DNA Damage Is Able to Induce Senescence in Tumor Cells in Vitro and in Vivo

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ABSTRACT

Often the use of cytotoxic drugs in cancer therapy results in stable disease rather than regression of the tumor, and this is typically seen as a failure of treatment. We now show that DNA damage is able to induce senescence in tumor cells expressing wild-type p53. We also show that cytotoxics are capable of inducing senescence in tumor tissue in vivo. Our results suggest that p53 and p21 play a central role in the onset of senescence, whereas p16INK4a function may be involved in maintaining senescence. Thus, like apoptosis, senescence appears to be a p53-induced cellular response to DNA damage and an important factor in determining treatment outcome.

INTRODUCTION

Re replicative or cellular senescence, a process leading to irreversible arrest of cell division, was first described in cultures of human fibroblasts that lost the ability to divide upon continuous subculture (1). Since then, replicative senescence has been shown in various mammalian tissues in culture and in vivo (2, 3). Contrary to normal somatic cells, most tumors have extended or infinite life spans. Cellular and viral oncogenes, or the loss of tumor suppressors, are involved in the transformation and immortalization of primary cells. Inactivation of the p53 and p16INK4a tumor suppressors is among the most common events in human cancers (4–8). Li-Fraumeni patients, who inherit a germ line mutation in one p53 allele, are predisposed to cancer because of loss of the remaining wild-type allele (9). Similarly, p53 knockout mice are highly susceptible to tumor development (10). p53 reacts to DNA damage by activating transcription-dependent and -independent pathways that lead to cell cycle arrest or apoptosis, thereby preventing proliferation of cells with a damaged genome. One of the genes transcriptionally activated by p53 is the p21 gene (11). p21 inhibits the phosphorylation of Rb by cyclin D-CDK4 and cyclin E-CDK2 (12, 13). Whether cells undergo apoptosis or cell cycle arrest after p53 induction is dependent on a variety of factors, including cell type, Rb status, and the expression of certain oncogenes and tumor suppressors (14). There is evidence that the transcriptional activity of p53 also plays an important role in the induction of cellular senescence, most likely via transactivation of the p21 gene (15). Consistent with this, tetracycline-induced expression of p21 in human bladder cells induced senescence (16). Similarly, carcinoma cells engineered to overexpress wild-type p53 undergo senescence (17, 18). DNA damage by a variety of compounds has been shown to induce cellular senescence in normal human diploid fibroblasts and also involved p53, p21, and p16INK4a (19–21).

Like p53, p16INK4a is not required for normal development, but is a potent tumor suppressor. p16INK4a was first identified as a CDK4-binding protein (22) and inhibits CDK4 and CDK6 by binding in competition with cyclin D (23, 24), preventing phosphorylation of Rb. The p16INK4a gene is often a site of allelic loss in many human malignancies (25, 26) and is mutated in a wide variety of primary tumors (27) and families with familial atypical multiple mole/melanoma (28, 29).

We have previously reported that human colon and ovarian carcinoma cells lacking or expressing mutant p53 treated with topoisomerase inhibitors undergo rapid apoptosis after S-G2 arrest. However, in carcinoma cells expressing wild-type p53, the response was characterized mainly by prolonged cell cycle arrest (30).

Topoisomerase inhibitors are commonly used anticancer drugs, and like most cytotoxics are used to induce DNA damage in tumor cells, activating p53 and leading to cell cycle arrest or apoptosis (31, 32). It has also been shown that these drugs are potent inducers of a reversible senescence-like state in normal human fibroblasts (33).

In the present study we investigated whether topoisomerase inhibitors were able to induce senescence in colon, ovarian, and breast adenocarcinoma cell lines expressing wild-type p53. We show that DNA damage induced by clinically relevant concentrations of topoisomerase inhibitors is able to induce irreversible growth arrest involving p53, p21, and p16INK4a. These cells had the morphological and biochemical characteristics of cellular senescence. The onset of the senescence program was coincident with increased levels of p53 and p21, whereas p16INK4a levels remained unchanged during treatment. When cells were recultured in drug-free medium, the p53 and p21 levels decreased to normal. However, p16INK4a levels increased soon after drug withdrawal, and cells remained senescent. These data suggest that p53 and p21 play a central role in the onset of senescence, whereas p16INK4a may be involved in maintaining senescence. Furthermore, isogenic cell line pairs either lacking p53 or p21 were unable to induce the senescence program in contrast to the parental cell line after treatment. Thus, senescence may not be just a characteristic of aging cells, but like the apoptotic pathway is a p53-induced cellular response to DNA damage that acts to prevent the proliferation of damaged cells.

We believe that our observations could have profound clinical implications and explain some of the controversy concerning p53 as a prognostic factor. Generally, stable disease is classified as treatment failure, whereas this may not be the case if tumors of patients with stable disease contain senescent cells, which have lost the capacity to proliferate as a result of treatment. Consistent with this, we show that 15 of 36 breast tumor resections after neoadjuvant chemotherapy stained positive for senescence-associated β-galactosidase activity and corresponded with high p16INK4a staining and low p53 staining, whereas we observed only a few individual cells staining positive in 2 of 20 untreated breast tumor resections, suggesting that the senescence observed in these tumors is attributable to chemotherapy treatment. Thus, DNA damage-induced premature cellular senescence may indeed be a relevant factor in determining treatment outcome.
**MATERIALS AND METHODS**

**Cell Culture and Drug Treatment.** LS174T and HCA-7 colon, MCF-7 breast, and A2780 ovarian carcinoma cells were obtained from the Imperial Cancer Research Fund cell culture laboratory. The HCT116 parental, with wild-type p53 and functional p21, and the p53- and p21-null HCT116 cell lines were a kind gift of Prof. Vogelstein (34). All cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies) and kept at 37°C in a humidified atmosphere containing 5% CO₂. Before drug treatment, cells were allowed to attach for 24 h. SN-38 and VP-16 (Sigma Chemical Co.) were dissolved in DMSO (Sigma Chemical Co.) and 50% ethanol, respectively, and stock solutions were diluted in medium just before treatment. For prolonged exposures, medium was replaced every 24 h, and detached cells were harvested and resuspended in the fresh drug medium.

**Flow Cytometry.** The DNA content of the cells was determined using a rapid one-step DNA-staining technique, with the fluorescent dye propidium iodide (Sigma Chemical Co.), and apoptotic cells were discriminated by controlled extraction of low-molecular weight DNA as described previously (30). Fluorescence was measured using a FACSscan, and results were analyzed using Lysys II software (Becton Dickinson). anti-BrdUrd-FITC (Becton Dickinson) staining was performed as described by the supplier.

**Western Blotting.** Cells were harvested and washed twice with ice-cold PBS. Cells were counted and lysed in Laemmli buffer [62.5 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 1% (w/v) SDS, 5% (w/v) 2-mercaptoethanol, 0.0025% (w/v) bromphenol blue; Sigma Chemical Co.]. Samples were separated on 7.5–17.5% SDS-PAGE gels, transferred onto polyvinylidene difluoride membrane (Bio-Rad), and immunostained. The following primary antibodies were used: Pab240 (hybridoma supernatant) for p53, anti-p21 (Oncogene Research Products), G175-405 anti-p16INK4a and Ab-5 anti-Rb (PharMingen), and horseradish peroxidase-labeled rabbit antirabbit antibody (Dako) as secondary antibody. Proteins were visualized using the ECL detection system (Amersham).

**SA-β-gal Activity.** Cells were stained for β-galactosidase activity as described by Dimri et al. (2). Briefly, cells were seeded in 6-well plates containing coverslips. After the appropriate exposure, the cells were washed twice with PBS, fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS, and washed twice in PBS. Cells were stained overnight in X-gal staining solution [1 mg/ml X-gal, 40 mM citric acid/sodium phosphate (pH 6.0), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, 2 mM MgCl₂]. Cryosections of snap-frozen breast tumor biopsies, from untreated patients or patients who had received neoadjuvant CAF, were fixed for 1 min in 1% formalin in PBS and stained as described above.

**Immunohistochemistry.** Cryosections were incubated overnight in PBS at 60°C and left in methanol containing 0.03% hydrogen peroxide for 20 min. After being washed in PBS three times, cells were incubated for 1 h with primary antibody: DO-7 (DAKO) for p53 and G175-405 for p16INK4a (PharMingen). The sections were washed and exposed to biotinylated goat antimouse antibody for 30 min, followed by streptavidin-conjugated horseradish peroxidase for 30 min. After being washed, sections were incubated with diamobenzidine (0.2 mg/ml in PBS containing 0.03% hydrogen peroxide) for 30 min. After a thorough wash with tap water, sections were counterstained with hematoxylin (2 min) and washed again with tap water. Sections were scored for intensity as follows: −, no staining; +, low staining; ++, medium staining; ++++, high staining.

**RESULTS**

**SN-38 and VP-16 Cause Either Prolonged Cell Cycle Arrest or Apoptosis in Tumor Cells Expressing Wild-Type p53.** In these studies we used LS174T and HCA-7 colon, MCF-7 breast, and A2780 ovarian adenocarcinoma cells, all expressing wild-type p53. Clinically relevant concentrations of the topoisomerase I and II inhibitors SN-38 (the active metabolite of CPT-11) and VP-16 (etoposide) were used as DNA-damaging agents. Growth inhibition after exposure to the drugs was observed in all cell lines investigated. In the LS174T, MCF-7, and A2780 cell lines, growth inhibition was characterized by growth arrest, whereas in HCA7 cells this could be attributed to cell death (Fig. 1a).

The response of LS174T cells to SN-38 was characterized mainly by prolonged G1 arrest, although some cells were arrested in the S and G2-M. A percentage of cells, however, underwent apoptosis, which was maximal after 72 h (Fig. 1b). In A2780 cells, no apoptosis could be observed by analysis of DNA content and microscopic examination, and cells were predominantly accumulated in G1 (Fig. 1b). A similar pattern was observed in MCF-7 cells, although again some cells underwent apoptosis, which peaked at 24 h of treatment, with the cell cycle distribution remaining constant thereafter (Fig. 1b). In HCA-7 cells, the response to SN-38 treatment was characterized mainly by apoptosis. At 24 h of exposure, transient cell cycle arrest could be observed in G1, after which cells progressed to G2-M and died by apoptosis (Fig. 1b).

Exposure to VP-16 had similar effects. In LS174T, A2780, and MCF-7 cells, the response was cell cycle arrest, whereas HCA-7 cells died by apoptosis. Contrary to SN-38 exposure, there was little evidence of apoptosis with VP-16 in LS174T cells either by FACS analysis or by microscopic examination. Accumulation in G1 was much more profound, whereas the S phase was depopulated (Fig. 1b). In A2780 cells treated with VP-16, some apoptosis could be observed (not shown).

**Cell Cycle Arrest in LS174T Cells Is Irreversible.** We next investigated whether withdrawal of the drug would result in release of the cell cycle block. LS174T cells were incubated with SN-38 (6.25–100 ng/ml) for 168 h, after which the cells were recultured in drug-free medium. There was no growth after drug withdrawal: the cell counts remained constant for 4 weeks, and cells excluded trypan blue (up to 168 h; Fig. 2a and data not shown) at each concentration of the initial SN-38 exposure. Apoptosis could not be observed by flow cytometric analysis of DNA content or cellular morphology, and there was no redistribution of the cell cycle (Fig. 2b).

During treatment, cells exposed to low SN-38 concentrations progressed through G1 and S phase, followed by accumulation in the G2-M phase of the cell cycle. Cells exposed to intermediate levels of the drug arrested in late S and G2-M phase, whereas cells exposed to the highest SN-38 doses were retained in G1 phase (30). After drug withdrawal and reculturing in drug-free medium, the cell cycle distribution remained the same. At low concentrations, cells remained arrested in the G2-M phase, at intermediate concentrations in S phase, and at the highest concentrations in the G1 phase of the cell cycle (Fig. 2b). To investigate when cell cycle arrest became irreversible, LS174T cells were exposed to 100 ng/ml SN-38 for 24–168 h, followed by a 48-h washout period in drug-free medium and 30 min of pulse-chase labeling with BrdUrd. FACS analysis of dual staining for DNA content and FITC-anti-BrdUrd to visualize incorporation of BrdUrd into newly synthesized DNA showed that 24 h of exposure to SN-38 was sufficient to irreversibly arrest LS174T cells (Fig. 2c). No BrdUrd incorporation could be observed in the longer incubations (not shown).

**Expression of p53, p21, and p16INK4a in LS174T Cells.** SN-38 treatment resulted in increased p53 protein levels and the transcriptional activation of p21; p21 levels increased within 4–8 h up to a maximum at 24 h, after which they remained constant (Fig. 3a). In contrast, overall Rb levels decreased with time, although the relative abundance of the hypophosphorylated form increased with time until only the hypophosphorylated form was present (Fig. 3a). During SN-38 exposure, there was no change in the protein levels of p16INK4a (Fig. 3a). However, when after 7 days of exposure to SN-38 the drug was withdrawn and the cells were recultured in drug-free medium, the levels of p53 decreased dramatically, which resulted in a gradual decrease in p21 levels (Fig. 3b). This was mirrored by an increase in...
p16\(^{INK4a}\). Levels of p16\(^{INK4a}\) started increasing 8 h after drug withdrawal and continued to increase to a maximum at 7 days and remained elevated to 4 weeks (Fig. 3).

**DNA Damage Induces a State Resembling Cellular Senescence in Tumor Cells.** The observation that the LS174T, A2780, and MCF-7 cells seemed permanently arrested, resisted apoptosis, and had an enlarged and flattened morphology (results for LS174T cells shown in Fig. 4; results for A2780 and MCF-7 cells not shown)
together with the prolonged expression of p53 and p21 in the LS174T cells were an indication that the response to DNA damage was induction of a senescence-like state. It has been shown in several cell types that senescent, but not presenescent or terminally differentiated cells stain positive for SA-β-gal activity at pH 6 (2). To investigate whether the irreversible cell cycle arrest observed could be characterized as senescence, we stained treated cells for SA-β-gal. In LS174T cells, increased β-galactosidase staining relative to control was observed 24 h after treatment with SN-38 at all concentrations investigated, and the number of SA-β-gal-positive cells continued to increase up to 120 h of exposure (100 ng/ml SN-38; Fig. 4 and results not shown). MCF-7 and A2780 cells also stained positive for β-galactosidase activity (Fig. 4b), although the increase was slower than in LS174T cells. Treatment with VP-16 produced a similar staining pattern, although the number of SA-β-gal-positive cells was generally greater than in the case of SN-38 (results for LS174T cells shown in Fig. 4b, and results not shown). Even in surviving HCA-7 cells, in which the main response to treatment was apoptosis, an increasing number of SA-β-gal-positive cells could be observed (Fig. 4b). When LS174T cells were stained for SA-β-gal after a 48-h washout period, the number of cells that stained positive (Fig. 4c) was similar the number of cells that staining positive during SN-38 treatment (Fig. 4a), consistent with the fact that senescence is an irreversible process.

**p53 and p21 Are Required for DNA Damage-induced Senescence in Tumor Cells.** To further assess the requirement for p53, p21, and p16 in the process of DNA damage-induced senescence, we examined the consequences of SN-38 treatment in the HCT116 colon carcinoma cell line, which expresses wild-type p53 but has a methylated p16 promoter, and its isogenic derivatives, which are null for either p53 or p21. The HCT116 cell lines were exposed to 6 ng/ml SN-38 for 24 h and stained for SA-β-gal on day 7. As can be seen in Fig. 5a, the HCT116 parental cell line expressing wild-type p53 showed enlarged and flattened morphology and SA-β-gal staining in the majority of cells, whereas these characteristics are absent in the DMSO control. There was no significant induction of apoptosis in these cells, no sub-G1 peak with FACS analysis, or cleavage of caspase 3 (results not shown). In the p53- and p21-null isogenic HCT116 cell lines (Fig. 5, b and c), the morphology of the SN-38-treated cells was similar to that of the DMSO controls, and SA-β-gal staining was observed in only a few individual cells, showing that the loss of either p53 or p21 abrogates the induction of senescence. On day 7, the majority of the p53- and p21-null cells were in the early stages of apoptosis, as cells with a sub-G1 content and caspase 3 cleavage could be observed (results not shown), similar to the pattern observed in p53 mutant cell lines.

In contrast to the LS174T cells, no induction of p16 could be observed in the HCT116 cells, most likely because of the hypermethylated p16 promoter. However, the p53 and p21 levels were maintained in this background (results not shown).

**DNA Damage Is Able to Induce Senescence in Vivo.** To investigate whether senescence could be induced in vivo in response to DNA-damaging agents, newly sectioned material from frozen archival breast tumors from patients who had received neoadjuvant chemotherapy was stained for SA-β-gal activity and p53 and p16INK4a protein levels. Tumor sections from patients who had not been treated with surgery before were stained as controls. All treated patients had received CAF, and tumors had been resected 12–87 days after chemotherapy. One of the difficulties of the SA-β-gal protocol is that it is based on enzymatic activity, which is difficult to show in archival material. Although not all tumor cells in positive sections showed staining, 15 of 36 tumors (41%) stained positive for SA-β-gal (Table 1 and Fig. 6a), whereas sections of normal tissue and normal tissue surrounding positive tumors were completely negative (Table 1 and example in Fig. 6a). Tumor sections of patients who had not received chemotherapy showed SA-β-gal staining in 2 of 20 cases (10%: P = 0.014). The staining in the untreated tumor sections was in isolated individual cells, unlike the patches of positive cells in the SA-β-gal-positive treated tumor sections. These data indicate that the SA-β-gal staining observed in the tumor sections is most likely attributable to chemotherapy treatment.

SA-β-gal staining was associated with low p53 staining (P = 0.019, Fisher’s exact test; example in Fig. 6b) and high p16INK4a staining (P = 0.006, Fisher’s exact test; example in Fig. 6c) compared with SA-β-gal-negative tumors (Table 1). Generally, the SA-β-gal-negative tumors overexpressed p53 and/or had low levels of the p16INK4a protein (Table 1). In normal tissue, no overexpression of p53 or p16INK4a was observed (Table 1).

**DISCUSSION**

In this study we show that human carcinoma cell lines expressing wild-type p53 respond to DNA damage by inducing cellular senescence or apoptosis. Exposure of LS174T and HCT116 colon, MCF-7 breast, and A2780 ovarian carcinoma cells to topoisomerase I and II inhibitors resulted in prolonged cell cycle arrest predominantly in the G1 phase of the cell cycle. The HCA-7 colon adenocarcinoma cells responded mainly by apoptosis. The fact that the LS174T and MCF-7 cell lines did not respond to treatment by apoptosis was not attributable to a defect in their apoptotic pathway: both cell lines rapidly underwent p53-dependent apoptosis after exposure to 5,6-dichloro-1-β-d-ribofuranosyl-benzimidazole in our previous study (35).

Cell cycle arrest became irreversible within 24 h of exposure and was characterized by increased levels of p53 and p21, resulting in the increased relative abundance of the hypophosphorylated form of Rb. Our data are consistent with a model in which DNA damage leads to p53-dependent transcriptional activation of p21 (11), which may sustain arrest by association with G1 and G2 cyclins, CDKs, and proliferating cell nuclear antigen (12, 13, 36, 37) in inhibitory complexes. In HCT116 cell lines in which either the p53 or p21 function was compromised, DNA damage-induced senescence was abrogated, confirming the necessity of these proteins in this process.

Drug withdrawal resulted in the decrease of p53 and p21 levels in...
the LS174T cells, but was mirrored by increases in the levels of p16\textsuperscript{INK4a}, which by its interaction with CDK4 and CDK6 can also prevent phosphorylation of Rb and the related p107 and p130 proteins, thus maintaining cell cycle arrest (22, 38, 39). Similar patterns were observed in senescing normal human diploid fibroblasts and fibroblasts treated with DNA-damaging agents leading to senescence (21, 23). However, when cells are unable to express p16, as in the case of the HCT116 cell line, which has a methylated p16\textsuperscript{INK4a} promoter, the increased levels of p53 and p21 upon exposure to SN-38 were maintained after drug withdrawal, thus maintaining the senescent phenotype.

At the cellular level, in response to treatment with SN-38 and VP-16 cell morphology became flattened and enlarged, indicating that DNA damage induced senescence in LS174T, MCF-7, A2780, and the parental HCT116 cells. This was strengthened by the finding that after exposure to SN-38 and VP-16, all cell lines investigated stained positive for \(\beta\)-galactosidase activity at pH 6. Senescence was observed only in p53 wild-type cells. p53-null or mutant ovarian and colon cell lines respond to topoisomerase inhibitors by apoptosis after transient S-G\(_2\) arrest (30). This and the fact that p53 levels increased during drug exposure and returned to normal after drug withdrawal suggests that p53 is required for the induction...
overexpression of p21 has been shown to block entry into mitosis (42–44).

Often the use of cytotoxics in the clinic does not result in regression of the tumor, but rather results in stable disease, which has typically been seen as a failure of treatment. This may not be the case if tumors contain senescent cells in response to treatment and may explain some of the controversy around p53 as a prognostic marker because nonresponders include both progressive and stable disease. As we have shown here, cytotoxics are capable of inducing senescence in tumor cell cultures and tumor tissue in vivo. Fifteen of 36 breast tumor resections after neoadjuvant chemotherapy stained positive for SA-β-gal (41%) and had low p53 and high p16 

\(^{INK4a}\) staining, whereas only 2 of 20 untreated tumors showed any SA-β-gal staining, suggesting that senescence may indeed be a relevant process in determining treatment outcome. Therefore, p53 may prove to be a better prognostic factor if stable disease is grouped with the responders.

The relationship between stable disease and survival is not well documented, and we have not been able to find any report investigating p53 status in relation to stable disease. However, one of the few reports that have split the nonresponders in stable and progressive disease found that there was no significant difference with regard to survival between patients with regression of the tumor and stable disease (45). Furthermore, the prognostic role of p53 seems much less well defined after resection compared with resection and adjuvant chemotherapy (46). Our data suggest that tumor cells expressing wild-type p53 prevent proliferation when under stress by inducing either apoptosis or accelerated cellular senescence. This latter process appears to require wild-type p53 and p21 for induction, whereas p16 

\(^{INK4a}\) may be involved in its maintenance.

Our data are the first showing that senescence may be a normal cellular response to DNA damage in tumor cells. We believe that our observations could have profound clinical implications and explain some of the controversy concerning p53 as a prognostic factor.

In the clinic, replicative senescence appears to be a relevant factor in determining treatment outcome and warrants further investigation, especially regarding p53 as a prognostic factor and of stable disease in relation to survival after chemotherapy. It would also be extremely important to investigate whether there is any correlation between tumor type or stage of progression and the cellular response to drug treatment, i.e., apoptosis or senescence. Such information could be of great value in choosing an appropriate chemotherapeutic strategy. It is also tantalizing to compare a recent report in which the use of topoisomerase inhibitors in normal human fibroblasts resulted in reversible senescence (33) with our data that tumor cells expressing

### Table 1 DNA damage is able to induce senescence in vivo

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SA-β-gal positive, n (%)</th>
<th>SA-β-gal negative, n (%)</th>
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<tbody>
<tr>
<td>Treated tumor</td>
<td>15 (41)</td>
<td>21 (59)</td>
</tr>
<tr>
<td>p53 +++++</td>
<td>3 (20)</td>
<td>13 (61)</td>
</tr>
<tr>
<td>p16 +++++</td>
<td>13 (87)</td>
<td>8 (38)</td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
<td>5 (100)</td>
</tr>
<tr>
<td>Untreated tumor</td>
<td>2 (10)</td>
<td>18 (90)</td>
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\(^{a}\) Unpublished observations.
wild-type p53 respond to similar treatment by irreversible senescence. In this light, it is encouraging that normal tissue of CAF-treated patients was completely negative for SA-β-gal. Although the molecular basis for this difference between tumor and normal tissue is unknown and should be investigated further, such potential selectivity may be exploited clinically.

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