Inactivation of p53 Increases the Cytotoxicity of Camptothecin in Human Colon HCT116 and Breast MCF-7 Cancer Cells

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ABSTRACT

Camptothecin (CPT) derivatives are topoisomerase I (top1) inhibitors recently introduced as clinical agents. To explore the role of p53 in CPT-induced cytotoxicity, we examined CPT effects in two isogenic pairs of human cancer cell lines, MCF-7 breast carcinoma and HCT116 colon carcinoma cells, in which p53 function had been disrupted by transfection with the human papillomavirus type-16 E6 gene. Clonogenic survival assays showed that both MCF-7/E6 and HCT116/E6 cells were more sensitive to CPT. No differences in top1 protein levels and activity analyzed by a novel *in vitro* oligonucleotide assay were observed in the E6 transfectants. Also, CPT showed comparable top1 cleavable complex formation *in vivo*, as determined by DNA single-strand breaks and DNA protein cross-links. These results suggest that p53 can protect against CPT-induced cytotoxicity and that this protection is mediated downstream of CPT-induced DNA damage. Flow cytometry analyses showed that CPT can induce G_1 arrest in cells with normal p53. This G_1 arrest was markedly reduced in the p53-deficient cells. These results demonstrate a critical role of p53 as a G_1 checkpoint regulator after CPT-induced DNA damage and suggest a rationale for the selectivity of CPT toward tumors with p53 mutations.

INTRODUCTION

The nuclear protein p53 is important in the etiology of cancer and is mutated in more than half of all human tumors (1). Loss of p53 function leads to gene amplification and the development of aneuploidy (2), suggesting that p53 acts as a "guardian of the genome" (3–5). In some cell types, the G_1 arrest induced by p53 in response to DNA damaging agents is believed to allow additional time for the cell to repair its DNA. In addition, DNA repair of at least some types of DNA lesions might also be under the influence of p53 (6, 7). p53 has also been implicated in G_2-M checkpoints control (8). Alternatively, p53 can activate an apoptotic response to DNA damage (9, 10). These opposing functions of p53 can influence the response of human cancers to DNA-damaging agents in different ways.

In hematopoietic and lymphoid cells types, the loss of p53 leads to an inability to trigger apoptosis in response to DNA-damaging agents (11–13). In epithelial tumor cell lines, the ability to trigger apoptosis by IR[2] is also often lost when p53 is disrupted, whereas in primary mouse fibroblast cells, there are no differences in the radioresistance of the p53 null cells *versus* the normal cells (14). A relationship between *p53* gene status and chemosensitivity of cancer cells has recently been reviewed (15).

CPT is an antitumor drug extracted from the Chinese tree *Camptotheca acuminata* (16) and is a specific inhibitor of DNA top1 (17). CPT derivatives have been approved as clinical agents and are active against many tumors, including colorectal, ovarian, non-small cell lung, breast cancer, and mesothelioma (18). CPT and the derivatives of CPT are also active against multidrug-resistant tumor cell lines (19–21). We examined the effect of CPT on p53 alteration in colon and breast cancer cell lines.

In the present study, we used MCF-7 and HCT116 cell lines, both of which contain functionally intact wt p53 and do not appear to undergo apoptosis following γ-irradiation (7) or CPT treatment. p53 function was disrupted in these cell lines by constitutive high-level expression of the human papillomavirus type-16 E6 gene, which stimulates the degradation of p53 through a ubiquitin pathway (22, 23). We found that cells lacking p53 function showed increased sensitivity to CPT (16). The mechanism of the increased sensitivity in MCF-7/E6 and HCT116/E6 was not related to alterations in top1 protein levels, top1 activity, or initial DNA damage produced by CPT. Differences in cell cycle analyses suggested that G_1 delay controlled by p53 plays a role in CPT-induced cytotoxicity.

MATERIALS AND METHODS

Cell Culture and Drugs. The breast carcinoma MCF-7 and colon carcinoma HCT116 cell lines were cultured in RPMI 1640 (Life Technologies, Inc.) containing 10% serum and 2 mM L-glutamine. Cells transfected with either control vector and vector containing overexpressed HPV-16 *E6* were grown in the same medium containing G418. CPT was a gift from Drs. Monroe E. Wall and Mansukh C. Wani of the Research Triangle Institute (Research Triangle Park, NC). Nocodazole was purchased by Sigma Chemical Co. (St. Louis, MO). Stock solutions of CPT were prepared in 10 mM DMSO and further diluted in

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[2] The abbreviations used are: IR, ionizing radiation; CPT, camptothecin; top1, topoisomerase I; SSB, single-strand break; DPC, DNA protein cross-link; wt, wild-type.

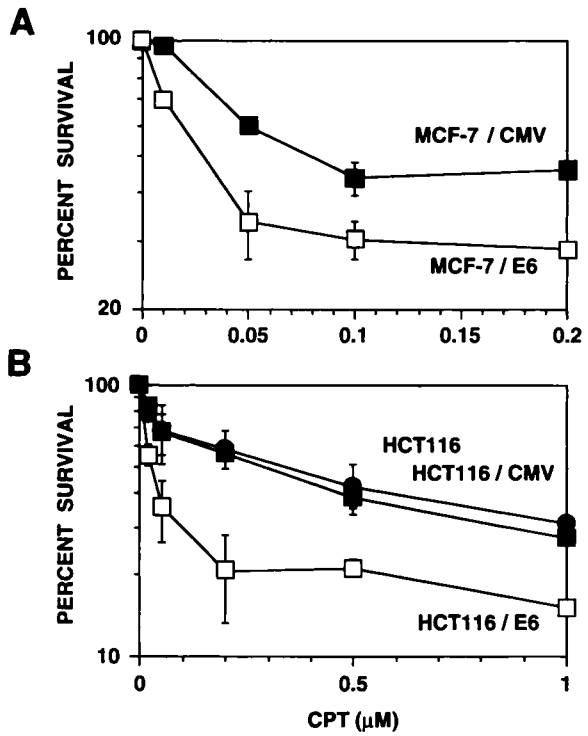

Fig. 1 Clonogenic survival of MCF-7 (*A*) and HCT116 (*B*) cell lines transfected with control pCMV.3 vector (■) or the human papilloma virus type-16 *E6* (□) containing plasmid after treatment with CPT for 8 h. Mean ±SD of three independent experiments is shown. ●, HCT 116.

Table 1 IC_{50} for HCT116 and MCF-7 cell lines treated with CPT

Cell lines	CPT (μM)
HCT116	0.30
HCT116/CMV	0.30
HCT116/E6	0.03
MCF-7/CMV	0.08
MCF-7/E6	0.03
MCF-7/mup53	0.025

IC_{50} values were determined from the cytotoxicity dose-response curves (shown in Fig. 1) after 8 h-drug exposure.

water immediately prior to each experiment. α-[^{32} P] cordycepin was purchased from DuPont NEN Research Products (Boston, MA).

Clonogenic Assays. Cells were plated in log phase into 25-cm^2 culture flasks with 5 ml of medium for 24 h, rinsed with fresh medium, and then treated with various doses of CPT for 8 h. Cells were then washed twice with prewarmed PBS, trypsinized, diluted, and 500 cells were seeded per flask in triplicate. Colonies were allowed to grow for 10–14 days. Cells were then washed with PBS, fixed with methanol (95%), and stained with 0.005% methylene blue (24). Survival fraction for each cell line was expressed as the ratio of plating efficiency of treated cells to that of the untreated control cells. The plating efficiency of the control cells was between 50–60%. The IC_{50} was calculated as the drug concentration that inhibited colony formation to 50% of the control cells.

DNA Cleavage Assays. An oligonucleotide containing a single high-affinity cleavage site for topI activity was used to assay topI sensitivity to CPT (24). The 32 mer oligonucleotide was labeled at the 3′-terminus of the scissile strand with α-[^{32} P] cordycepin and terminal transferase (DuPont, Wilmington, DE) as described previously (24). The oligonucleotide was incubated with nuclear extract at 37°C for 10 min in the presence of CPT. Reactions were terminated by adding 1% SDS. Samples were denatured by adding 4 volumes of loading buffer containing 90% formamide, 0.1% xylene cyanol, 0.1% bromphenol blue, and 10 mM EDTA (pH 8.0) and were separated in a denaturing gel (16% polyacrylamide and 7 M urea)that was run at 52°C. topI-mediated cleavage generated a DNA band corresponding to a cleaved (19 mer) product from the uncleaved substrate (33 mer). The radioactivity of the cleaved and the uncleaved products was quantitated using a PhosphorImager and IMAGE QUANT Ver. 3.22 (Molecular Dynamics, Sunnyvale, CA).

Measurement of DPCs and DNA SSBs. Alkaline elution assays were performed as described (25, 26). Briefly, MCF-7 and HCT116 cell lines were labeled with 0.04 μCi/ml [^{14}C]-thymidine for 24 h. CEM cells were used as internal standard and were labeled with 0.2 μCi/ml methyl-[^{3}H]-thymidine. Cells were chased for an additional 20 h with radioisotope-free medium prior to drug treatments and were harvested after drug treatment into ice-cold HBSS containing CPT to avoid reversal of cleavable complexes (27). For measuring DNA SSBs, only internal standard CEM cells and calibrator cells were irradiated on ice with 3 Gy. Cells were loaded onto polycarbonate filters (2 μm pore size; Poretics Corp., Livermore, CA) and lysed with SDS lysis solution (0.1 M Glycine, 2% SDS, and 0.025 M Na_2EDTA, pH 10) containing 0.5 mg/ml proteinase K (Boehringer Mannheim, Indianapolis, IN). After one wash with 0.02 M EDTA (pH 10), DNA was eluted in tetrapropylammoniumhydroxide (Pr_4NOH, pH 12.1) at a flow rate of 0.03–0.04 ml/min. Elutions were performed with Pr_4NOH (pH 12.1) containing 0.1% SDS at a flow rate of 0.12–0.16 ml/min. The SSB frequency (expressed in rad equivalents) can be calculated by the formula:

$$SSB = [\log(r_1/r_0)/\log(R_x/r_0)] \times 300 \text{ rads}$$

Where r_1 is the DNA retention for drug-treated cells, r_0 the DNA retention for untreated cells, and R_x the DNA retention for irradiated cells that are used as a calibrator.

For the DPC assays, cells were irradiated on ice with 30 Gy immediately before elution and were kept on ice until they were loaded onto protein-adsorbing filters (Metricel, 0.8 μm pore size; Gelman Science, Inc., Ann Arbor, MI). Cells were lysed with SDS lysis solution (0.1 M glycine, 2% SDS, and 0.025 M Na_2EDTA, pH 10) without proteinase K. After one wash with 0.02 M EDTA (pH 10), DNA was eluted in tetrapropylammoniumhydroxide at a flow rate of 0.03–0.04 ml/min. Elutions were performed with Pr_4NOH (pH 12.1) containing 0.1% SDS at a flow rate of 0.12–0.16 ml/min.

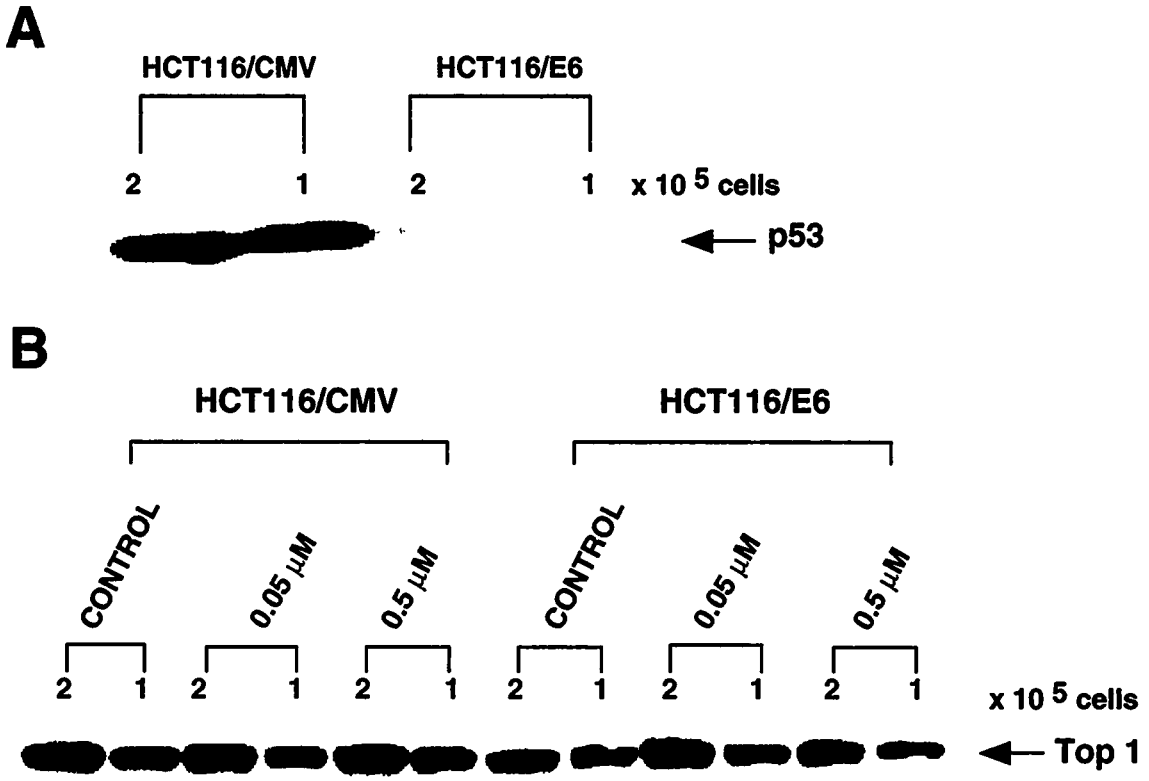

Fig. 2 Expression of p53 and topl in HCT116/CMV and HCT116/E6 cells. Cell lysates from 2 × 10^5 (*2*) and 1 × 10^5 (*1*) cells were resolved on 12% Tris/glycine/SDS gels. Western blotting was performed using monoclonal anti-human topl (*B*) and p53 mouse (D0-1) (*A*) antibodies (Santa Cruz Biotechnology, Inc.). *B*, 0.05 μM and 0.5 μM CPT treatment for 8 h.

The DPCs (in rad = equivalents) are calculated using the formula:

$$DPC = [(1\text{-}r)^{-1} - (1\text{-}r_0)^{-1}] \times 3000$$

where r is the retention for drug-treated cells and r_0 the retention for cells that have not been drug-treated.

Filter Elution Assays for the Measurement of DNA Fragmentation. DNA fragmentation was measured by filter elution as described previously (26, 28). Briefly, reaction mixtures containing 0.5 × 10^6 [^{14}C]-thymidine-labeled cells were deposited on protein-adsorbing filter (Metricel, Gelman Science, Ann Harbor, MI) and washed with 5 ml of nucleus buffer. This fraction (W) was collected and lysis was performed with 5 ml LS10 (2 M NaCl, 0.04 M Na_2EDTA, 0.2% sarkosyl, pH 10) followed by washing with 5 ml of 0.02 M Na_2EDTA, pH 10. The lysis (L) with EDTA (E) fractions were collected. All fractions (W, L, and E) and filter (F) were counted by liquid scintillation. DNA fragmentation was calculated as the percentage of DNA eluting from the filter as follows:

$$\text{DNA fragmentation} = 100 \times (W + L + E)/(W + L + E + F)$$

Each value was normalized to the untreated sample. All experiments were repeated at least two or three times.

Flow Cytometry. Samples were prepared for flow cytometry as described previously (7). Briefly, cells were fixed in ice-cold 70% ethanol, washed with PBS, treated with RNase (Boehringer Mannheim) at 37°C for 30 min, and stained with 50 μg/ml propidium iodide (Sigma). Cell cycle analysis was performed using a Becton Dickinson fluorescence-activated cell analyzer. Cells (15,000) were analyzed for each point, and quantitation of cell cycle distribution was performed using the SOBR model program provided by the manufacturer.

RESULTS

Abrogation of wt p53 Function by Human Papillomavirus Type-16 E6 Transfection Sensitizes MCF-7 and HCT116 to CPT. The human papilloma virus type-16 E6 gene product, which stimulates the degradation of p53 through an ubiquitin-dependent pathway (22, 23) was used as a mean of inhibiting p53 function in MCF-7 and HCT116 cells (7, 29). The *E6* gene was transfected into MCF-7 or HCT116 cells in a plasmid containing the CMV promoter, which allows constitutive high-level expression of the transgene. In additional experiments, we used a dominant-negative mutant p53 transgene (143 Ala to Val cloned into a PCMV plasmid) to inhibit p53 function in MCF-7 cells (7). The generation and characterization of the MCF-7 and HCT116 transfectants have been described previously (7, 29, 30). The survival of MCF-7 and HCT116 cell lines was assessed in clonogenic survival assays after an 8-h treatment with CPT. The cytotoxicity of CPT depends mostly upon

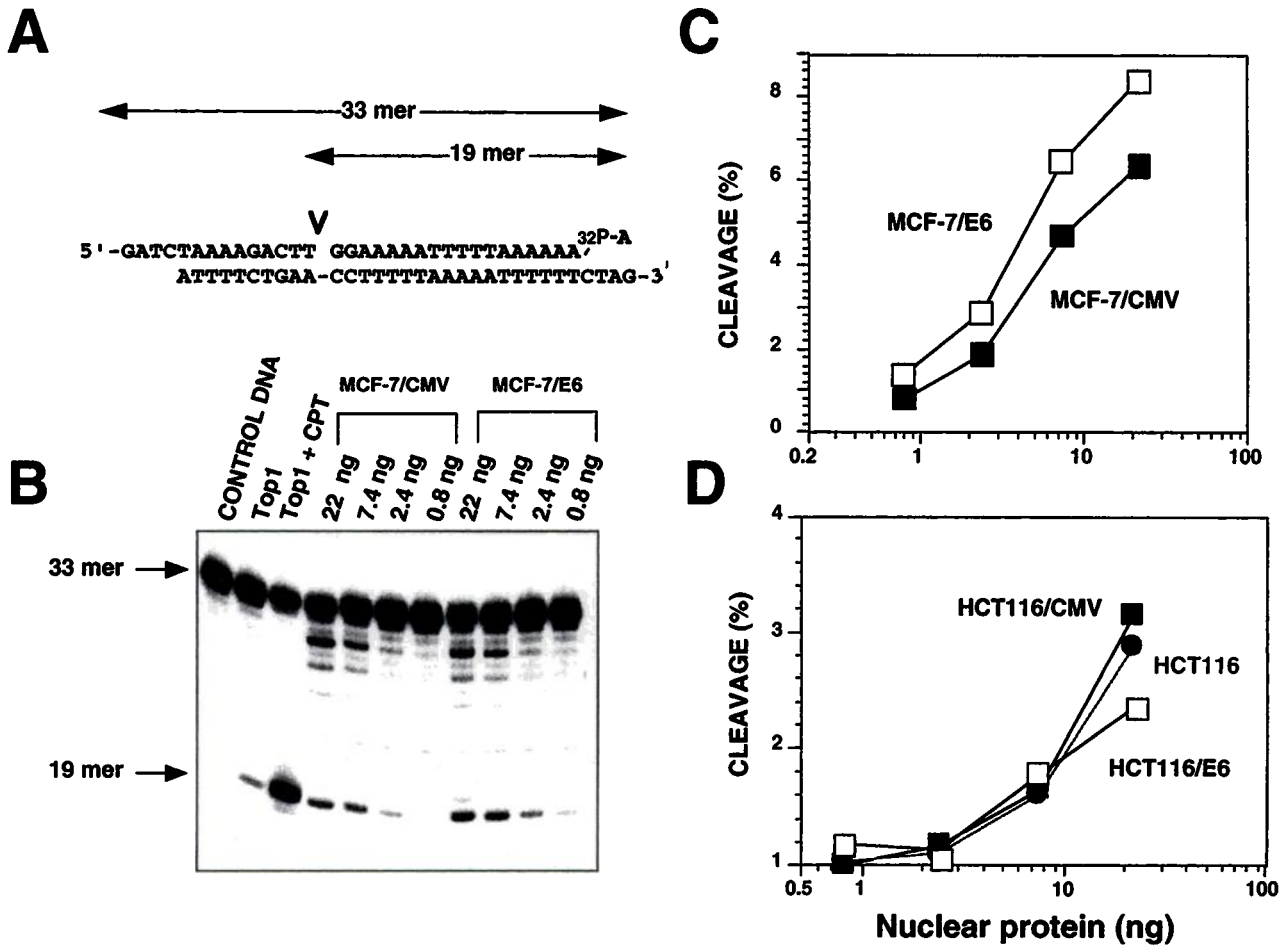

Fig. 3 DNA cleavage activity of topl from MCF-7 and HCT116 nuclear extracts. *A*, the oligonucleotide with a single cleavage site for topl. *B*, PhosphorImager image of a typical experiment of samples electrophoresed into a 16% polyacrylamide denaturing gel. In *C* and *D*, the amounts of uncleaved and cleaved bands were quantitated using a PhosphorImager. The percentage of cleavage *versus* different amounts of nuclear proteins extracted from MCF-7 (*C*) and HCT116 (*D*) was plotted.

the percentage of cells in the S phase at the time of drug exposure (31). Fig. 1 shows that at the IC_{50} dose of CPT, MCF-7/E6 cells (Fig. 1*A*) were approximately 2–3-fold and HCT116/E6 cells (Fig. 1*B*) were approximately 10-fold more sensitive than control CMV transfectants, whereas differences approximately 2-fold were seen in the plateau levels (Fig. 1) between the E6 and CMV transfectants. The actual IC_{50} values determined from the cytotoxicity curves are shown in Table 1. In both MCF-7/E6 and HCT116/E6 cell lines, 0.03 μM CPT was used to achieve 50% cytotoxicity. Enhanced sensitivity was not limited to the E6-transfected cell lines but was also observed when MCF-7 cells were transfected with a mu-p53 transgene, arguing against a spurious property of the E6 gene product in this sensitization. These results show that disruption of p53 function in MCF-7 and HCT116 cells increases the cytotoxicity of CPT.

topl Protein Levels Are the Same in HCT116/CMV and HCT116/E6 Cell Lines. The basal amounts of p53 were measured in HCT116/CMV and HCT116/E6 cell lines, and clear differences in the protein levels were seen (Fig 2*A*). These results are consistent with the ability of the E6 gene to degrade p53 protein. The impaired response to γ-ray-induced p53 by E6 transfection was also seen in MCF-7 (7). We measured topl protein levels in the transfected cells before and after CPT treatment. HCT116/CMV and HCT116/E6 cells were treated with 0.05 μM and 0.5 μM CPT for 8 h, and topl protein levels were analyzed by Western blotting (Fig. 2*B*). topl protein levels were found not to change significantly with respect to CPT treatment or the expression of the E6 gene product. These results suggest that topl, the cellular target for CPT, in contrast to p53, was not affected by transfection with E6.

CPT-induced topl DNA Cleavage Activity Was Not Affected by E6 Transfection. An *in vitro* assay was used to investigate whether E6 affected topl activity and CPT-induced cleavage. We have reported previously (24) that a 33-mer oligonucleotide containing a high-affinity topl binding site (Fig. 3*A*) can be used to determine CPT-induced topl-mediated DNA cleavage activity using crude nuclear extracts. topl cleaves the labeled 33 mer to produce a 19-mer cleaved product that can be separated by DNA denaturing electrophoresis (Fig. 3*B*), and

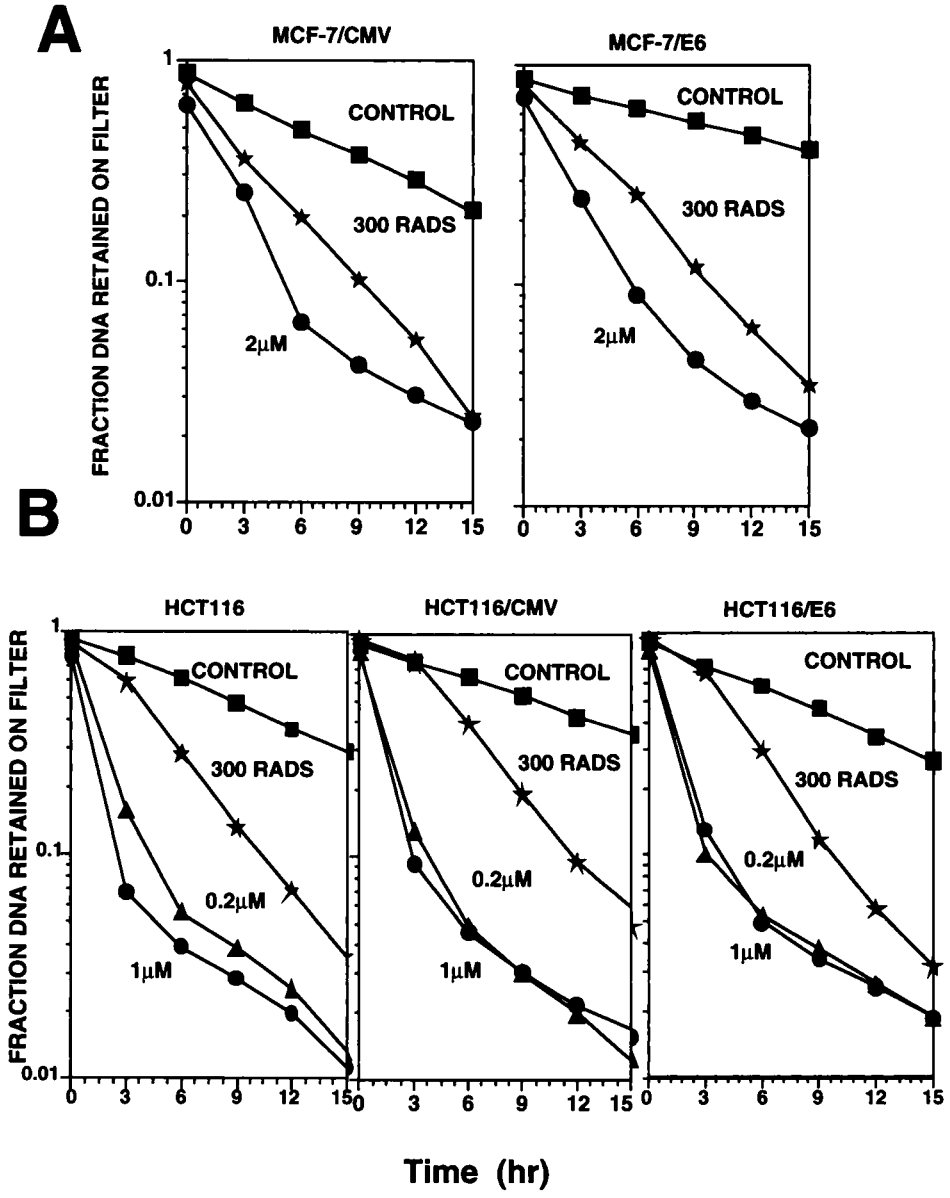

Fig. 4 Measurement of CPT-induced DNA SSBs in MCF-7 (*A*) and HCT116 (*B*) cell lines by alkaline elution. Alkaline elution curves of typical experiments are shown. CPT concentrations are indicated in μM. The cells were treated for 1 h.

CPT-induced DNA cleavage can be quantitated by PhosphorImager and plotted *versus* nuclear protein concentration. MCF-7/E6 nuclear extracts produced approximately 1.5-fold more CPT-induced cleavage activity than MCF-7/CMV (Fig. 3*C*) whereas nuclear extracts from HCT116/E6 cells had approximately 1.5-fold less topl cleavage activity than HCT116 and HCT116/CMV nuclear extracts (panel 3D). Although differences in topl cleavage activity were slightly altered in the E6 transfectant, it is clear that such changes did not correlate completely with the survival data.

To assess topl activity *in vivo*, CPT-induced SSBs and DPCs were measured by alkaline elution. The induction of SSB by CPT in the MCF-7 cells (Fig. 4*A*) and HCT116 cell lines pairs (Fig 4*B*) was calculated in all of the cell lines and was not significantly different (Table 2). DPCs were measured in HCT116/CMV and HCT116/E6 cells after 1-h treatments with different concentrations of CPT (Fig. 5). HCT116/E6 produced slightly less CPT-induced DPC than HCT116/CMV cells (Table 2). The amount of initial damage induced by CPT was not significantly affected by the p53 status for the MCF-7 and HCT116 cell lines. These results demonstrate that the increased CPT sensitivity of the E6 transfected cells is not a direct result of differences of topl-mediated DNA damage.

DNA Fragmentation Was Not Affected by E6 Transfection. The human papilloma virus 16 E6 gene has been reported to sensitize human epithelial cells to apoptosis by mitomycin C and staurosporine (32). Therefore, the possibility that E6 would sensitize HCT116 cells to CPT-induced apoptosis was investigated using a DNA fragmentation assay (28). Cells were treated for 8 h with CPT, and DNA fragmentation was assayed

Table 2 Measurement of CPT-induced DNA SSBs and DPCs

Cell lines	CPT (μM)	SSB (rad equivalent)	DPC (rad equivalent)
MCF-7/CMV	2	404	ND[a]
MCF-7/E6	2	409	ND
HCT116	0.2	485	ND
	1	536	ND
HCT116/CMV	0.2	609	ND
	1	628	848
HCT116/E6	0.2	436	ND
	1	432	696

[a] ND, not determined.

after 24 and 48 h. Neither HCT116/CMV nor HCT116/E6 cells showed any substantial DNA fragmentation for at least 48 h after CPT treatment (Table 3). Morphological studies also failed to show apoptosis in normal, CMV-, and E6-transfected cells (data not shown), indicating that MCF-7 and HCT116 carcinoma cells did not die by rapid apoptosis following CPT treatment.

Cell Cycle Changes in CPT-treated Cells. The requirement for a functional p53 in the G_1 checkpoint after IRs is well known (33), although its role after exposure to CPT is not yet established. As shown in Fig. 6, in the presence of nocodazole, which prevents progression through mitosis, both HCT116/CMV and HCT116/E6 cell lines were arrested in G_2-M. After an 8-h treatment with CPT and 16 h of postincubation, a significant fraction of HCT116/CMV cells that were in G_1 at the onset of treatment remained in G_1 at the end of the 24-h protocol. This can be inferred from the preservation of the G_1 peak when nocodazole was included. On the other hand, in the case of similarly treated HCT116/E6, the G_1 population was very small, suggesting that these cells are deficient in the checkpoint that normally limits the entry of drug-treated cells into S phase. These results suggest that CPT can activate a G_1 checkpoint and that abrogation of the p53 function by E6 inactivates the CPT-induced G_1 checkpoint. Similar results have been seen with γ-radiation (7). However, this is the first report of a p53-dependent G_1 checkpoint in response to CPT.

Transfection with E6 slightly increased the S-phase fraction in control cells compared to CMV. The percentage of S-phase cells was 26–28% in HCT116/E6, whereas it was 20–22% in HCT116/CMV cells. This S-phase increase is not significant to account for the differences in cytotoxicity.

DISCUSSION

To investigate the role of p53 in CPT-induced cytotoxicity, we have examined MCF-7 and HCT116 cell lines in which p53 function was disrupted by transfection with the human papillomavirus type-16 E6 gene. This study shows that these two sets of isogenic cell lines with p53 alteration are more sensitive to CPT when compared to parental- or control-transfected cells. p53 disruption did not affect topI protein levels or assessable levels of DNA damage (cleavable complexes) in the presence of CPT. These results suggest that p53 plays a role in the downstream events from which CPT-induced topI DNA damage is translated into cell death (34).

Some cell types with inactivated p53 are more sensitive to other chemotherapeutic agents, including cisplatin and alkylating agents such as melphalan (7, 35). Similar sensitization has been reported for UV (36). One plausible explanation is that the loss of p53 results in impaired repair of DNA damage produced by these agents. Evidence linking p53 to the repair of cisplatin-introduced DNA adducts was shown using a platinated reporter plasmid (7). Also, Smith *et al.* have shown that RKO cells lacking functional p53 have reduced ability to repair a UV-damaged reporter plasmid. The pathways by which CPT-induced damage is repaired are not known. CPT forms a ternary complex with topI and DNA that prevents the religation of the topI-induced nicks in DNA. CPT-induced cytotoxicity seems to depend upon ongoing DNA replication. Collision between an advancing replication fork and CPT-induced DNA SSBs probably results in irreversible fork arrest and production of DNA double-strand breaks (16); CPT can then induce p53 only when the DNA breaks are replication associated (37). p53 might be involved in the recognition and repair of CPT-induced damage. In favor of this hypothesis, enhanced binding of wt p53 to its consensus DNA sequence was seen after the exposure of NIH-3T3 mouse fibroblasts to CPT (38). wt p53 and the COOH-terminal 75 amino acid of p53 show an increased binding affinity for damaged DNA by restriction digestion, DNase I treatment, or IR (39, 40). DNA strand breaks may be sufficient to cause an increase in p53 binding activity. The binding of p53 to gene-regulatory sequences activates the transcription of growth-arrest genes *e.g.*, *gadd45* (33) and *WAF1/CIP1* (41), represses cell cycle genes, and/or halts DNA replication.

A single unrepaired lesion (39) may be sufficient to activate the G_1 arrest response in human and rodent cells. Huang *et al.* (39) proposed that the p53-dependent G_1 arrest is partly due to the inefficient repair of the double-strand breaks and that this mechanism prevents the proliferation of cells with structural chromosome changes. A breakage of a single dicentric chromosome in a cell expressing wt p53 also produces a prolonged G_1 arrest (42). Thus, it is possible that CPT, which is known to be selectively toxic to replicating cells (17, 34), can also directly

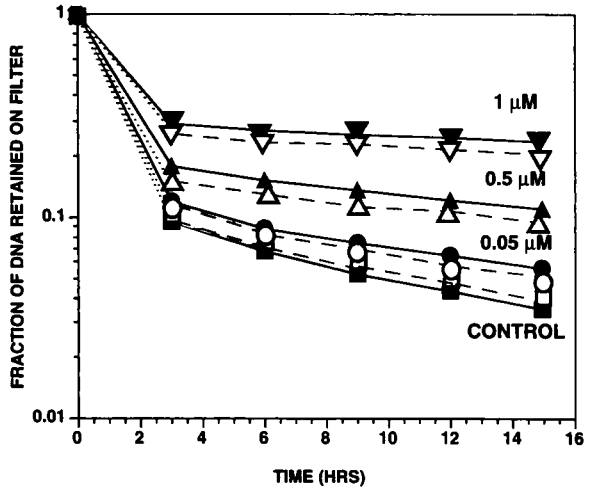

Fig. 5 Measurement of DPCs induced by CPT in HCT116/CMV and HCT116/E6 after 1 h of CPT treatment. *Solid lines* and *filled symbols*, HCT116/CMV cells; *dashed lines* and *open symbols*, HCT116/E6 cells.

Table 3 DNA fragmentation in HCT116 cell lines treated with CPT

	CPT (μM)	DNA fragmentation (%)		
		HCT116	HCT116/CMV	HCT116/E6
24 h	0.05	2.1	1.7	1.37
	0.2	3.32	4.6	1.35
	1	5.9	7.1	2.34
48 h	0.05	1.5	1.3	5.7
	0.2	1.64	6.6	10.9
	1	10.4	12.5	12.1

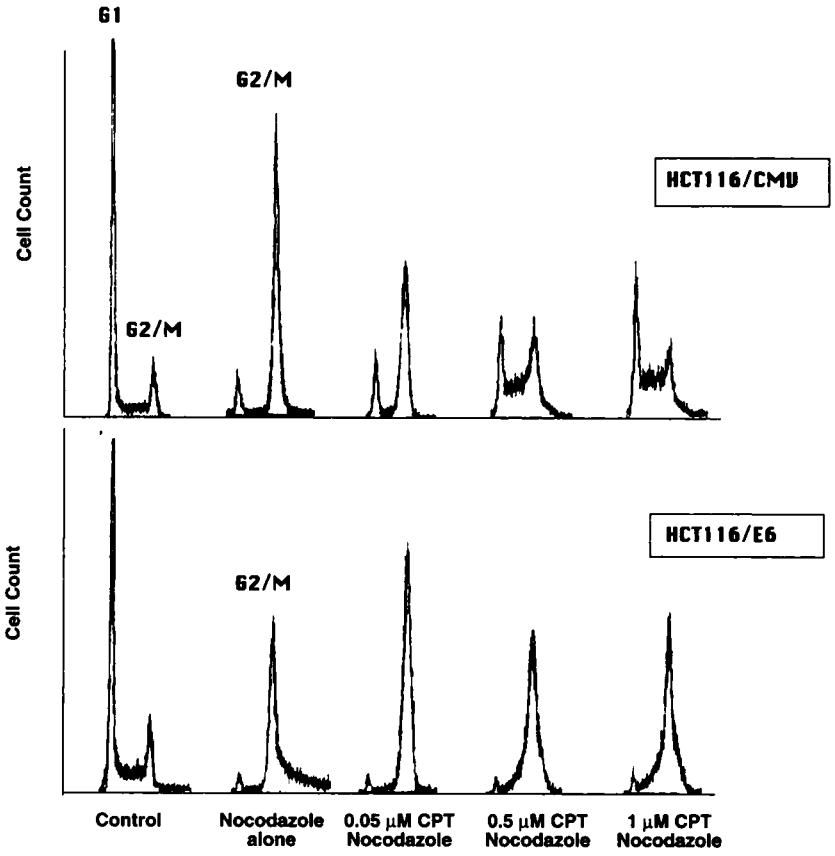

Fig. 6 Flow cytometry analysis of HCT116/CMV and HCT116/E6 cell lines in the presence of nocodazole and CPT. Cells were treated with CPT for 8 h in the presence of 0.4 μg/ml nocodazole, and CPT was then removed. Cells were washed and resuspended in fresh medium containing nocodazole. Cell cycle profiles were analyzed after an additional 16 h.

activate G_1 arrest in cells that are initiating DNA replication. The association of topI with replication complexes would be consistent with this possibility (43).

In tumor cells that do not undergo apoptosis, our results indicate that p53 protects cells from DNA damage-induced cell death. Expression of the E6 oncoprotein results in a p53-deficient phenotype with a loss of the CPT-induced G_1 checkpoint function. Abrogation of this checkpoint may be responsible for an increase in sensitivity of p53-deficient cells to CPT when compared to cells with normal p53. This observation has implications for the treatment of cancers. Because p53 mutations are most commonly occurring in human malignancies, this provides a rationale for the relative selectivity of CPTs toward malignant cells with altered p53.

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