturer’s recommended concentration. Blots were visualized by enhanced chemiluminescence with the ECL Plus kit (Amersham, Piscataway, NJ) following the manufacturer’s recommendations.

**Reverse transcription-PCR.** RNA was prepared from appropriately treated cells following the manufacturer’s protocol with the RNeasy kit (Qiagen). Quantitative reverse transcription-PCR was done by reverse transcription with random hexamer primers (N6) using Omni-Script RT kit (Qiagen) and amplification of gene-specific signals with Cdc2/Cdk1 or GAPDH specific primers as reported previously in presence of [α-32P]ATP (NE, Boston, MA: ref. 29). Sequences of Cdc2/Cdk1 primers were 5′-Cdc2/Cdk1 RT TACAGGTCAAGTGGTAGCCATGA and 3′-Cdc2/ Cdk1 RT CCAAGTATTTCCTCAGATCGATGA. Cdc2/Cdk1 signals were detected with the Cyclone Phosphoimager and normalized to the GAPDH signals.

**Colony escape assay.** H1299 cells were plated at 10% cell density and treated with 30 mmol/L camptothecin on the following day. After 3 days of drug exposure, the plates were rinsed and allowed to recover for an additional 4 days in fresh growth medium. After 4 days, the culture medium was replaced every 5 to 7 days with growth medium alone or with medium containing inhibitors (olomoucine, iso-olomoucine, roscovitine, and PNU112455A, Calbiochem, San Diego, CA) at the indicated concentrations. Colonies were scored from triplicate plates 14 to 21 days following chemotherapy exposure.

**Cdc2/Cdk1 and DN-Cdc2/Cdk1 cloning.** Cdc2/Cdk1 was generated by PCR from cDNA with primers 5′-R1-Cdc2/Cdk1 and 3′-R3-Cdc2/Cdk1 (see primer sequences below), introducing EcoRI and PstI sites at the 5′ and 3′ ends of the fragment. The PCR product was digested and inserted into the corresponding EcoRI and PstI sites in the pS7G(F) vector. The primers 5′-BamKozac and 3′-Pst-Cdc2/Cdk1 were used in PCR to generate the Cdc2/ Cdk1 fragment with a Kozac sequence and two flag tags upstream of the 5′ start site. This was inserted into the pCR1 vector with the topo-TA cloning kit (Invitrogen, Carlsbad, CA) and subcloned into the pcDNA3.1 vector. The DN-Cdc2/Cdk1 was generated through PCR mutagenesis using two oligonucleotides that overlapped the mutation (5′-DN-Cdc2/Cdk1 and 3′- DN-Cdc2/Cdk1 primers). Plasmids were transfected into H1299 cells with Effectene and stable transformants were selected in 600 μg/mL G418. Primers were 5′-RI-Cdc2/Cdk1 TTTAATTTTTGAGATTTACACAAATTA- GAAA, 3′-Pst-Cdc2/Cdk1 CCTCTCGAGATTTTATGATGGATCTTTGCG- CAT, 5′-BamKozac CGGATTGCAGCCACCATGCAGAACAGAAG-CCA, 3′-DN-Cdc2/Cdk1 TAAATGGCTCAATTTTGGCTTGG, and 3′-DN-Cdc2/Cdk1 GCGAGGGGCTATAATGCAGAC.

**Frozen section immunohistochemical staining.** Cryostat sections of normal or cancerous lung tissues were fixed in cold acetone for 5 minutes, air dried, and incubated with a nonspecific antibody, the anti-Cdc2/p34 antibody (clone 17, Santa Cruz Biotechnology), or the anti-Ki-67 antibody (MIB-1, DakoCytoamation, Carpinteria, CA) for 40 minutes at room temperature. Blocking with nonspecific antibodies was done for selected antibodies. After two PBS rinses, immunolocalization was done via a modified avidin-biotin immunoperoxidase technique with nickel chloride–enhanced 3,3′-diaminobenzidine chromogen. Finally, sections were counterstained with hematoxylin.

**Image reproduction and processing.** Images of gel and digital photographs were generated with Adobe Photoshop 7.0 and Canvas 6.

**Results**

Escape from replicative arrest and ACS occurs spontaneously in H1299 cells exposed to chemotherapeutic agents. H1299 cells (p16RNR3A/+/pRb/deleted-p53) were selected for these studies, because p53 and p16 have been shown previously as barriers to reversibility in cellular senescence (30). We have found that between 5% and 90% of viable H1299 cells will assume the senescence phenotype after a 3-day exposure to moderated doses (1-3 × IC50) of a variety of chemotherapeutic agents: cisplatin (10-30%), camptothecin (85-90%), etoposide (40-60%),
paclitaxel (2-5%), and vindesine (20-40%). For example, 4 days following the release of camptothecin into fresh medium, nearly 90% of the remaining cells show enlarged, flattened morphology, increased cytoplasmic granularity, and expression of the replicative senescence marker SA-β-gal (see Fig. 1A, ACS). These cells (referred here as ACS cells) do not further divide as confirmed by the retention of PKH2 fluorescence signal for 4 days following labeling compared with 10-fold loss of fluorescent signal in proliferating H1299 cells (Fig. 1B).

Importantly, when ACS cells are followed for 18 to 24 additional days, infrequent cells reenter the cell cycle and replicate into colonies. We estimate that this event occurs in 1 of 10^6 cells based on colony formation from a known number of ACS cell. Under microscopy, the majority of these colonies consist of pleomorphic cell populations, with infrequent cells reassuming the senescence morphology (Fig. 1A, SE10 and SE12). We postulated that a subset of these colonies derived from ACS cells that have circumvented replicative senescence. We have termed the cells in these colonies senescence escape cells.

Senescence escape cells do not manifest primary resistance to camptothecin and have a gene expression profile more similar to ACS cells than to parental cells. We first characterized the sensitivity of senescence escape cells to camptothecin in Alamar blue reduction assays and found that the IC_{50} of four independent colonies ranges between 32 and 40 nmol/L, insignificantly different from an IC_{50} of 35 nmol/L in untreated H1299 cells. This finding suggests that the senescence escape cells do not represent H1299 cells with primary camptothecin resistance. We proceeded to characterize the expression of SA-β-gal in these senescence escape cells (Fig. 1A, SE10 and SE12). SA-β-gal, which we have observed to be restricted to ACS cells and absent in proliferating H1299 cells or cells arrested in low serum

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**Figure 1.** Characterization of senescence escape H1299 cells. A, light microscopy of untreated H1299 cells, ACS cells (4-day recovery following camptothecin treatment), and two senescence escape colonies cells stained with 5-bromo-4-chloro-3-indolyl-β-d-galactoside at pH 6.0. B, density plot of untreated H1299 cells or ACS cells labeled with PKH2 followed for 4 days. Red, immediately following PKH2; blue, 4 days following PKH2. C, RPA's were carried out on total RNA from untreated H1299 cells (H), cells arrested in 0.5% serum (SA), cells following 1 day treatment with camptothecin (PA), ACS cells, and cells derived from three senescence escape colonies (SE9, SE10, and SE12). Protected signals against specific probes (indicated) are normalized with respect to both GAPDH and L22 signals. D, paired Student's t test comparing cumulative RNA levels of 66 genes in the indicated cells with respect to those in ACS cells. Means (■) and 95% confidence intervals (bars) of the differences. *, P < 0.05, two-tailed.
concentration, was found to be present in 2% to 5% of cells derived from several colonies. The unexpected presence of SA-β-gal further supports that these senescence escape cells derived from previously arrested ACS cells.

We next used gene expression profiling based on quantitative RPAs to determine whether the senescence escape cells resemble the ACS cells. In designing these assays, we asked whether stress-responsive and cell cycle–related genes are differentially expressed among the senescence escape and other H1299 cell populations. We selected eight sets of RPA probes targeting 66 genes for this analysis (listed in Material and Methods). The RPA signals were normalized against the GAPDH and L22 RNA levels. For each of these 66 genes, the RNA levels in three individual senescence escape colonies (SE9, SE10, and SE12) were compared with the RNA levels in the following H1299-derived populations: (a) untreated cells; (b) cells arrested in low serum concentration; (c) cells following 1 day of camptothecin treatment (pulse arrest); and (d) cells arrested in ACS. The results for 8 of the 66 genes are displayed in Fig. 1A. The statistical analysis of these data was done based on paired two-tailed Student’s t test comparisons between the RNA profiles of these 66 genes using an online statistical program (see Materials and Methods). The gene expression levels of ACS cells were used as standard for these comparisons. The results of these statistical comparisons are depicted in Fig. 1D. The expression profiles of cells derived from all three senescence escape colonies closely resembled the profile of ACS cells, with SE9 and SE10 reaching statistical significance based on the 95% confidence intervals around the cumulative mean differences (P < 0.05). The expression profile of the pulse-arrested cells also resembles that of ACS cells but does not reach statistical significance. In contrast, profiles from untreated cells (H1299) and from cells arrested in low serum medium did not resemble the profile of ACS cells. When taken together with their ACS-like morphology, their ectopic expression of SA-β-gal, and the unaltered camptothecin IC_{50}, the RNA expression data provide additional evidence that senescence escape cells originated from ACS cells and infer that senescence escape cells have bypassed the terminal arrest program. Of note, the senescence escape cells can be reinduced into ACS by camptothecin albeit to lesser extent (30-50%). They are also prone to cell death by mitotic catastrophe (data not shown).

H1299 cells respond to camptothecin through a caffeine-sensitive G2-M cell cycle arrest and up-regulate Cdc2/Cdk1 protein level on escape from ACS. H1299 cells arrest at G2-M in response to treatment by camptothecin (Fig. 2A, left). The G2-M arrest of these cells by camptothecin is mediated by ATM/ATR as indicated by the ability of caffeine pretreatment (5 mmol/L) to relieve the camptothecin-induced G2-M cell cycle block (Fig. 2A, right). In contrast, pretreatment of H1299 cells with the selective inhibitor of p38MAPK SB203580 had no effect on the G2-M arrest (data not shown).

To examine the known downstream effectors of the ATM/ATR pathway, Western blot analysis was carried out to determine the levels of Chk1, Cdc25, Cdc2/Cdk1, and phosphorylated Cdc2/Cdk1 (Y15; Fig. 2B). Chk1 and Cdc25 levels did not differ significantly in untreated cells (lane H), low serum–arrested cells, or cells that were at 1, 2, or 4 days following 3 days of camptothecin treatment. Phosphorylated Cdc2/Cdk1, indicative of the inactive kinase, was found to be up-regulated on day 1 following camptothecin and slowly diminished over the subsequent 3 days, suggesting that phosphorylation mediates the early inactivation of Cdc2/Cdk1. The overall Cdc2/Cdk1 protein level eventually declined to a nadir level in the ACS cells. In contrast to the overall levels of Cdc2/Cdk1 in ACS cells, we found a significant increase in the overall levels of Cdc2/Cdk1 in senescence escape cells. This increase was observed in 4 of senescence escape colonies in an initial experiment (Fig. 2B) and in 12 of 13 colonies in a follow-up experiment (Fig. 2C). In the follow-up experiment, Cdc2/Cdk1 was found to be 3- to 15-fold increased in the senescence escape cells relative to the level in either untreated H1299 or ACS cells. The Cdc2/Cdk1 kinase activity was measured in selected clones and found to be 2.5- to 3-fold and 7.5- to 9-fold higher relative to untreated H1299 and ACS cells, respectively (Fig. 2C).

Cyclin B1, the principle activator of Cdc2/Cdk1 kinase, was increased 1- to 3-fold and 2- to 6-fold in senescence escape cells compared with untreated H1299 and ACS cells, suggesting that the observed increase in Cdc2/Cdk1 kinase activity may also reflect the cyclin B1 level (Fig. 2D). Cyclin D1 and Cdk2 levels were not significantly altered in the senescence escape cells, indicating that a global increase in the cell division kinases does not occur. Finally, levels of the tumor suppressor proteins p16 and p21 remained undetectable and unchanged in these cells (data not shown). In summary, these results suggest that the selective overexpression of Cdc2/Cdk1 and the deregulation of the G2 damage pathway may represent mechanisms through which ACS programs are bypassed in the senescence escape cells.

Cdc2/Cdk1 protein overexpression is mediated by both transcriptional and post-transcriptional processes. We proceeded to determine the Cdc2/Cdk1 RNA level in these cells by quantitative reverse transcription-PCR (Fig. 3). Cdc2/Cdk1 RNA levels were found to be 2.8- to 6.5-fold higher in the senescence escape cells than in the ACS cells; however, they were 1- to 2.4-fold higher than replicating H1299 cells despite much higher Cdc2/Cdk1 protein levels (Fig. 2B). Northern blot and RPA analyses corroborated with this result (data not shown). These findings suggest that the observed increase of Cdc2/Cdk1 in senescence escape cells is controlled by both transcription and post-transcriptional processes.

Inhibition of Cdc2/Cdk1 kinase arrests senescence escape cells and disrupts escape of ACS cells. If the aberrant expression of Cdc2/Cdk1 kinase enables ACS cells to bypass the G2-M checkpoint and to reenter the cell cycle, we postulate that the selective inhibition of Cdc2/Cdk1 kinase would cause senescence escape cells to rearrest. To address this possibility, we treated H1299 and senescence escape cells with Cdc2/Cdk1 inhibitor olomoucine and its inactive isomer iso-olomoucine (Fig. 4A–C). Forty-eight to 72 hours following exposure to 100 μmol/L olomoucine, the senescence escape cells from two independent clones (SE21 and SE26) were found to rapidly rearrest and die (Fig. 4C) from a mechanism unrelated to apoptosis (data not shown; absence of chromatin fragmentation, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling, and Annexin V stain). Morphologically, the rearrested cells detach and form cell clumps with indistinct cytoplasmic borders (Fig. 4A, 50 and 100 μmol/L). Their cell cycle profile was difficult to characterize due to aggregation in the fluorescence-activated cell sorting analyzer. In contrast to its effect on senescence escape cells, olomoucine at the same concentrations induces growth retardation over at least 7 days in senescence escape cells treated with iso-olomoucine exhibited replicative arrest or morphologic alteration. We determined the percentage of cells that express SA-β-gal in response to these various treatments 36 hours following the treatment with
olomoucine when nearly 100% of cells remain viable (Fig. 4B). In situ SA-β-gal assay was done by scoring 500 to 3,000 cells from six to eight consecutive microscopy fields. Olomoucine profoundly increased the percentage of SA-β-gal-expressing senescence escape cells while minimally altered the SA-β-gal expression in H1299 cells.

Because olomoucine may also have activity against Cdk2 and extracellular signal-regulated kinase, we also used the siRNA approach to selectively inhibit Cdc2/Cdk1 expression (Fig. 4D). We transfected senescence escape cells with 100 nmol/L of a mixture of siRNA directed against either the bacterial β-gal (LacZ) or the Cdc2/Cdk1 RNA. Forty-eight hours following transfection, senescence escape cells transfected with Cdc2/Cdk1-siRNA, but not with LacZ-siRNA, were found to exhibit the same morphologic alteration as olomoucine-treated senescence escape cells. This result confirms the selective effect of Cdc2/Cdk1 inhibition in cells that have bypassed ACS.

Senescence escape cells can be reinduced into ACS with camptothecin. To examine if reinduced senescence can be promoted by the down-regulation of Cdc2/Cdk1, we transfected cells from two independent senescence escape colonies with Cdc2/Cdk1-siRNA and LacZ-siRNA. Thirty-six hours following transfection, the cells were treated with camptothecin for 3 days and scored for both viability and SA-β-gal expression following 4-day recovery (Fig. 4E). The protein level of Cdc2/Cdk1 was determined

Figure 2. Mechanism of H1299 arrest by camptothecin and escape from ACS. A, cell cycle profile of untreated H1299 cells (H1299) or cells 48 hours following treatment with 60 nmol/L camptothecin (Campto), 5 mmol/L caffeine, or a combination of camptothecin and caffeine. B, Western blot analysis of indicated protein expression in untreated cells, cells arrested in 0.5% serum, cells 1 or 2 days following camptothecin (1d or 2d), ACS cells, and four independent senescence escape colonies (7, 9, 10, and 12). Similar levels of β-actin immunoreactivity indicate similar amounts of protein loaded in each lane. C, Cdc2/Cdk1 protein expression and kinase activity (bottom of each lane) in untreated cells, ACS cells, and 13 unselected senescence escape colonies. D, levels of cyclin B1, cyclin D1, and Cdk2 in untreated cells, 1 day pulse-treated cells (P), ACS cells, and senescence escape colonies (7, 9, 10, and 12).

Figure 3. Quantitative reverse transcription-PCR of Cdc2/Cdk1 RNA level in untreated H1299 cells, ACS cells, and senescence escape colonies 7, 9, 10, 12, and 21.
immediately before camptothecin and was found to be knocked down specifically by Cdc2/Cdk1-siRNA in a concentration-dependent manner (bottom). Both percentages of viable and SA-β-gal expression cells were found to be similarly reduced 4 days following chemotherapy recovery. At 100 nmol/L Cdc2/Cdk1-siRNA, phenotypically viable ACS cells were rarely found. LacZ-siRNA had no effect on the viability or the SA-β-gal or protein expression of the senescence escape cells. These results suggest that the senescence escape cells depend on the activity of Cdc2/Cdk1 for viability and that down-regulation of Cdc2/Cdk1 does not promote therapy-induced senescence but rather elicits cell death.

Activities of Cdc2/Cdk1 modulate escape from ACS. We also hypothesized that escape from ACS could be modulated by manipulation of Cdc2/Cdk1 activity or level. To examine the possibility that inhibition of Cdc2/Cdk1 in senescent cells could disrupt escape from the terminal arrest, we exposed camptothecin-treated cells to various pharmacologic compounds 4 days following their release into unmodified medium. The number of senescence escape colonies was quantified (Fig. 5A) and expressed as a percentage of colonies formed in camptothecin-treated cells receiving no other drugs. Concentration-dependent reduction of senescence escape colony formation was observed with Cdc2/Cdk1 inhibitors, olomoucine and roscovitine, but not with DMSO, iso-olomoucine, and PNU112455A, a selective inhibitor of Cdk2/Cdk5. The results here confirm that inhibitors of Cdc2/Cdk1 could disrupt escape from the ACS program triggered by chemotherapy likely through mediating cell death in senescence escape cells (Fig. 4C and E).

We further examined the effect of ectopic expression of Cdc2/Cdk1 on senescence escape colony formation. We isolated H1299 cells stably transfected with blank plasmids or plasmids encoding either FLAG-tagged Cdc2/Cdk1 or kinase-defective (DN) Cdc2/Cdk1 mutant (D146N). Antibiotic-resistant cells were characterized for their Cdc2/Cdk1 expression and tested for colony formation following camptothecin treatment (Fig. 5B). Ectopic Cdc2/Cdk1 overexpression promoted colony formation by ~50% in three independent experiments. The expression of D146N Cdc2/Cdk1,
tumor. We also tested for the expression of Cdc2/Cdk1 in a subset of these samples (see Fig. 6). Interestingly, nuclear Cdc2/Cdk1 stain was significantly elevated in tumor cells relative to their normal lung cells in patients 2 and 3 (patient 2 shown). In both of these patients, the tumors have reduced mildly in size following chemotherapy and the cancer cells can be assumed to be growth arrested. In two untreated patients (patient 5 shown), the Cdc2/Cdk1 expression was mainly confined to the basal epithelial layer of the tumor specimen where the cells are proliferating rapidly and not in all cell layers of the tumor. In general, we have observed a correlation between the expression of Cdc2/Cdk1 expression pattern and that of Ki-67 expression pattern (data not shown), suggesting that Cdc2/Cdk1-expressing cells are resuming cell proliferation. Although this study is preliminary, it shows that ACS is an in vivo response to chemotherapy in lung cancer.

**Discussion**

ACS is induced in H1299 cells despite the absence of p53 and p16, the primary modulators of physiologic and therapy-related senescence. An abbreviated exposure to a moderate dose of camptothecin arrests H1299 cells at G2-M through biphasic regulation of Cdc2/Cdk1, initially a transient inhibitory phosphorylation within the first 2 days and then a more sustained reduction of its mRNA and protein (Figs. 2 and 3). Recently, intact p53 function was found to be necessary for topoisoomerase I-induced G2-M arrest and ACS in immortalized and primary glioblastoma cell lines (31). In this study, arrest and senescence was restricted to cells with intact p53, where Cdc2/Cdk1 regulation in the first 7 days seems to be biphasic as well. Apoptosis was the dominant response when p53 was inactivated either through E6 expression or by mutation. In the p53-dysfunctional cells, Cdc2/Cdk1 regulation was primarily mediated through sustained inhibitory phosphorylation, whereas Cdc2/Cdk1 protein remained at a relatively constant level. In senescent fibroblasts and in doxorubicin-arrested HCT116 cells, reduction of Cdc2/Cdk1 by both transcription and protein regulation has also been observed (32, 33). These studies raise the possibility that sustained down-regulation of Cdc2/Cdk1 protein level may be required for prolonged cell cycle arrest and possibly for the senescence response. In contrast to the study discussed above, our finding here suggests that p53 is not the sole determinant for prolonged cell cycle arrest in lung cancer cells and that other p53-independent mechanisms can mediate ACS through Cdc2/Cdk1. In the glioblastoma model, it is noteworthy that therapy-induced arrest was also reversible despite the presence of p53, suggesting that p53 does not irreversibly reenforce ACS.

This and other studies show that prolonged cell cycle arrest and ACS can be reversible for a subset of cells (31). In our system, ACS cells can escape through up-regulation of Cdc2/Cdk1. Remarkably, our data suggest that the senescence escape cells are uniquely dependent on the Cdc2/Cdk1 kinase activity, such that selective inhibition of the Cdc2/Cdk1 kinase by pharmacologic inhibitors or through siRNA knockdown results in cell death, hence reducing escape. These effects were not observed in untreated H1299 cells. We therefore postulate the presence of survival mechanisms that may be directly coupled to Cdc2/Cdk1 such that these survival pathways enable rare ACS cells to remain viable and escape. As such, in senescence escape cells, elevated Cdc2/Cdk1 activates these survival mechanisms and the inhibition of Cdc2/Cdk1 results in cell death. One candidate of this mechanism is the survival checkpoint mediated by survivin, known to be regulated by Cdc2/Cdk1 through
phosphorylation (34, 35). Survivin, a homologue of the baculovirus inhibitor of apoptosis, is overexpressed in many human cancers (36) and survivin interferes with caspase-9-dependent apoptosis at mitosis (34). Another candidate is the phosphatidylinositol 3'-kinase/AKT pathway, which regulates cyclin B1 expression and Cdc2/Cdk1 activation (37). The phosphatidylinositol 3'-kinase/AKT activation has been shown to mediate cell proliferation and survival (38). Geranylgeranyltransferase I inhibitors, currently being studied in human cancer, has been shown recently to target both phosphatidylinositol 3'-kinase/AKT and survivin pathways as possible mechanisms of its proapoptotic activity (39).

Defects in cell cycle regulatory proteins have been shown to be a frequent feature of human cancers. Rearrangement, amplification, and overexpression of cyclin D1, E, or A occur in both hematopoietic and somatic cancers (40). Elevated expression of Cdc2/Cdk1 variably correlating to cyclin B1 has been reported in nodal non-Hodgkin’s lymphomas, primary colorectal cancer, thyroid carcinomas, malignant breast lesions, and transforming Helicobacter pylori–associated mucosal lymphomas (41–45). The induction of Cdc2/Cdk1 may also be associated with therapy resistance (44). In a recent study, the inactivation of Cdc2/Cdk1 kinase was shown to be a requisite for p53-mediated G2-M arrest in EJ bladder carcinoma cells (46). The overexpression of a constitutively activated Cdc2/Cdk1 kinase could override the p53-mediated effect. In our ACS model, the overexpression of wild-type Cdc2/Cdk1 kinase, but not the dominant-negative kinase, promotes escape from ACS in EJ bladder carcinoma cells (46). In a recent study, the inactivation of Cdc2/Cdk1 kinase was shown to be a requisite for p53-mediated G2-M arrest in EJ bladder carcinoma cells (46). The overexpression of a constitutively activated Cdc2/Cdk1 kinase could override the p53-mediated effect. In our ACS model, the overexpression of wild-type Cdc2/Cdk1 kinase, but not the dominant-negative kinase, promotes escape from ACS in EJ bladder carcinoma cells (46). The overexpression of a constitutively activated Cdc2/Cdk1 kinase could override the p53-mediated effect. In our ACS model, the overexpression of wild-type Cdc2/Cdk1 kinase, but not the dominant-negative kinase, promotes escape from ACS in EJ bladder carcinoma cells (46).

In the glioblastoma cell lines, however, p16 status was found to be neither necessary nor sufficient for initiation and/or maintenance of topoisomerase I–induced ACS (31).

<p>| Table 1. Clinical parameters and outcome of patient who underwent neoadjuvant chemotherapy prior to surgery versus surgery alone |
|-------------------------------------------------|------------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Patient no.</th>
<th>TMN staging</th>
<th>Neoadjuvant therapy</th>
<th>Tumor pathology</th>
<th>SA-β-gal</th>
<th>Follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lung</td>
<td>Tumor</td>
<td>Lung</td>
</tr>
<tr>
<td>1</td>
<td>T2N0M0</td>
<td>Carboplatin/taxol × 3</td>
<td>Complete response</td>
<td>+/-</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>T2N1M0</td>
<td>Carboplatin/taxol × 3</td>
<td>Viable tumor</td>
<td>+/-</td>
<td>+++</td>
</tr>
<tr>
<td>3</td>
<td>T2N0M0</td>
<td>Carboplatin/taxol × 3</td>
<td>Viable tumor</td>
<td>+/-</td>
<td>+++</td>
</tr>
<tr>
<td>4</td>
<td>T2N0M0</td>
<td>None</td>
<td>Viable tumor</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>T2N0M0</td>
<td>None</td>
<td>Viable tumor</td>
<td>+/-</td>
<td>+/–</td>
</tr>
<tr>
<td>6</td>
<td>T2N0M0</td>
<td>None</td>
<td>Viable tumor</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

NOTE: +/-, scant/spotty positive.
The demonstration of the senescence marker SA-β-gal in treated breast cancers (26) and lung cancers (Fig. 6) implies that therapy-induced ACS is an in vivo mechanism of treatment response. Although its clinical significance remains to be understood, the model of tumor growth arrest via ACS following therapy correlates well with the clinical sequelae of chemotherapy treatment for solid tumors. For most somatic cancers, induction chemotherapy without concurrent radiation produces a 20% to 40% disease response rate, with >95% of these responses being partial tumor volume reduction (47, 48). For lung cancers, chemotherapy typically results in mild to moderate reduction in tumor volume with initial treatments before the tumor progression despite further therapy. We propose that growth arrest as a result of ACS accounts for the partial tumor response due to therapy. Clinical progression is encountered when a subset of the tumor cells are able to bypass the ACS program. Inhibitors of Cdk2/Cdk1 are presently being studied in the preclinical and clinical settings as antitumor agents (49, 50). The present study suggests that these agents need to be used in sequence with conventional chemotherapy agents or as a maintenance therapy. Further understanding of mechanisms of ACS and escape mechanisms will lead to novel targets for anticancer therapy.

Acknowledgments

Received 4/12/2004, revised 1/7/2005, accepted 1/16/2005.

Grant support: Department of Veterans Affairs (VA Merit Review) and NIH grant K08CA71928 (D.Y. Wu).

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Escape from Therapy-Induced Accelerated Cellular Senescence in p53-Null Lung Cancer Cells and in Human Lung Cancers


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