Differential Cytotoxic Pathways of Topoisomerase I and II Anticancer Agents after Overexpression of the E2F-1/DP-1 Transcription Factor Complex

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INTRODUCTION

The transcription factor E2F was originally characterized as a sequence-specific DNA-binding factor bound to the adenovirus E2A promoter (1). E2F-1 is the prototype of a family of E2F transcription factors that currently has six members. The DNA-binding and transactivating activity of E2F-1 is enhanced by heterodimerization to its partner DP-1 (2), which shares some structural homology with the E2F family. The heterodimer promotes transcription by binding to DNA at specific sequences and plays an important role in the transition from G1 to S phase (3–5). Among several targets for E2F-1 binding and induction are the S-phase genes DNA-polymerase α, proliferating cell nuclear antigen, thymidylate synthase, and CDC6 as well as the cell cycle-regulatory genes cdc2, cyclin A, cyclin E, and B-myb (6). Retinoblastoma protein binds to the E2F-1/DNA complex and actively represses transcription (7, 8). pRB is phosphorylated through G1 by cyclin D/ckd4-ckd6 (9), which decreases its binding capacity for E2F-1. Therefore the level of "free" E2F-1 increases and reaches a peak at the G1-S-phase transition, leading to activation/derepression of E2F-regulated genes.

The DNA topoisomerases are essential nuclear enzymes that are also specific targets for a number of anticancer agents (10). Topoisomerase I cleaves and relieves single-stranded DNA, thus relieving torsional stress, whereas topoisomerase II is able to cleave and religate double-stranded DNA, allowing strand passage. Topoisomerase II exists in two isoforms: (a) a 170-kDa α form that is up-regulated in S through G2-M-phase; and (b) a 180-kDa β form that is thought to be constantly expressed throughout the cell cycle (11). Topoisomerase II is the cellular target of several clinically important drugs such as the epipodophyllotoxin etoposide (VP-16) and the anthracycline doxorubicin (Adriamycin), whereas the camptothecins, several derivatives of which are now entering clinical trials, are substrates of topoisomerase I. Both camptothecin and etoposide produce DNA breaks by stabilizing a DNA-enzyme covalent complex that leads to the formation of DNA breaks (10). These drugs are termed topoisomerase I and II poisons because they convert their enzyme into a potent cellular toxin, leading to cell death in an as-yet-undefined manner (12). Because the toxicity of both topoisomerase I and II poisons is considered to be S-phase specific, their relationship to the transcription factor E2F-1 in cellular toxicity has been investigated in several studies. These studies have yielded varying results (13–16). In the first study, hypersensitivity to camptothecin and etoposide was
demonstrated in NIH-3T3 cells with a constitutive overexpression of NH2-terminal deletion mutant E2F-1d87, whereas no difference in sensitivity was found between cells that expressed full-length E2F-1 and those that did not (13). In the second study, hypersensitivity to etoposide but not to doxorubicin or the topoisomerase I poison topotecan was observed in 32D3 myeloid progenitor cells overexpressing E2F-1 or E2F-1/DP-1 (14). In a third study, all five E2F-1-transfected clones of HT-1080 cells were hypersensitive to both the camptothecin derivative SN38 and etoposide and doxorubicin (15), whereas in the most recent report, several human cancer cell lines were hypersensitive to etoposide and/or doxorubicin, whereas topoisomerase I-directed drugs were not studied (16). A confounding factor in the investigation of E2F-1 and sensitivity to the topoisomerase poisons is that most cell lines increase their apoptotic response after E2F-1 overexpression (4, 5, 17, 18). This itself could increase the cytotoxicity of topoisomerase-active drugs and thus mask a more specific underlying mechanism caused by E2F-1 induction. We have therefore used one of the uncommon E2F-1 transfectants that does not lead to any detectable apoptosis on E2F-1 overexpression to study in detail the relationship between E2F-1 and toxicity caused by DNA topoisomerase-directed drugs in an inducible system. Our results indicate that although the sensitivity to both topoisomerase I and II drugs increases on induction, we have therefore used one of the uncommon E2F-1 transfectants that does not lead to any detectable apoptosis on E2F-1 induction, this increase in sensitivity is caused by qualitatively different mechanisms.

MATERIALS AND METHODS

Cells. The p53+/+ and pRB+/- human osteosarcoma cell line U-2OS-TA that constitutively overexpresses the TetVP16 transactivator was primarily used. To be able to distinguish exogenous from endogenous protein, a construct with the influenza virus HA3 epitope (HA tag) fused to the NH2-terminal of the E2F-1 and DP-1 sequence was used. The expression vector pUHDHAE2F1 (19) was used to form the E2F-1 construct pUHDHAE2F1 (20), and the DP-1 construct pUHDHADP-1 was a kind gift from Dr. N. Heinz (University of Vermont, Burlington, VT). Before the stable transfection, U-2OS-TA cells were transiently transfected with the pUHDHAE2F1/DP-1 plasmids to confirm the activity of the transactivator (TetRVP16) and the inducibility of the TetO promoter in the pUHD constructs. The stable introduction of the exogenous E2F-1 and DP-1 genes was done by electroporation of 5 μg of the two pUHD 10-3 plasmids together with 0.5 μg of pBabepeu (21) encoding puromycin resistance. Cells were selected in medium containing 1 μg/ml puromycin for 10–14 days before single cell clones were picked. The inducibility of the clones was tested by immunostaining and Western blotting of cells grown in the presence or absence of tc using the 12CA5 antibody that recognizes the HA epitope (22). Cell lines conditionally expressing a full-length E2F-1 and DP-1 were expanded into cell lines, and the one designated UE1DP-1 was used for this study. UE1DP-1 cells were grown in RPMI 1640 containing 25 mM HEPES buffer (Life Technologies, Inc.) supplemented with 10% FCS, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 2.0 mM L-glutamine, 1.0 μg/ml puromycin, and 1.0 μg/ml tc. To check that the exogenous, HA-tagged E2F-1 also possessed E2F-1 activity, an oligonucleotide containing the E2F-1 binding region from the adenoviral E2 promoter (1) was used in a gel retardation assay. The DNA binding activity was markedly increased in protein extracts from UE1DP-1/tc− cells at 16 h without tc, compared to UE1DP-1/tc+ cells (data not shown). Furthermore, the transactivation activity of E2F-1 was tested on an E2F-dependent promoter using the E2F3CAT (8) and pGL3TATA6xE2F (a kind gift from Dr. A. Fattaey; ONXY Pharmaceuticals, Richmond, CA) luciferase plasmids as reporter constructs and pSVCAT and pCMVluc as internal standards. In this assay, a 50-fold transactivation was observed in UE1DP-1/tc− cells at 24 h without tc compared with UE1DP-1/tc+ cells (data not shown). Other cells used included the mouse fibroblast cell line NIH/3T3 and HL-60 cells obtained from ATCC (Manassas, VA).

Preparation of Whole Cell Lysates and Nuclear Extracts. All cells were harvested at subconfluence. Cells were centrifuged for 5 min at 1,200 × g, and the supernatant was discarded. When preparing whole cell lysates, the pellet was resuspended in lysis buffer [0.1% NP40, 50 mM HEPES (pH 7.0), 250 mM NaCl, 3 μg/ml aprotinin, 3 μg/ml leupeptin, 150 μg/ml phenylmethylsulfonyl fluoride, 1 mM DTT, and 5 mM EDTA] and incubated on ice for 30 min. Extracts were then centrifuged for 20 min at 20,000 × g at 4°C, and then the pellet was discarded. Immediately thereafter, loading buffer [0.0625 M Tris-HCl (pH 6.8), 2% SDS, 17% glycerol, and 0.05% bromophenol blue] was added (1:1, v/v). Samples that were not used for Western blotting immediately after preparation were kept at −80°C in 50% glycerol. Nuclear extracts were prepared as described previously (23).

Cell Cycle Analysis. The cell cycle distribution was evaluated by fluorescence-activated cell-sorting analysis. In addition, acceleration of G1 phase as the time required for 50% of the cells to enter S phase was measured by labeling cells with BrdUrd after synchronization in mitosis by nocodazole block. BrdUrd (100 μM) was added to cells replated after synchronization, and cells were evaluated by fluorescence microscopy after staining with FITC-coupled anti-BrdUrd antibody. Ten fields of approximately 50 cells were counted to establish the fraction of cells entering S phase.

DNA Synthesis. DNA synthesis was measured by thymidine incorporation. After 24 h in either tc+ or tc− medium, cells were incubated with 2 μCi/ml [3H]thymidine for 1 h and centrifuged at 500 × g. The pellet was resuspended in 0.3 M NaOH and lysed for 15 min on ice. This solution was mixed with an equal volume of 20% trichloroacetic acid, kept on ice for an additional 15 min, and filtered through a Whatman GF/C glass microfiber filter by applying vacuum. Nonincorporated [3H]thymidine was washed out from the filter with 10% trichloroacetic acid followed by 96% ethanol. After drying, the filters were immersed in scintillation fluid and counted.

Western Blotting and Immunological Reagents. Protein extracts (25 μg/lane) were separated by SDS-PAGE and
blotted onto nitrocellulose membranes by semidry blotting using a three-buffer system [buffer 1 (pH 10.4), 0.3 M Tris and 10% methanol; buffer 2 (pH 10.4), 0.025 M Tris and 10% methanol; buffer 3 (pH 7.6), 0.025 M Tris, 0.040 M glycine, and 10% methanol]. Filters were then blocked in 5% nonfat dry milk in TBST (0.1 M Tris, 1.5 M NaCl, and 0.5% Tween 20 adjusted to pH 8.2 with HCl). The filters were subsequently probed with the relevant primary antibodies followed by horseradish peroxidase-coupled secondary antibodies and developed by an enhanced chemiluminescence system (Amersham, Little Chalfont, United Kingdom). Monoclonal antibody toward topoisomerase I was a generous gift from Dr. Y-C. Cheng (Yale University, New Haven, CT; Ref. 24), monoclonal antibody toward the COOH-terminal region of topoisomerase IIα was commercially obtained from Cambridge Research Biochemicals (Cheshire, United Kingdom), and a polyclonal antibody toward the COOH-terminal region of topoisomerase IIβ was purchased from BioTrend (Cologne, Germany). Monoclonal antibody to PARP and mdm2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal E2F-1 antibody KH20 (2) was used.

Transfection Studies. UE1DP-1 cells were transiently transfected by the calcium phosphate method with 10 μg of one of three reporter plasmids (−1200TOP2LUC, −562TOP2LUC, or −295TOP2LUC; Ref. 25) alone or in combination with 5 μg of pSV-β-gal (Promega, Madison, WI) as an internal control. Using the same method, U-2OS-TA cells were transfected with 3 μg of E2F-1 and/or 2 μg of cdk2 or cdk2-dn (a dominant negative version of cdk2) expression vector. The DNA amount was always adjusted to 20 μg with empty expression vector (pCMV). At 12 h posttransfection, UE1DP-1 cells were split into two groups, and half of the cells were reseeded in tc+ media, and the other half of the cells were reseeded in tc− media. After a 24-h incubation, lysates from UE1DP-1 cells were prepared in Reporter Lysis Buffer (Promega), whereas transfected U-2OS-TA cells were harvested 36 h posttransfection. Luciferase activity measurements were performed using the Luciferase Assay System (Promega) according to the manufacturer’s instructions. Escherichia coli β-Gal activity was measured using O-nitrophenyl-β-d-galactopyranoside as substrate.

Alkaline Filter Elution Assay. The alkaline filter elution assay was used to measure DNA SSBs and DSBs as described by Kohn (26), with slight modifications as described in Ref. 23. In the SSB assay, 3H-labeled L1210 cells used as internal standard were exposed to 100 μM H2O2 for 60 min on ice, corresponding to an irradiation dose of 300 rad (27). UE1DP-1 cells labeled for 24 h with 3H-thymidine were incubated in medium supplemented with the indicated drug at 37°C for 1 h, washed in 10 ml of ice-cold PBS, and then lysed on the filter (Nucleopore; 2.0 μm pore size) with 5 ml of SDS-EDTA lysis solution (2% SDS, 0.1 M glycine, and 0.025 M Na2EDTA) at pH 10 followed by the addition of 1.5 ml of SDS-EDTA lysis solution supplemented with 0.5 mg/ml proteinase K (Sigma, St. Louis, MO). Mixing of standard and experimental cells was done immediately before lysis. DNA was eluted with tetrapropyl-ammoniumhydroxide-EDTA (pH 12.1) containing 0.1% SDS at a rate of 0.125 ml/min. Fractions were collected at 20 min intervals for 2 h. Filters were treated with 400 μl of 1 M HCl for 1 h at 60°C and cooled, and 1 ml of 0.4 M NaOH was added before scintillation counting. The extent of DNA fragmentation was measured as the relative amount of radioactivity on the filters and transformed into rad equivalents by use of an empirical formula (28). The same assay was used to measure DNA DSBs with the modifications that only test UE1DP-1 cells were labeled with 3H-thymidine, and the elution was performed at pH 9.6 (26). Because the sensitivity of the DSB assay is lower than that of the SSB assay, it was necessary to increase the incubation period to 3 h for etoposide and 12 h for camptothecin.

DNA Relaxation Assay. Topoisomerase I catalytic activity was measured by relaxation of supercoiled plasmid DNA without ATP. NaCl nuclear extracts (0.35 ml) were incubated with 0.5 μg of pBR322 DNA in a reaction mixture consisting of 35 mM Tris-HCl (pH 8.0), 72 mM KCl, 5 mM MgCl2, 5 mM DTT, 5 mM spermidine, and 0.01% (w/v) BSA at 37°C in a final volume of 20 μl. The reaction was stopped by a 60-min incubation with 0.5% (w/v) SDS and 200 ng/ml proteinase K. Samples were then run on a 1% agarose gel, stained with ethidium bromide, and photographed under UV light.

kDNA Decatenation Assay. Topoisomerase II catalytic activity was measured using 3H-thymidine-labeled kDNA purified from Crithidia fasciculata (ATCC). Briefly, 0.35 mM NaCl nuclear extracts from UE1DP-1/tc− cells at 24 h of induction and from UE1DP-1/tc+ cells of decreasing protein concentration were incubated with 0.2 μg of kDNA for 15 min at 37°C in buffer containing 50 mM Tris-Cl (pH 8), 120 mM KCl, 10 mM MgCl2, 0.5 mM ATP, 0.5 mM DTT, and 30 μg BSA/μl in a final volume of 20 μl. After the addition of stop buffer/loading dye mix, samples were loaded on 1% agarose/0.5% ethidium bromide gels and run in Tris-borate EDTA buffer containing 0.5 μg/ml ethidium bromide at 100 V for approximately 50 min. Gels were photographed under UV light, loading wells were cut out, and scintillation was counted.

Apoptosis Assay. Apoptosis was measured five different ways, namely by: (a) the appearance of DNA ladders; (b) a quantitative ELISA assay; (c) flow cytometric detection of sub-G1 pools; (d) PARP cleavage assay by Western blot; and (e) morphological assessment after ethidium bromide and acridine orange staining. In the DNA ladder assay, UE1DP-1 cells were incubated with or without tc for 5 days, and then floating cells were collected, centrifuged for 5 min at 500 × g, and resuspended in 400 μl of lysis buffer (10 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1% SDS, and 0.5 mg/ml proteinase K). After 16 h at 42°C, the lysate was homogenized by pipetting, and DNA was extracted with phenol/chloroform and precipitated in ethanol. The pellet was resuspended in 50 μl of Tris EDTA buffer and treated with DNase for 1 h at 37°C, and 3 μg of DNA were loaded per lane on a 1.5% agarose gel and electrophoresed in Tris-acetate EDTA buffer for 1 h at 100 V. HL-60 cells (ATCC) with and without etoposide treatment were used as internal control cells because these cells easily apoptose. In all four of the other assays, 3 ml of UE1DP-1 cell suspension at 2 × 105 cells/ml were seeded in wells with or without tc. Drug was added after 24 h, and after an additional 24 h with drug, the wells were washed, and medium containing tc was added to all wells. After an additional 72 h, the different assays were conducted. The Cell Death Detection ELISA PLUS kit (Boehringer

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Mannheim, Mannheim, Germany) was used in the quantitative ELISA assay according to the manufacturer’s instructions. Flow cytometry was as described above, whereas PARP cleavage and morphology were performed using previously described standard techniques (29, 30).

Clonogenic Assay. Cytotoxicity was measured in a 3-week clonogenic assay, as described previously (31). Exponentially growing cells were washed three times in RPMI 1640 to remove tc and incubated with or without tc for 24 h. Cells were then incubated with drug for 1 (etoposide) or 3 (crama- thecin and cisplatin) h and washed in RPMI 1640 before plating. Cells were plated in media containing 0.3% agar on top of a feeder layer of 0.5% agar and 2.5% sheep RBCs to which 1.0 μg/ml puromycin and 1.0 μg/ml tc were added.

RESULTS

Protein Expression: E2F-1/DP-1 Overexpression Induces Higher Levels of Topoisomerase IIα but not Topoisomerase IIβ or Topoisomerase I. The expression of the proteins encoded by the pUDH vectors was induced by the removal of tc from the medium. Transcription of cyclins A and E has been described to be up-regulated by E2F-1, and after a nocodazole block reduced the time to reaching S phase. However, no differences were observed in the amount of topoisomerase IIβ (Fig. 1A) or topoisomerase I (Fig. 1C) after E2F-1/DP-1 overexpression. The level of mdm2 protein was also unchanged (data not shown).

Cell Cycle Analysis: UE1DP-1/tc− Cells Show the Expected Increase in S Phase. Using fluorescence-activated cell-sorting analysis, UE1DP-1/tc− cells at 24 h showed an increase in S phase from 49.5% to 62.9% (13.4%; SD of three experiments was 2%) compared with UE1DP-1/tc+ cells. Concomitantly, a decrease in G1 from 38.2% to 29.8% and a decrease in G2/M from 12.2% to 6.6% were observed. Furthermore, induction of E2F-1 in UE1DP-1/tc− cells after a nocodazole block reduced the time spent before entering S phase by 25%, namely from 16 to 12 h (data not shown). Thus, not only is E2F-1 overexposed in UE1DP-1/tc− cells as shown in Fig. 1A, it is also functional in that the expected increase in S-phase fraction occurs.

DNA Synthesis: UE1DP-1/tc− Cells Show Increased Thymidine Incorporation. Activity of DNA synthesis was measured as the incorporation of [3H]thymidine at 24 h after E2F-1/DP-1 induction showed a mean increase of 1.79-fold (median, 1.76-fold) from five separate experiments in UE1DP-1/tc− cells compared with UE1DP-1/tc+ cells. This confirms the increase in UE1DP-1/tc− cells as mentioned above.

Topoisomerase IIα Promoter Studies: E2F-1 Overexpression Up-Regulates the Topoisomerase IIα Promoter via a Nonspecific S-Phase-mediated Mechanism. To examine whether the increased topoisomerase IIα protein levels were a result of enhanced promoter activity of the topoisomerase IIα gene, UE1DP-1 cells were transiently transfected with reporter plasmids expressing firefly luciferase under control of the human topoisomerase IIα promoter. These experiments showed that reporter plasmids carrying topoisomerase IIα promoter sequences 1200, 562, or 295 bp upstream of the transcription start site were all induced 2–3-fold on tc removal (Fig. 2), which corresponds to the 2–3-fold increase in topoisomerase IIα protein levels shown in Fig. 1B. This induction was further con-
Overexpression of E2F-1 induces topoisomerase IIα promoter activity 2–3-fold through an S-phase-mediated mechanism. A, a 2–3-fold increase in expression is observed in three different topoisomerase IIα promoter constructs (−1200, −562, and −295) transfected to UE1DP-1/tc− cells 24 h after tc removal. B, dominant negative cdk2 suppresses E2F-1-induced stimulation of the human topoisomerase IIα promoter. Parental TA-U2OS cells were transiently transfected with pCMV-E2F-1 (2 μg), pCMV-cdk2 (3 μg), and pCMV-cdk2-dn (3 μg), as indicated, together with −295TOP2LUC (10 μg) and pSV-β-gal (5 μg), and the DNA amount was adjusted to 20 μg with empty expression vector (pCMV-neo). Luciferase and β-gal activities were measured 24 h posttransfection. The values represent relative promoter activation and are the averages of three independent experiments done in duplicate (error bars, ± SE).

Fig. 3 UE1DP-1/tc− cells are hypersensitive to etoposide and camptothecin but not to cisplatin. Representative clonogenic assays demonstrate increased cytotoxicity of etoposide (A) and camptothecin (B) but no difference in cisplatin cytotoxicity (C) in UE1DP-1/tc− cells. Incubation with drug after the 24 h tc removal was 1 h for etoposide and 3 h for camptothecin and cisplatin. For relative IC50 values in repeated experiments, see Table 1.

Cytotoxicity: UE1DP-1/tc− Cells Are Hypersensitive to Camptothecin and Etoposide but not to Cisplatin. Direct cytotoxicity to the cells of overexpressing E2F-1/DP-1 for 24 h was measured by trypan blue exclusion. On average, approximately 9% of both the induced (tc−) and the noninduced (tc+) cells stained blue, indicating that induction of E2F-1 did not affect the short-term viability of the cells. The plating efficiency for UE1DP-1/tc− and tc+ cells as well as for nontransfected U-2OS-TA cells was equal and stable at 3% in the 10^2–10^5 cells/ml range. In clonogenic assays, UE1DP-1/tc− cells were hypersensitive to both the topoisomerase II-directed drug etoposide (Fig. 3A) and the topoisomerase I-targeted agent camptothecin (Fig. 3B). Thus, in repeated experiments, UE1DP-1/tc− cells were 2.5–6-fold hypersensitive to etoposide compared with tc+ cells in experiments using a 1-h drug incubation and 1.3–7-fold hypersensitive to camptothecin using a 3-h drug incubation (Table 1). In contrast, no difference in sensitivity between tc− and tc+ cells was observed when the cells were treated with cisplatin, a drug that does not show a cell cycle-specific toxicity (Fig. 3C).

Apoptosis: UE1DP-1/tc− Cells Show Increased Apoptosis after Camptothecin Treatment but not after Exposure
Cells were exposed to drug for 1 h (etoposide) or 3 h (camptothecin), washed, and plated. We first established that E2F-1/DP-1 overexpression (18, 19). To obtain quantitative results, we then used an ELISA assay. As shown in Fig. 4, camptothecin induced a marked increase in apoptosis in UE1DP-1/tc− cells was observed as compared with tc+ cells. However, no difference in apoptosis between UE1DP-1/tc− and tc+ cells was observed after either etoposide (A) or cisplatin treatment (C). All experiments were independently conducted three times with similar results.

### Table 1 Clonogenic sensitivity of EU1DP-1/tc− and tc+ cells

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<tr>
<th>Etoposide IC50 (μM)</th>
<th>Camptothecin IC50 (μM)</th>
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<tr>
<td>tc+</td>
<td>tc−</td>
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<tr>
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Fig. 4 Increased apoptosis in UE1DP-1/tc− cells after camptothecin treatment but not after etoposide or cisplatin treatment in the ELISA assay. The quantitative Cell Death Detection ELISA kit was used to measure apoptosis. After camptothecin treatment, a marked increase in apoptosis in UE1DP-1/tc− cells was observed as compared with tc+ cells (B). However, no difference in apoptosis between UE1DP-1/tc− and tc+ cells was observed after either etoposide (A) or cisplatin treatment (C). All experiments were independently conducted three times with similar results.

**Fig. 4** Increased apoptosis in UE1DP-1/tc− cells after camptothecin treatment but not after etoposide or cisplatin treatment in the ELISA assay. The quantitative Cell Death Detection ELISA kit was used to measure apoptosis. After camptothecin treatment, a marked increase in apoptosis in UE1DP-1/tc− cells was observed as compared with tc+ cells (B). However, no difference in apoptosis between UE1DP-1/tc− and tc+ cells was observed after either etoposide (A) or cisplatin treatment (C). All experiments were independently conducted three times with similar results.

**to Etoposide or Cisplatin.** We first established that E2F-1/DP-1 overexpression itself does not induce apoptosis in UE1DP-1/tc− cells by equal DNA laddering (data not shown), an event reported to occur in approximately every fifth cell line with forced E2F-1 overexpression (18, 19). To obtain quantitative results, we then used an ELISA assay. As shown in Fig. 4B, camptothecin induced a marked increase in apoptosis in UE1DP-1/tc− cells, whereas neither etoposide (Fig. 4A) nor cisplatin (Fig. 4C) showed any difference between UE1DP-1/tc− and UE1DP-1/tc+ cells. Furthermore, no difference in apoptosis after etoposide treatment was observed in UE1DP-1/tc− cells compared with tc+ cells in the DNA fragmentation assay (data not shown). To verify the results in the ELISA assay showing different responses to camptothecin and etoposide in UE1DP-1/tc− compared with tc+ cells, we used three more assays, namely, flow cytometry, PARP cleavage, and morphology. As shown in Fig. 5, all three assays confirmed the findings of the ELISA assay. Thus, it is reasonable to state that this differential apoptotic response to camptothecin is not an artifact. Furthermore, the assays in Fig. 5 also confirm that induction of E2F-1/DP-1 in UE1DP-1/tc− cells does not in itself lead to apoptosis.

**Alkaline Elution:** UE1DP-1/tc− Cells Exhibit More DNA SSBs and DSBs after Etoposide Treatment but not after Camptothecin Treatment. Etoposide-induced DNA SSBs increased 1.5–2-fold in UE1DP-1/tc− cells in all four separate experiments (Table 2). This is illustrated in Fig. 6, where the number of SSBs (as determined by an increased elution rate) is higher in UE1DP-1/tc− cells at both 0.5 and 2.5 μM etoposide (VP-16). In contrast, camptothecin only increased DNA SSBs in UE1DP-1/tc− cells in one of five separate experiments (data not shown). Because the lethal damage is presumed to be caused by DNA DSBs (10), these were also measured. In two separate experiments, etoposide caused a 50% relative decrease in filter retention in UE1DP-1/tc− cells compared with tc+ cells, indicating a significant increase in DSBs in these cells consistent with the increase in SSBs. Because the lack of increase of SSBs by camptothecin was unexpected, the DSBs induced by this drug were studied in more detail. Five independent experiments using camptothecin concentrations from 1–20 μM all demonstrated equal filter retentions in UE1DP-1/tc− and tc− cells (data not shown). Thus, no increase in DSBs was observed after camptothecin exposure in UE1DP-1/tc− cells compared with tc+ cells, which is in accordance with the SSB results.

**Topoisomerase I and II Catalytic Assays: No Change in Either Assay in UE1DP-1/tc− Cells.** No difference in topoisomerase I catalytic activity between UE1DP-1/tc− and tc+ cells was observed 24 h after tc removal (Fig. 7A) in three separate experiments from two different nuclear extractions using a DNA relaxation assay. This is in accordance with the unchanged enzyme level shown in Fig. 1D. Further-
more, as shown in Fig. 7B, there was no difference in the catalytic activity of topoisomerase II using a kDNA decatenation assay. This was the case in all eight separate experiments using three different nuclear extractions. This result was unexpected, considering the 2–3-fold increase in topoisomerase IIα content in UE1DP-1/tc− cells demonstrated in Fig. 1B.

**DISCUSSION**

The topoisomerase I and II poisons are believed to exert their cytotoxicity by creating DNA breaks after trapping of their respective enzyme to DNA in a drug-enzyme-DNA cleavable complex (10). DNA breaks, both single and double-stranded, occur when one of these cleavable complexes collides with a replication fork, the so-called road block model (10). These DNA breaks then lead to cell death in as yet undetermined manner. An increase in DNA breaks and their consequent cytotoxicity caused by a given dose of a topoisomerase poison can therefore theoretically be caused by either an increase in enzyme levels leading to more cleavable complexes being formed or an increase in DNA replication increasing the number of available replication forks or a combination of these two factors. The topoisomerase I poisons such as the camptothecins (topotecan and irinotecan) and the topoisomerase II poisons such as the epipodophyllotoxins (etoposide and teniposide) and the anthracyclines (daunorubicin and doxorubicin) are considered to be
levels in G1 and S phase (34). Furthermore, the increase in etoposide (VP-16)-induced DNA SSBs in UE1DP-1/tc- cells. Incubation with etoposide was for 1 h at 37°C after the 24 h E2F-1/DP-1 induction. For conversion of SSBs to rad equivalents, see Table 2.

Fig. 6 Increased DNA SSBs in UE1DP-1/tc- cells after etoposide treatment. Representative alkaline elution assay demonstrating an increase in etoposide (VP-16)-induced DNA SSBs in UE1DP-1/tc- cells. Incubation with etoposide was for 1 h at 37°C after the 24 h E2F-1/DP-1 induction. For conversion of SSBs to rad equivalents, see Table 2.

S-phase-specific agents, with the topoisomerase I drugs being more tightly linked to this cell cycle phase than the topoisomerase II drugs. As mentioned in the “Introduction,” several studies have commented on E2F-1 overexpression and sensitivity of topoisomerase I- and/or topoisomerase II-directed drugs with somewhat varying results (13–16). We therefore wished to study this question in more detail using a cell line with wild-type p53 and retinoblastoma that did not increase its apoptotic rate after E2F-1 induction.

Although clonogenic cytotoxicity toward camptothecin was increased in UE1DP-1/tc- cells (Fig. 3B; Table 1), this was not due to an increase in enzyme level (Fig. 1D) or activity (Fig. 7A), a result that is supported by the unaltered topoisomerase I levels in G1 and S phase (34). Furthermore, the increase in cytotoxicity caused by camptothecin is not explained by an increase in the amount of DNA SSBs or DSBs. This indicates, at least in UE1DP-1 cells, that E2F-1/DP-1 overexpression increases the cells’ apoptotic response and clonogenic toxicity by a post-DNA damage pathway that remains to be elucidated. Interestingly, a similar difference in apoptotic response to camptothecin in Werner Syndrome-deficient cells compared with control immortalized cells has recently been reported (35). The cytotoxic post-DNA damage mechanisms of topoisomerase I-directed drugs are the focus of much current interest (36–38), and the inducible system provided by UE1DP-1/tc- cells would be an exciting model for the study of this field.

A major difference between the α and β isof orm of topoisomerase II is expression. Topoisomerase Iα activity is primarily associated with proliferating cells and decreases progressively as cells are induced to differentiate or are deprived of serum (39). Levels of topoisomerase Iα enzyme also change within the cell cycle, with low levels during G0-G1 and accumulation during S phase and G2 to reach maximal levels during mitosis (40), and this accumulation is obtained by enhancement of both transcription and mRNA stability during S phase (41, 42). The topoisomerase Iβ gene is constitutively expressed in proliferating as well as differentiated tissue, and its transcription rate is more or less constant throughout the cell cycle (43). Thus, the 2–3-fold increase in topoisomerase Iα and the lack of change in topoisomerase Iβ levels found in UE1DP-1/tc- cells (Fig. 1, B and C) are compatible with the increase in S phase in these cells. Furthermore, UE1DP-1/tc- cells had an increase in etoposide-induced DNA SSBs (Fig. 6; Table 2) and cytotoxicity (Fig. 3A; Table 1), which agrees with the increase in the content of the α isof orm. However, we found no difference between UE1DP-1/tc+ and tc- cells when we measured the catalytic activity of topoisomerase II (Fig. 7B), which is in marked contrast to the aforementioned increase in enzyme levels and etoposide-induced DNA breaks. A possible explanation for this discrepancy could be the known lower level of phosphorylation of the α isof orm in S phase compared with the G2-M phase (40). Although the effects of phosphorylation on the activity and biological function of topoisomerase II are still a matter for debate (44), several studies have suggested that phosphorylation of purified topoisomerase II enhances its activity (44–46). It is thus likely that the increase in topoisomerase Iα number is sufficient to increase the number of formed cleavable complexes

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that lead to DNA SSBs and cell death, but that the low level of phosphorylation at this stage entails that the enzyme’s catalytic activity is unchanged. This would agree with the finding that phosphorylation did not affect reaction steps that preceded hydrolysis of the enzyme’s high energy ATP cofactor including DNA cleavage/religation (45, 46). Another possibility is that the increase in pRb caused by the increase in S phase binds and inactivates topoisomerase IIα (47). The most unexpected result was the lack of increased apoptosis in UE1DP-1/tc cells exposed to etoposide demonstrated both by electrophoretic assay (data not shown) and four other assays (Figs. 4 and 5). This result further stresses the recently reviewed discrepancy between apoptosis and clonogenic survival (48). Previous studies on other cell lines have shown an increased apoptotic response to etoposide in cells with E2F-1 overexpression (14, 16). However, in contrast to UE1DP-1 cells, these cell lines increase their apoptotic rate on E2F-1 induction. This suggests that etoposide only induces apoptosis in E2F-1-overexpressing cells if these cells are already “primed” by an increased apoptosis due to the E2F-1 overexpression itself. At any rate, in UE1DP-1/tc cells, the increased cytotoxicity of etoposide is apoptosis independent and can be explained solely by the increase in topoisomerase IIα in these cells. We therefore wished to establish the mechanism of this E2F-1-induced increase of this isoform. As mentioned in the “Introduction,” a large number of genes that are up-regulated in response to cell cycle entry are controlled by the retinoblastoma/E2F pathway, and their promoters contain binding sites for the E2F family of transcription factors (5′-TTCC/GG/CCGC-3′). Because the topoisomerase IIα promoter does not have an E2F-1 binding sequence, it is tempting to postulate that the increase in its activity after E2F-1 overexpression (Fig. 2A) leading to overexpression of topoisomerase IIα protein is due to the E2F-1-induced S-phase entry rather than to a more specific interaction with the promoter. To test this, we cotransfected a dominant negative version of cdk2 (cdk2-dn) known to induce a G1 arrest downstream of E2F activation (33). This suppressed the topoisomerase IIα promoter even in the presence of ectopic E2F-1 expression (Fig. 2B). We can therefore conclude that the increased cytotoxicity to etoposide in UE1DP-1 cells after the increase in topoisomerase IIα promoter activity and protein levels is most likely due only to the increase in S phase caused by E2F-1 overexpression.

In conclusion, both the S-phase-specific topoisomerase I and topoisomerase II drugs camptothecin and etoposide increase their cytotoxicity in a clonogenic assay after increasing the S-phase fraction of cells after overexpression of the E2F-1/DP-1 transcription factor. However, whereas this may be explained by an increase in enzyme level and DNA damage in the case of etoposide and topoisomerase IIα, camptothecin appears to exert its S-phase-induced cytotoxicity via a post-DNA damage apoptotic pathway. Further elucidation of the latter result should be of interest in studying the mechanisms of cell death cause by topoisomerase I-directed anticancer agents.

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