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Apoptotic Death of Tumor Cells Correlates with Chemosensitivity, Independent of p53 or Bcl-2

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Abstract

p53 has been implicated as a determinant of chemosensitivity and radiosensitivity. We measured chemosensitivity of human tumor cell lines (n = 11), with or without wild-type p53, following exposure to clinically useful chemotherapeutic drugs (n = 4). Chemosensitivity and apoptosis induction were correlated independently of p53 status or Bcl-2 protein levels in vitro. Wild-type p53 correlated with chemosensitivity in ovarian carcinoma and some Burkitt’s lymphoma cell lines, but not in leukemia or lung cancer. Bcl-2 levels correlated with chemoresistance only in Burkitt’s lymphoma. p53-dependent p21WAF1/CIP1 induction and cell cycle arrest occurred at sublethal doses of chemotherapy, whereas at lethal doses of chemotherapy apoptotic death was observed, consistent with models proposing a relationship between the level of DNA damage versus survival or death. Loss of apoptosis induction was observed in drug-resistant ML-1 and HL-60 leukemia cells, without changes in p53 or Bcl-2. Targeted loss of p53 protein in H460 lung cancer cells using HPV-16 E6 inhibited the etoposide-induced G1 checkpoint but did not decrease chemosensitivity. Our studies suggest that the simple measurement of apoptosis induction may be a useful predictor of chemosensitivity, at least in vitro, and confirm that p53 status and Bcl-2 expression may be useful predictors of chemosensitivity in certain cell types.

Introduction

The emergence of chemotherapeutic drug resistance is a major clinical problem and reason for failure in the therapy of patients with cancer (1). There are many mechanisms of resistance to chemotherapy including the overexpression of the MDR1 p-glycoprotein family (2), overexpression of bcl-2 (3), BCR-ABL (4), or MDM2 (5) proteins, or underexpression of DNA topoisomerase II (6). Recent studies have provided some evidence that mutations of the p53 tumor suppressor gene associated with decreased sensitivity of Burkitt’s lymphoma cells to treatment with ionizing radiation and DNA-damaging chemotherapeutic agents (7). In addition, the targeted disruption of p53 in mice led to a radiosensitive phenotype (8, 9). Studies have also suggested that the p53 tumor suppressor gene is required for efficient execution of a common cell death program induced by chemotherapeutic agents (10, 11). The pathway of p53-mediated apoptosis is poorly understood, but its disruption by SV40 large T antigen, HPV-16 E6, or by mutations of the p53 gene, which are very common in human tumors, contributes not only to the tumorigenic process but to the acquisition of drug resistance (11-13).

In the present studies, we tested the hypothesis that induction of the p53 pathway may be an important determinant of chemosensitivity. Several epithelial and hematopoietic tumor cell types were treated with one of four clinically useful chemotherapeutic agents. In addition to the role of p53 induction, we also evaluated the induction of apoptosis, by both sublethal and lethal doses of chemotherapeutic agents in the same tumor cells, as potential predictors of chemosensitivity. At sublethal doses of chemotherapeutic drugs, the p53 pathway was induced, resulting in cell cycle arrest, but little or no DNA degradation was observed. At lethal doses of chemotherapeutic agents, substantial amounts of degraded DNA were observed. When the tumor cells expressed no p53, or contained mutant p53 genes, DNA degradation was still found following exposure to lethal doses of chemotherapeutic drugs. Little or no apoptosis was observed at sublethal doses of chemotherapy regardless of p53 status. Tumor cell lines containing an intact wild-type p53 pathway were not necessarily more sensitive to treatment by DNA-damaging chemotherapeutic agents as compared with cell lines containing mutant or no p53, i.e., leukemia or lung cell lines without wild-type p53 were more sensitive to chemotherapeutic agents, whereas ovarian carcinoma and some Burkitt’s lymphoma cell lines were more sensitive if they expressed wild-type p53. Disruption of p53 function by HPV-16 E6 did not lead to chemoresistance. Additionally, tumor cell lines selected for resistance to chemotherapy still had intact p53 function. However, such resistant tumor cell lines lost the ability to undergo apoptosis when exposed to chemotherapeutic agents at previously lethal doses. Our results suggest that the simple in vitro measurement of apoptosis induction following chemotherapeutic drug exposure may be a useful marker of chemosensitivity, independent of p53 status.

Materials and Methods

Tumor Cell Lines and Culture Conditions. The human leukemia cell lines ML-1 and HL-60, a gift from Michael B. Kastan (Johns Hopkins University, Baltimore, MD), were maintained in RPMI 1640 supplemented with 10% FBS, 1% penicillin, and streptomycin.

The abbreviations used are: FBS, fetal bovine serum; CMV, cytomegalovirus.
Apoptotic Death of Tumor Cells

lines Fowler, CA46, and RAMOS, a gift from Kishor Bhatia, Patrick M. O’Connor, and Ian Magrath (National Cancer Institute, Bethesda, MD), and the human lung cancer cell lines H460, U1752, and H209, a gift from Stephen B. Baylin (Johns Hopkins University, Baltimore, MD), were also maintained in RPMI 1640. The human ovarian cancer cell lines PA-1, OVCAR3, and SKOV3 were obtained from American Type Culture Collection (Rockville, MD) and maintained in basal modified Eagle’s medium, RPMI 1640 with 10 μg/ml insulin, and McCoy’s 5A medium, respectively. The chemotherapeutic agents Adriamycin, carboplatinum, etoposide, and cytoxan were obtained from the Oncology Outpatient Pharmacy at the University of Pennsylvania Comprehensive Cancer Center. Cells were grown in the presence of various concentrations of chemotherapeutic agents for different durations as indicated in the individual figure legends.

**Western Blot Analysis.** Following incubation of tumor cell lines either in the absence or presence of chemotherapeutic agents, total cellular proteins were harvested in SDS-PAGE loading buffer and electrophoresed through 15% denaturing polyacrylamide gels as previously described (14). Immunoblotting for p53, p21WAF1/CIP1, and Bcl-2 expression was performed using mouse anti-human p53 monoclonal pAb1801 (Oncogene Science) to detect human p53, the mouse anti-human WAF1 monoclonal EA10 (Oncogene Science) to detect human p21WAF1/CIP1 proteins, and the mouse anti-human Bcl-2 monoclonal 124 (DAKO) to detect human Bcl-2 proteins as described previously (14, 15).

**Determination of Cell Cycle Arrest.** The proportion of tumor cells engaged in new DNA synthesis was determined by measuring [3H]thymidine incorporation into cellular DNA following treatment of tumor cell lines with various concentrations of Adriamycin and etoposide as previously described (14).

**Determination of Tumor Cell Viability and Chemosensitivity.** For nonadherent tumor cell lines, cell viability was determined using trypan blue exclusion on day 7 following treatment with various chemotherapeutic agents used at different concentrations. Adherent tumor cell lines were plated in 24-well plates and selected in the presence of different concentrations of several chemotherapeutic agents. Clonogenic survival was determined at 7–10 days after drug exposure using Wright’s stain of the cells which remained attached to the plates. Viability testing was performed in triplicate. Using the percentage of viability for nonadherent cells and percentage of clonogenic survival for adherent tumor cell lines, we determined the dosage of each chemotherapeutic agent which resulted in a 50% loss of viability relative to untreated cells (IDso).

**Analysis of DNA Integrity.** In the initial experiments we evaluated tumor cell lines for gross morphological changes including nuclear fragmentation using the DNA reactive dye 4,6-diamidino-2-phenylindole staining and fluorescence microscopy as described (16). We also varied time of exposure to chemotherapeutic drugs as well as drug concentration to determine the optimum assay conditions. For evaluation of tumor cells for the presence of cellular DNA fragmentation, cells were treated at different doses (as indicated in the figure legends) of chemotherapeutic drugs for 3 days, harvested, and the DNA was extracted and visualized as described previously (17, 18). In brief, 5 × 10⁶ cells were lysed by incubation in 1.0 ml 5 mM Tris (pH 7.4), 5 mM EDTA, and 0.5% Triton X-100 for 2 h at 4°C. The lysate was centrifuged at 27,000 × g for 20 min to remove large molecular weight undegraded DNA. The supernatant containing degraded DNA, RNA, and cellular proteins was treated with 200 mg/ml proteinase K for 1 h at 50°C, phenol/chloroform extracted, ethanol precipitated, and treated with 1 mg/ml boiled pancreatic RNase A for 1 h at 50°C. DNA integrity was assessed following agarose gel electrophoresis and ethidium bromide staining.

**Selection of Chemosensitive Tumor Cell Lines.** ML-1 and HL-60 were seeded at 1 × 10⁵ cells/ml in 24-well plates, and selected with various concentrations of either Adriamycin or etoposide. After 1 month, resistant pooled clones were viable. Resistant HL-60 clones were propagated in 10% FBS-supplemented RPMI 1640 media containing either 0.012 μg/ml Adriamycin or 0.2 μM etoposide, and resistant ML-1 clones were propagated in 10% FBS-supplemented RPMI 1640 media containing either 0.01 μg/ml Adriamycin or 0.2 μM etoposide.

**Targeting p53 for Destruction.** To generate a tumor cell line in which p53 function was lost, H460 non-small cell lung cancer cells were transfected with an HPV 16 E6 expression plasmid, which expresses E6 protein constitutively driven by the CMV immediate early promoter. H460 cells were transfected with either pcMV-neo-bam (gift from Bert Vogelstein, Johns Hopkins University, Baltimore, MD) or pcMV16E6 (gift from Kathleen R. Cho, Johns Hopkins University, Baltimore, MD) as previously described (19). Individual clones were isolated following selection in the presence of 500 μg/ml G418 (Life Technologies, Inc.) as described (20) and tested for p53 and p21WAF1/CIP1 inducibility following treatment with Adriamycin as previously described (14).

**Results**

**Chemosensitivity versus p53 Status.** To test the hypothesis that p53 status is a key determinant of tumor cell sensitivity to chemotherapeutic agents, we treated a panel of human tumor cell lines (n = 11) with one of four clinically useful chemotherapeutic agents. We measured clonogenic survival at multiple drug concentrations and used this to calculate the dose at which 50% of the tumor cells were nonviable (IDso) with each drug (Fig. 1). In preliminary experiments with nonadherent tumor cell lines, trypan blue exclusion was measured on a daily basis following addition of drugs until a steady state of percentage of viability was reached (data not shown). Fig. 1 shows the ID50 for 11 tumor cell lines containing either wild-type p53 (n = 5) or mutant (or nonexpressed) p53 (n = 6), determined in the presence of either Adriamycin (A), etoposide (B), carboplatinum (C), or cytoxan (D). To assess the role of p53 in tumors from different tissue origins, we chose previously characterized tumor cell lines of leukemic origin (ML-1 and HL-60), lymphoma origin (Fowler, CA46, and RAMOS), ovarian carcinoma (PA-1, OVCAR3, and SKOV3), and lung cancer (H460, U1752, and H209) to determine chemosensitivity (Fig. 1). Each category contained at least one wild-type p53-containing tumor cell line and at least one tumor cell line which expressed either mutant p53 or no p53 protein, as indicated in the legend to Fig. 1. Prior to determination of the IDso’s, the status of p53 was confirmed in each tumor cell line by checking for induction of both p53
The determination of $ID_{50}$'s for tumor cell lines with drugs which kill by diverse mechanisms allowed examination of the role of p53 in the same tumor cells either in the presence or absence of p53 induction. Table 1 lists $ID_{50}$ values for the cell lines shown in Fig. 1. In the leukemia cell lines ML-1 and HL-60, there was no apparent difference in $ID_{50}$ for Adriamycin, etoposide, or carboplatinum. Thus, for these two cell lines, the presence of wild-type p53 in ML-1 or the absence of p53 expression in HL-60 did not alter sensitivity to drugs which induced p53 in ML-1. The $ID_{50}$ with cytoxan was 3-fold higher for HL-60 as compared with ML-1 (Fig. 1D and Table 1); cytoxan has not been reported to induce p53 and did not in our wild-type p53-containing cell lines (data not shown). In lung cancer, $ID_{50}$ appeared to be influenced more by histological type than by p53 status, i.e., the wild-type p53-expressing non-small cell lung cancer cell lines H460 and U1752 were both significantly more resistant to all drugs tested than the small cell lung cancer cell line H209 which expresses mutant p53 (Fig. 1 and Table 1). This appeared to be the case regardless of whether p53 could be induced by a particular agent. In Burkitt's lymphoma cell lines, we observed a similar relationship between p53 status and chemosensitivity as has previously been reported (7). The Burkitt's cell line CA46, which expresses mutant p53, was more resistant to all agents tested as compared with the wild-type p53-expressing Burkitt's cell line Fowler (Fig. 1 and Table 1). However, a less consistent relationship was obtained if the $ID_{50}$'s with the Fowler cell line were compared with the $ID_{50}$ obtained with another mutant p53-expressing Burkitt's cell line RAMOS (Fig. 1 and Table 1), i.e., RAMOS was more sensitive than Fowler with Adriamycin and etoposide (Fig. 1, A and B), but appeared to be more resistant than Fowler following treatment with carboplatinum or cytoxan (Fig. 1, C and D). In three ovarian carcinoma cell lines, wild-type p53 status correlated with chemosensitivity with all drugs tested. Thus, wild-type p53-containing PA-1 cells were more sensitive to all four drugs used than the two cell lines OVCAR3 and SKOV3, which do not express wild-type p53 (Fig. 1 and Table 1).

Evaluation of the results as a function of the tissue type of origin did not provide a consistent pattern. For example, although the leukemia cell lines tested were more sensitive to all drugs than tumor cell lines derived from other tissues, there were examples of resistant lines within otherwise sensitive groups of tumor cell lines derived from other tissues, i.e., CA46 or SKOV3. Interestingly, both of these tumor cell lines which lack wild-type p53 protein displayed resistance to all drugs used compared to the corresponding wild-type p53-expressing tumor cells derived from the same tissue type. There was in general a similarity in the relative $ID_{50}$'s with a particular cell line to all agents used. In other words, ML-1, HL-60, PA-1, and H209 were generally sensitive to all four drugs, whereas CA46, SKOV3, H460, and U1752 were more resistant to the same drugs. Therefore, although there were examples where p53 status appeared to correlate with chemosensitivity, i.e., in the ovarian and some lymphoma cell lines, the discrepancies observed with other cell lines would suggest that perhaps factors other than p53 status alone (such as cell type, the particular drug, or the expression of MDR1, Bcl-2, or other proteins) might play an important role in predicting chemosensitivity. In this regard, we checked Bcl-2 expression in this panel of tumor cell lines, and p21WAF1/CIP1 proteins following incubation in the presence of either Adriamycin or etoposide, as previously described (7, 14). As expected, cell lines that expressed mutant p53 did not up-regulate expression of either p53 or p21WAF1/CIP1, whereas tumor cell lines that expressed wild-type p53 up-regulated both (data not shown).

![Graphs showing chemosensitivity of human tumor cell lines versus p53 status.](https://example.com/graph1.png)

**Fig. 1** Chemosensitivity of human tumor cell lines versus p53 status. Histogram of mean $ID_{50}$ values ± SD (Y-axis) in the presence of the chemotherapeutic agents Adriamycin (A), etoposide (B), carboplatinum (C), or cytoxan (D) are shown for a panel of human tumor cell lines (X-axis) derived from diverse tissue origins: leukemia (ML-1 and HL-60), Burkitt's lymphoma (Fowler, RAMOS, and CA46), ovarian carcinoma (PA-1, OVCAR3, and SKOV3), or lung cancer (H460, U1752, and H209). p53 status was designated as wild-type (ML-1, Fowler, PA-1, and H460), mutant (RAMOS, CA46, OVCAR, SKOV3, and H209), or no p53 expression (HL-60). H209 demonstrated the presence of p53 overexpression as well as DNA damage-inducible p21WAF1/CIP1, suggesting the presence of a wild-type allele.
Apoptotic Death of Tumor Cells

626

Table 1 ID50 as function of p53 status of tumor cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>p53*</th>
<th>Adriamycin (µg/ml)</th>
<th>Etoposide (µM)</th>
<th>Carboplatin (µM)</th>
<th>Cytoxan (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ML-1</td>
<td>w.t.</td>
<td>0.04 ± 0.02</td>
<td>0.11 ± 0.01</td>
<td>4.63 ± 0.38</td>
<td>13.9 ± 1.13</td>
</tr>
<tr>
<td>Fowler</td>
<td>w.t.</td>
<td>0.17 ± 0.02</td>
<td>2.5 ± 1.5</td>
<td>15.0 ± 1.1</td>
<td>400 ± 204</td>
</tr>
<tr>
<td>PA-1</td>
<td>w.t.</td>
<td>0.08 ± 0.05</td>
<td>0.33 ± 0.09</td>
<td>5.0 ± 0.0</td>
<td>360 ± 170</td>
</tr>
<tr>
<td>H460</td>
<td>w.t.</td>
<td>0.53 ± 0.09</td>
<td>2.7 ± 1.35</td>
<td>61.3 ± 15.1</td>
<td>1190 ± 700</td>
</tr>
<tr>
<td>U1752</td>
<td>w.t/m</td>
<td>0.39 ± 0.09</td>
<td>2.91 ± 1.64</td>
<td>45.0 ± 15.0</td>
<td>1650 ± 980</td>
</tr>
<tr>
<td>HL-60</td>
<td>m</td>
<td>0.03 ± 0.02</td>
<td>0.09 ± 0.02</td>
<td>3.53 ± 0.23</td>
<td>37.5 ± 7.50</td>
</tr>
<tr>
<td>RAMOS</td>
<td>m</td>
<td>0.15 ± 0.03</td>
<td>0.90 ± 0.5</td>
<td>20.2 ± 14.1</td>
<td>860 ± 280</td>
</tr>
<tr>
<td>CA46</td>
<td>m</td>
<td>0.45 ± 0.04</td>
<td>7.70 ± 3.4</td>
<td>84.0 ± 54.0</td>
<td>3893 ± 300</td>
</tr>
<tr>
<td>OVCAR3</td>
<td>m</td>
<td>0.14 ± 0.06</td>
<td>0.70 ± 0.1</td>
<td>7.50 ± 2.5</td>
<td>404 ± 270</td>
</tr>
<tr>
<td>SKOV3</td>
<td>m</td>
<td>0.55 ± 0.25</td>
<td>6.4 ± 0.0</td>
<td>53.3 ± 9.4</td>
<td>2440 ± 1430</td>
</tr>
<tr>
<td>H209</td>
<td>m</td>
<td>0.10 ± 0.07</td>
<td>0.64 ± 0.17</td>
<td>12.5 ± 5.4</td>
<td>207 ± 25</td>
</tr>
</tbody>
</table>

*The p53 status of the listed cell lines has been previously reported (7, 22, 45, 46).

w.t., wild type; m, mutant; HL-60 and SKOV3 express no p53 protein.

Fig. 2 Bcl-2 expression in a panel of human tumor cell lines of diverse tissue origin. Western blot analysis of ML-1 (Lane 1), HL-60 (Lane 2), Fowler (Lane 3), RAMOS (Lane 4), CA46 (Lane 5), PA-1 (Lane 6), OVCAR3 (Lane 7), SKOV3 (Lane 8), H460 (Lane 9), U1752 (Lane 10), and H209 (Lane 11) tumor cells for expression of Bcl-2 protein (arrow).

cell lines and found no correlation between chemosensitivity (Fig. 1) and Bcl-2 expression level (Fig. 2).

Cell Death versus Arrest following p53 Induction. Mammalian cells respond to DNA damage by undergoing either cell cycle arrest or death. Induction of the tumor suppressor p53 following DNA damage has been correlated with this cell cycle arrest (21) or death by apoptosis (9). The identification of the cyclin-dependent kinase inhibitor p21WAF1/CIP1 as a target for transcriptional activation by p53 has provided a molecular mechanism for the cell cycle arrest following DNA damage (14, 19). However, the mechanism of p53-mediated apoptosis, and the coordination and control of the decision to undergo cell cycle arrest versus apoptosis remains unknown. It has been proposed that p53-dependent cell death may be an important determinant of cytotoxicity following administration of chemotherapeutic agents (10). We hypothesized that both cell cycle arrest and cell death may occur simultaneously at varying extents depending on the degree of DNA damage. We examined this hypothesis by treating two wild-type p53-expressing tumor cell lines (ML-1 and Fowler) with sublethal versus lethal doses (based on ID50’s in Fig. 1 and Table 1) of the chemotherapeutic agent Adriamycin, and then by measuring the consequences with respect to p53 induction, p21WAF1/CIP1 induction, cell cycle arrest, and the induction of cell death as measured by DNA fragmentation (Fig. 3).

Both wild-type p53-containing cell lines ML-1 and Fowler induced p53, p21WAF1/CIP1, underwent cell cycle arrest and extensive DNA degradation following treatment with a lethal dose of Adriamycin (Fig. 3, Lanes 4 and 8 versus Lanes 1 and 5). At a sublethal dose, p21WAF1/CIP1 protein was induced (Fig. 3, Lanes 2 and 6 versus Lanes 1 and 5), and cell cycle arrest was observed, i.e., 40 and 50% of control [3H]thymidine incorporation with ML-1 and Fowler treated with the sublethal doses 0.0125 and 0.05 µg/ml, respectively. Induction of p53 protein was not appreciated by Western blot analysis at these low doses of Adriamycin, but with increasing doses, there was an increase in p53 expression, p21WAF1/CIP1 expression, and an accompanying increase in the degree of cell cycle arrest (Fig. 3). These results appear to be consistent with the hypothesis that both cell cycle arrest and cell death may occur simultaneously at lethal doses of DNA-damaging chemotherapeutic agents. Furthermore, the occurrence of cell cycle arrest at sublethal doses is consistent with the idea that repair allowing cell survival may be occurring. We tested HL-60 cells (which express no p53 protein) with a lethal dose of Adriamycin and likewise observed simultaneous cell cycle arrest and endonucleolytic DNA cleavage (data not shown). These results suggested the possibility that comparing the extent of apoptosis induced by a particular dose of chemotherapeutic agent may reflect lethality to such an agent, rather than simply measuring cell cycle arrest or p53 induction. Additionally, the results in Figs. 1 and 3 suggested that although under some circumstances there may be a correlation between p53 induction, apoptosis induction, and clonogenic survival, measuring cell death may provide an independent prediction about cytotoxicity, particularly in tumor cell lines which lack wild-type p53, or under circumstances where p53-mediated cell cycle arrest occurs and permits cell survival.

Apoptosis Induction versus Chemosensitivity. Our earlier studies showed that DNA fragmentation occurred following treatment of ML-1, HL-60, and Fowler cells with lethal doses of Adriamycin (Fig. 3; data not shown), whereas sublethal doses appeared to induce p21WAF1/CIP1-associated growth arrest in wild-type p53-expressing tumor cell lines without significant DNA degradation (Fig. 3). We therefore evaluated the use of apoptosis induction as an independent marker of chemosensi-
though this greater sensitivity was suggested by the lower ID_{50} cell line, suggesting that HL-60 may be more sensitive to various chemotherapeutic drugs (Fig. 4; data not shown). In the Burkitt’s lymphoma cell lines, we observed either extensive DNA fragmentation, little DNA fragmentation, or no DNA fragmentation in the RAMOS, Fowler, and CA46 cell lines, respectively (Fig. 4B). At the concentrations of Adriamycin (0.0125 or 0.05 μg/ml) and etoposide (0.2 or 1.0 μM) used, the observed patterns of DNA fragmentation would be predicted if apoptosis induction correlated with the cytotoxicity/clonogenic survival; i.e., CA46 which was resistant to the doses of Adriamycin and etoposide used (Fig. 1 and Table 1) did not produce any detectable DNA fragmentation (Fig. 4B), RAMOS which was sensitive to the doses of Adriamycin and etoposide used (Fig. 1 and Table 1) did not produce any detectable DNA fragmentation (Fig. 4B), and an intermediate pattern of DNA fragmentation was observed with the Fowler cell line which displayed intermediate sensitivity to Adriamycin and etoposide (Figs. 1 and 4B). If wild-type p53 expression and the ability to undergo either p53-mediated cell cycle arrest or cell death alone could accurately predict chemosensitivity, we should not have observed extensive cell death in the mutant p53-expressing RAMOS cell line. It is possible that p53 status may account for the observed differences in cytotoxicity between the Fowler and CA46 cell lines; i.e., Fowler which contains wild-type p53 was more sensitive to DNA-damaging agents and underwent more extensive DNA fragmentation as compared with mutant p53-containing CA46, in which no DNA fragmentation was observed at the doses used in Fig. 4B. It is also possible that in the Burkitt’s lymphoma cell lines Bcl-2 expression levels are more meaningful in terms of predicting chemosensitivity, i.e., there appears to be a perfect correlation between Bcl-2 expression levels and apoptosis induction in this group of tumor cell lines and both the relative chemosensitivities and apoptosis induction (Figs. 1, 2, and 4B).

In ovarian carcinoma cell lines, we observed similar patterns of apoptosis induction relative to ID_{50} values (Figs. 1 and 4C). Extensive, little, or no DNA fragmentation was observed following exposure to either Adriamycin or etoposide of the human ovarian carcinoma cell lines PA-1, OVCAR3, and SKOV3, respectively (Fig. 4C), and this correlated perfectly with the relative chemosensitivities of these cell lines to Adriamycin and etoposide (Fig. 1 and Table 1). In the case of ovarian carcinoma, the relative chemosensitivities also correlated with p53 status; i.e., the most sensitive cell line PA-1 expressed wild-type p53, whereas the more resistant cell lines OVCAR3 and SKOV3 lacked wild-type p53. However, the relative resistance of SKOV3 versus OVCAR3 is the opposite of what might be predicted based on acquisition of a gain of function mutant of p53; i.e., OVCAR3 contains a mutant p53 (248_{Arg}^{*}_{Glu})...
SKOV3 contains no detectable p53 mRNA (22). A similar mutation at codon 248/Glu→Asp has been shown to confer a dominant negative property affecting wild-type p53’s function in mediating sequence-specific transcriptional activation (23). None of the ovarian tumor cell lines expressed Bcl-2 protein at detectable levels (Fig. 2).

In lung cancer cell lines, we again observed a correlation between apoptosis induction and chemosensitivity; i.e., the most sensitive cell line H209 produced extensive DNA fragmentation following treatment with Adriamycin or etoposide, whereas apoptosis induction was not detectable in the cell lines H460 or U1752 at doses of Adriamycin or etoposide well below their ID₅₀’s (Fig. 4D and Table 1). In the lung cancer group, neither chemosensitivity nor apoptosis induction correlated with p53 status. Thus, although H209 expressed mutant p53, this cell line was relatively more sensitive to all drugs tested as compared with wild-type p53-expressing H460 and U1752. In the case of lung cancer cell lines, it is likely that other factors such as histological subtype (small cell lung cancer versus non-small cell lung cancer) may have a more significant relationship to chemosensitivity than p53 status. There was no correlation between the Bcl-2 oncogene expression level and chemosensitivity in the lung cancer cell lines, i.e., the most sensitive cell line, H209, expressed the highest levels of Bcl-2 (Fig. 2). Thus, in lung cancer cell lines, as in leukemia cell lines, the Bcl-2 expression level appears to negatively correlate with chemosensitivity.

These results suggest that the simple in vitro measurements of either nuclear condensation or DNA fragmentation following chemotherapeutic drug exposure reflect the much more labor-intensive and time-consuming determination of ID₅₀ to assess chemosensitivity. It remains to be determined whether any of these in vitro measurements mimic the in vivo situation.

Loss of Apoptosis Induction versus Chemoresistance. To further explore the relationship between apoptosis induction and chemosensitivity, we selected chemotherapy-resistant tumor cell lines. Our hypothesis was that if apoptosis induction is indeed a good marker of chemosensitivity, then its levels should decrease in the same cell lines selected for drug resistance. Fig. 5 shows the degree of apoptosis induction obtained with HL-60 parental cells either untreated (a, b, and k; Lane 1) or treated with low doses of either Adriamycin (c, d, and k; Lane 2) or etoposide (e, f, and k; Lane 3) as compared with levels of apoptosis induction observed in the presence of Adriamycin or etoposide in HL-60 cells selected for resistance to either Adriamycin (g, h, and k; Lane 4) or etoposide (i, j, and k; Lane 5), respectively.
Fig. 5  Loss of apoptosis induction in HL-60 cells selected for resistance to Adriamycin or etoposide. Phase microscopy (a, c, e, g, and i) or UV fluorescence microscopy (b, d, f, h, and j) was performed at ×400, and analysis of DNA integrity (k) was carried out for parental HL-60 cells untreated (a, b, and k; Lane 1) or incubated either in the presence of 0.024 μg/ml Adriamycin (c, d, and k; Lane 2) or 0.2 μM etoposide (e, f, and k; Lane 3), HL-60 cells selected and growing in the presence of 0.024 μg/ml Adriamycin (g, h, and k; Lane 4), or selected and growing in the presence of 0.2 μM etoposide (i, j, and k; Lane 5).
respectively. We observed a loss of apoptosis induction in drug-resistant HL-60 cells, suggesting that the measurement of apoptosis induction may be a reliable indicator of the relative sensitivity or resistance of tumor cell lines to chemotherapeutic agents. We observed a similar effect on the loss of apoptosis induction in ML-1 cells selected for resistance to either Adria- 
mycin or etoposide (Table 2). The Adriamycin- and etoposide-resis-
tant ML-1 cells still induced p53 and p21^WAF1/CIP1 proteins fol-
lowing treatment with etoposide or Adriamycin, respectively
(data not shown). Thus, the acquired drug resistance of ML-1 cells
was not due to a loss of transcriptionally active wild-type
p53.

Loss of p53 Induction versus Chemoresistance. We
further evaluated the relative status of p53 in determin-
ing chemosensitivity by introducing an HPV 16 E6 expres-
sion vector into wild-type p53-expressing tumor cell lines. Fig.
6A shows a Western blot analysis of p53 and p21^WAF1/CIP1
expression in the absence (Lanes 1, 3, 5, 7, 9, and 11) or follow-
ing incubation in the presence (Lanes 2, 4, 6, 8, 10, and 12) of Adriamycin of parental H460 lung cancer cells (Lanes 1
and 2), two individual G418-resistant clones (Lanes 3–6), two
individual clones transfected with HPV 16 E6 expression vector
and selected for G418 resistance (Lanes 7–10), and pooled
clones transfected with HPV E6 and selected in the presence of
G418 (Lanes 11 and 12). As expected, Adriamycin induced both
p53 and p21^WAF1/CIP1 expression in the parental H460 cells
and individual clones selected only for G418 resistance (Fig.
6A, Lanes 1–6). Pooled clones and one of two clones shown which
were transfected with HPV 16 E6 still induced p53 and p21^WAF1/CIP1 following Adriamycin treatment (Fig. 6A, Lanes
7, 8, 11, and 12). However, clone 6, shown in Fig. 6A, Lanes 9
and 10, failed to induce either p53 or p21^WAF1/CIP1 following
Adriamycin treatment. Since this clone failed to induce p53, we
used it as a test of whether loss of p53 induction leads either to
loss of the p53-mediated G1 checkpoint or to chemoresistance.
Fig. 6B shows that treatment of H460 lung cancer cells, trans-
fected with the pCMV-neo-bam vector and selected for G418
resistance, with the DNA-damaging agent etoposide led to
inhibition of DNA synthesis, whereas treatment of H460 cells,
transfected with pCMV16E6 and selected for G418 resistance,
with etoposide failed to inhibit new DNA synthesis, except at
very high doses. We then determined the ID50 in the presence
of either Adriamycin or etoposide for the parental H460 cell line,
the same G418-resistant H460 cell line, and the same H460

clonettransfected with HPV 16 E6 which failed to induce p53 or
arrest following treatment with chemotherapy. The results in
Table 3 suggest that with either Adriamycin or etoposide, loss of
p53 induction did not lead to chemotherapeutic drug resistance,
in fact the ID50’s were lower for both drugs as compared with
cells that could induce p53 or undergo cell cycle arrest. We
found similar results using a second independently isolated
G418-resistant HPV16 E6-transfected H460 cell line (H460-
E6–8, Table 3).

Discussion
A clinically favorable response to cancer therapy often
requires that the agents used induce tumor cell death. Under-
standing the pathways of cell death has therefore become not
only of interest in understanding the control of cell number
during tissue development and homeostasis, the immune re-
response, but also an important goal for the development of
anticancer therapy (24, 25). The available clinically useful cytotoxic agents provide excellent tools for manipulating the cellular pathways which result in cell death, with the hope of understanding the underlying mechanisms. Cell death pathways may be regulated negatively by certain oncoproteins such as Bcl-2 (26, 27) or viral oncoproteins such as adenoviral E1B 19K protein (28, 29), and positively by certain tumor suppressor genes such as p53 (30, 31) or viral oncoproteins such as adenoviral E1A (32). These regulators are thought to relay signals into a final common pathway of protease and endonuclease activity by as yet undefined mechanisms, resulting in the phenotype of apoptosis (33, 34). It has been suggested that a major pathway of cell death following the administration of chemotherapy or radiation involves activation of p53-mediated apoptosis (9–11). Both bax and Fas/APO1 have been identified as downstream targets of p53 transcription, although both of their precise roles in apoptosis induction following DNA damage or p53 induction remains unclear (35–37). A correlation has been found between bax induction following γ-irradiation and apoptosis induction in wild-type p53-expressing cell lines (38).

The results presented here illustrate several significant relationships between the measurement of in vitro cytotoxicity and expression of Bcl-2 protein, wild-type p53 induction, and apoptosis induction. In addition, the results show that tumor cells can undergo p53-dependent cell cycle arrest and apoptosis simultaneously following treatment with chemotherapy.

The most consistent relationship in all tumor cell types tested was a correlation between chemosensitivity and apoptosis induction, regardless of either the p53 status or Bcl-2 expression level. Bcl-2 levels correlated with relative chemoresistance in human ovarian carcinoma and some lymphoma cell lines. Bcl-2 levels correlated with relative chemoresistance in human mammary epithelial cells to mitomycin C (42). These relationships between p53 status, apoptosis induction, and chemosensitivity by comparing drug-sensitive versus drug-resistant tumor cells following selection, as well as by evaluating the role of p53 by introducing an HPV 16 E6 expression vector into tumor cell lines, thereby targeting p53 for degradation via the ubiquitin pathway. Such cell lines have been generated in the past to evaluate the role of p53 in mediating a G, checkpoint following DNA damage as well as to study the role of p53 in determining radiosensitivity or chemosensitivity (39–42). In our studies, we found that HL-60 cells selected for either Adria- mycin or etoposide resistance were much less likely to undergo apoptosis in the presence of chemotherapy as compared to parental cells. This resistance to apoptosis induction in the Adria- mycin- or etoposide-resistant HL-60 cells was not p53 dependent, since HL-60 already expressed no wild-type p53, and was not due to overexpression of MDR1 (data not shown). p53-independent apoptosis has also recently been reported following treatment of human colon tumor cell lines with γ-radiation or a human prostate cancer cell line with various chemotherapeutic agents (18, 43), and has previously been reported following treatment of leukemia cell lines with either topoisomerase I or II poisons (44). We observed similar results in ML-1 cells selected for resistance to either Adriamycin or etoposide (Table 2). We looked for differences in the threshold or level of p53/p21 induction following treatment of drug-resistant cells, but found no evidence for such underlying mechanisms (data not shown).

We also observed that targeting p53 for degradation in wild-type p53-expressing lung cancer cells inhibited the DNA damage-induced G, arrest but did not alter chemosensitivity or the extent of apoptosis induction following Adriamycin or etoposide treatment. Similar results have been previously observed in colon cancer cell lines transfected with HPV 16 E6 and treated with either γ-radiation or camptothecin (39). However, a recent report provided evidence that a nontransformed human fibroblast cell line became more resistant to ionizing radiation following transfection of HPV 16 E6 (41), whereas other recent reports of E6 expression have found that loss of the G, checkpoint was associated with increased sensitivity to the human breast cancer cell line MCF7 or the human colon cancer cell line RKO cells to cis-platinum (40), or increased sensitivity of human mammary epithelial cells to mitomycin C (42). These different outcomes may be due to different cell types, differences between nontransformed and transformed cells, or differences in specific cytotoxic agents used.

Our results suggest that in vitro measurement of apoptosis induction may be useful as a general marker of chemosensitivity, whereas p53 status or Bcl-2 expression may be useful.

### Table 3. Loss of p53 and chemoresistance

<table>
<thead>
<tr>
<th>Cell line</th>
<th>p53</th>
<th>p21</th>
<th>Adriamycin (µg/ml)</th>
<th>Etoposide (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H460-P</td>
<td>+</td>
<td>+</td>
<td>0.45 ± 0.14</td>
<td>3.7 ± 0.75</td>
</tr>
<tr>
<td>H460-neo</td>
<td>+</td>
<td>+</td>
<td>0.34 ± 0.07</td>
<td>2.6 ± 0.53</td>
</tr>
<tr>
<td>H460-E6-6</td>
<td>-</td>
<td>-</td>
<td>0.23 ± 0.04</td>
<td>2.2 ± 0.23</td>
</tr>
<tr>
<td>H460-E6-8</td>
<td>-</td>
<td>-</td>
<td>0.18 ± 0.02</td>
<td>2.1 ± 0.20</td>
</tr>
</tbody>
</table>

*The expression of p53 and p21**WAF1/CIP1** proteins was either undetectable and not inducible (−) or detectable and inducible (+) before and after treatment of tumor cells with Adriamycin, respectively. H460-E6-6 and H460-E6-8 are two independent G418-resistant clones isolated following transfection of H460 cells with HPV16-E6. Immunoblotting was performed as described in "Materials and Methods."
markers of chemosensitivity in certain cell types. Future experiments should analyze larger series of tumor-derived cell lines, evaluate the effects of targeting wild-type p53-degradation in normal and tumor cell lines derived from different tissue origins, and examine the relationships between in vitro and in vivo chemosensitivity.

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References


Apoptotic death of tumor cells correlates with chemosensitivity, independent of p53 or bcl-2.

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