0026-895X/90/040471-10$03.00/0

MOLECULAR PHARMACOLOGY, 38:471-480

Development of a Stable Camptothecin-Resistant Subline of P388 Leukemia with Reduced Topoisomerase I Content

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Received April 12, 1990; Accepted July 20, 1990

SUMMARY

A camptothecin-resistant subline of P388 leukemia (P388/CPT) was developed by repeated transplantation of P388 cells in mice treated with therapeutic doses of camptothecin. In mice bearing the resistant tumor, a maximally tolerated dose of camptothecin produced no net reduction in tumor cell burden, in contrast to a 5-log cell kill in the parental P388 (P388/S). The IC_{50} of camptothecin, as determined by colony formation assays of cultured cells, was 8 times greater for the cloned P388/CPT cell line than for P388/S. P388/CPT cells were not cross-resistant to other antineoplastic agents, including topoisomerase II inhibitors. The type I topoisomerases purified from P388/CPT and P388/S cells were identical with respect to molecular weight, specific activity, *in vitro* camptothecin sensitivity, and DNA cleavage specificity. Camptothecin induced fewer protein-associated DNA single-strand breaks in the resistant cells than in the wild-type P388 cells. Topoisomerase I mRNA, immunoreactivity, and extractable enzymatic activity were 2–4 times lower for P388/CPT cells than for P388/S cells. As resistance to camptothecin developed, topoisomerase I extractable activity decreased, concomitant with an increase in topoisomerase II extractable activity. Furthermore, the appearance of camptothecin resistance was associated with specific rearrangements of the topoisomerase I gene. These results suggest that development of resistance to inhibitors of topoisomerase I can occur by down-regulation of the target enzyme, thus reducing the production of lethal enzyme-mediated DNA damage. The enhanced topoisomerase II activity in these cells suggests that resistance to camptothecin may be overcome by co-treatment with topoisomerase II inhibitors.

CPT, an alkaloid isolated from *Camptotheca acuminata* (1), is a potent inhibitor of eukaryotic DNA topoisomerase I (2). It stabilizes transient DNA-topoisomerase I complexes in mammalian cells (3, 4), and these complexes lead to permanent DNA damage and cytotoxicity when DNA replication occurs (5, 6). Analogs of CPT have been evaluated and structure-activity relationships have shown that derivatives that do not inhibit topoisomerase I lack antitumor activity (7–9). CPT and certain of these analogs demonstrate a broad spectrum and high degree of antitumor activity in transplantable rodent tumor models (10–12), and a water-soluble derivative of CPT with an improved therapeutic profile, SK&F 104864, is currently in phase I clinical trials (13, 14).

Resistance to CPT could result from several different mechanisms, including alterations in the intracellular target, topoisomerase I, and changes in the cellular uptake/efflux of the drug. Two described mammalian cell lines that are resistant to CPT contain a topoisomerase I that is resistant to inhibition by the drug in isolated enzyme assays; thus, qualitative changes in topoisomerase I can lead to CPT resistance (15, 16). The uptake/efflux of CPT in these cell lines was not altered; this common mechanism of drug resistance has not yet been demonstrated for CPT. The finding that multidrug-resistant cell lines that demonstrate increased efflux of various antineoplastic agents are not cross-resistant to CPT and its analogs suggests that CPT is not a substrate for the p170 glycoprotein (17–19).

Because the production of DNA-topoisomerase I complexes in cells by CPT leads to cytotoxicity when DNA replication occurs, one mechanism by which cells can achieve CPT resistance is by deletion of the topoisomerase I gene or other mechanisms leading to reduction in topoisomerase I levels. In yeast cells, it was shown that deletion of the gene for topoisomerase I results in complete CPT resistance, demonstrating that topoisomerase I is the relevant target for CPT and that the drug subverts the normal mechanism of the enzyme to produce lethal DNA damage (20, 21). Mammalian cells may not be able to delete topoisomerase I and remain viable, inasmuch as no such

This work was supported in part by Grant CA40884-01 from the National Cancer Institute.

ABBREVIATIONS: CPT, camptothecin; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; P388/S, parental P388 leukemia cells; P388/CPT$^+$, CPT-resistant P388 cells transplanted in CPT-treated mice; P388/CPT$^-$, CPT-resistant P388 cells transplanted in untreated mice; P388/S^c and P388/CPT^c, sensitive and resistant cells cloned *in vitro*; kb, kilobases.

cells have been described. However, we recently developed a subline of P388 leukemia by selecting for CPT-resistant cells in mice bearing P388 leukemia, and our studies suggest that resistance resulted from a rearrangement in the topoisomerase I gene, leading to reduced levels of enzyme content and activity (22). Herein, we define more precisely the biochemical characteristics of topoisomerase I isolated from these CPT-resistant cells and correlate alterations in enzyme content, mRNA, and gene rearrangements with the development of resistance.

Experimental Procedures

Materials. Scleroderma antiserum that cross-reacts with mammalian topoisomerase I was obtained from Dr. Gerd Maul of the Wistar Institute (23). Rabbit antibody raised to P388 topoisomerase II was obtained as described (24). Plasmid pDPT2789 was isolated as described (25). Plasmid λkTOP1-D2 containing human topoisomerase I sequences was provided by Dr. Leroy Liu, Johns Hopkins University School of Medicine, Baltimore, Maryland (26).

***In vivo* establishment of a CPT-resistant P388 subline.** Parental P388 leukemia cells (P388/S) were maintained by serial intraperitoneal implantation into DBA/2 mice, following standard screening protocols (27). The CPT-resistant subline (P388/CPT$^+$) was developed by serial intraperitoneal transplantation of 10^6 cells in B6D2F$_1$ mice treated intraperitoneally with CPT at 3 mg/kg on day 2 for four transplant generations, 3 mg/kg on day 1 for the next three transplant generations, 6 mg/kg on day 1 for the next 29 transplant generations, and 9 mg/kg on day 1 for subsequent transplant generations. Cells (10^6) were transplanted when ascites fluid was evident (generally 10–14 days after implant) and response to CPT was evaluated every two to five transplant generations. After 59 transplant generations in drug-treated mice, a subline (P388/CPT$^-$) was transplanted into untreated mice and evaluated for stability of resistance to CPT in the absence of drug exposure.

Dose-response characterization studies involved the intraperitoneal implantation of 10^6 cells of P388/S, P388/CPT$^+$, or P388/CPT$^-$ into groups of five or six B6D2F$_1$ mice, followed by treatment with CPT (0.75–12.0 mg/kg, intraperitoneally, as a suspension on days 1 and 5. Each dose-response study included a titration in untreated mice (10^5 to 10^2 cells/mouse) to assess differences in growth kinetics and drug-induced cell killing based on prolongation of median survival time relative to that of untreated controls. Sensitivity of P388/CPT$^+$ or P388/CPT$^-$ was assessed by comparing the increase in lifespan and the net change in tumor burden at the end of therapy at the optimal dose of drug with the same parameters obtained in concurrent dose-response studies using P388/S cells (17, 28).

***In vitro* cloning and maintenance of P388/CPT sublines.** Ascites fluid from CPT-treated animals at transplant generation 78 was aseptically removed; cells were washed three times by centrifugation in PBS, resuspended at 10^6 cells/ml in growth medium (RPMI 1640 supplemented with 20% heat-inactivated fetal calf serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 10 μM 2-mercaptoethanol), and maintained in a 5% CO_2 humidified incubator at 37° to establish a suspension culture. The cells were cloned by incubating 50 viable cells in growth medium containing 0.12% noble agar at 37° for 8 days. Individual clones were removed and grown as suspension cultures in growth medium. All *in vitro* experiments were conducted using clones that had undergone fewer than 30 passages in tissue culture.

Colony formation assays. Cloned cells of wild-type P388 (P388/S^c) and CPT-resistant P388 (P388/CPT^c) (5×10^4/ml) were treated continuously with CPT (3.1–316 nM) for 7 days at 37° in growth medium containing 0.12% noble agar. The resulting cell colonies were stained with 0.1% tetrazolium salts for 48–72 hr and counted using a Biotran III Automatic Totalizer (New Brunswick Scientific Co., Edison, NJ). Cloning efficiency was 2–3%. The mean ± standard deviation of triplicate samples was determined for each concentration.

Measurement of CPT-induced DNA lesions. The cloned P388/S^c and P388/CPT^c cells were assayed for CPT-induced DNA single-strand breakage by filter elution, as described for L1210 cells (3). Cultures were labeled for 16 hr with [*methyl*-^{14}C]thymidine (50 mCi/mmol; New England Nuclear Corp.) before drug treatment and assay. Proteinase K (0.5 mg/ml) was present in the lysis solution (3).

Purification of topoisomerase I from P388 leukemia cells. Topoisomerase I was purified from the P388/CPT$^-$ and the P388/S cell lines as described (16, 24, 25). Proteinase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM sodium bisulfite, and 1 mM benzamidine) were present in all solutions; some solutions contained additional inhibitors as noted. Cells (1.5×10^{10}) recovered from ascites were washed in PBS, the cytoplasmic membranes were disrupted by Dounce homogenization, and the nuclei were collected by centrifugation. The nuclei were incubated in nucleus extraction buffer (5 mM potassium phosphate, pH 7.0, 0.35 M NaCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 10% glycerol, 10 μg/ml soybean trypsin inhibitor, 50 μg/ml leupeptin) for 1 hr and centrifuged at $3000 \times g$ for 15 min at 4°. The resulting supernatant was adjusted to 1.5 M $(NH_4)_2SO_4$ and centrifuged again as above. Soluble proteins (supernatant) were applied to a phenyl-Sepharose column (Pharmacia) equilibrated with 1.5 M $(NH_4)_2SO_4$ in PS buffer (20 mM potassium phosphate, pH 7.0, 0.5 mM EDTA, 10% glycerol). The column was developed with a linear gradient of 1.5–0 M $(NH_4)_2SO_4$ in PS buffer. Fractions were collected and the topoisomerase I activity of each fraction was assayed (25). Fractions containing enzyme activity were pooled, dialyzed against HAPEQ buffer (20 mM potassium phosphate, pH 7.0, 10% glycerol, 1 mM EDTA), and applied to a hydroxylapatite column equilibrated with HAPEQ buffer. The column was developed with a linear gradient of 0.2–1.0 M potassium phosphate in HAPEQ. Active fractions were pooled, dialyzed against storage buffer (70 mM potassium sulfate, pH 7.0, 0.5 mM dithiothreitol, 0.1 mM EDTA, 50% glycerol), and stored at −20°. The specific activity of the purified enzymes from both sources was 2×10^8 units/mg (1 unit relaxes 50% of 0.1 μg of pDPT2789 plasmid DNA after 30 min at 37°).

Protein assay and immunoblotting. Protein concentrations were determined as described by Bradford (29); for samples containing SDS, protein concentrations were determined as described by Smith *et al.* (30). The samples for the immunoblotting analysis were prepared as follows. Cells were briefly washed and immediately lysed with SDS (5% SDS in PBS). The protein concentrations of the cell lysates were measured and lysates containing equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis (31). The proteins were transferred from the gels to nitrocellulose membranes as described (24). The nitrocellulose filters were probed with anti-human topoisomerase I antibody (scleroderma antiserum) or antitopoisomerase II antibody [either anti-topo II-α or anti-topo II-β (24, 32)] and then with ^{125}I-Protein A. Topoisomerase I and II immunoreactivity was determined by autoradiography. Relative amounts of immunoreactivity were quantitated by densitometric tracing of the autoradiogram.

DNA extraction and analysis. Cells (10^6) were lysed with 0.5% Nonidet P-40 in 10 mM Tris, pH 7.5, 10 mM NaCl, 1.5 mM $MgCl_2$. Nuclei were collected by centrifugation at $600 \times g$ for 10 min at 4°. The nuclei were lysed and incubated overnight at 37° in a buffer containing 10 mM Tris, pH 7.5, 20 mM EDTA, 0.5% SDS, and 200 μg/ml proteinase K. DNA was purified by two phenol extractions, two chloroform extractions, and precipitation with ethanol in the presence of 0.3 M sodium acetate. For DNA samples from cells of various transplant generations, cryopreserved samples of P388/CPT$^+$ cells were reimplanted into B6D2F$_1$ mice treated intraperitoneally with CPT at 9 mg/kg on day 1. The tumor cells were harvested in 10–14 days and DNA was extracted as described above. Electrophoresis in 1% agarose was performed as described previously (22). Probes were derived from the cDNA clone λkTOP1-D2, which contains HeLa cell topoisomerase I sequences (26). This partial cDNA is 2.7 kb in size, lacks 1.4 kb of the 5′ sequences from the complete topoisomerase I cDNA, and contains about 1.1 kb of non-coding sequence at the 3′ end. The cDNA was digested with *Hind*III to yield two fragments, a 5′ fragment of

approximately 0.9 kb, consisting of coding sequence, and a 1.8-kb 3′ fragment containing both coding and non-coding sequences. ^{32}P-labeled probes were prepared by nick-translation of these fragments.

RNA isolation and Northern blotting analysis. Isolation of total cellular RNA was carried out as described by Chirgwin *et al.* (33). Quantitation of RNA was by A_{260} measurement. Total RNA (30 μg) was electrophoresed and transferred to nitrocellulose membrane as described by Brill and Sternglanz (34). Blots containing transferred total RNA were probed with human topoisomerase I cDNA (nick-translated λkTOP1-D2) or human actin probes (22). Relative amounts of mRNA were quantitated by densitometric tracing of the autoradiogram.

Assay of extractable topoisomerase I and topoisomerase II activities. Nuclei were prepared and treated with nucleus extraction buffer (as described in Purification of topoisomerase I from P388 leukemia cells; see above). Extractable proteins were obtained after cellular debris was removed by centrifugation (3000 × *g* for 15 min at 4°), and total protein concentration of the extracts was measured (29). Topoisomerase I activity was assayed by measurement of ATP-independent relaxation of negatively supercoiled DNA, as described (25), using either pDPT2789 DNA or pBR322 DNA as substrates. Specific activity was determined by dividing the topoisomerase I activity by the total amount of protein in the extracts. In some cases, topoisomerase I activity was assayed using whole-cell extracts in addition to nuclear extracts. Topoisomerase II catalytic activity was assayed by ATP-dependent unknotting of bacteriophage P4 DNA, as described (35). Specific activity of topoisomerase II was determined as above.

Cleavage of DNA mediated by purified topoisomerase I. Two different substrates were used to study the CPT sensitivity of DNA cleavage by topoisomerase I, supercoiled plasmid DNA and singly end-labeled linear DNA. For the plasmid-nicking assay, 100 ng of supercoiled pDPT2789 DNA were mixed with 5 ng of purified topoisomerase I and drug for 30 min, to allow the formation of enzyme-DNA covalent complexes. The complexes were trapped by addition of SDS and the covalently bound enzyme was digested with proteinase K. The resultant nicked plasmid was analyzed by agarose gel electrophoresis and quantitated by ethidium bromide staining, followed by densitometric tracing of the photographic negative of the stained gel, as described (25). For the sequence-specific DNA cleavage assay, a 1049-base pair *Ava*I-*Bam*HI fragment of pDPT2789 was labeled with ^{32}P at the 3′ end of the *Ava*I site (36). The formation and treatment of the enzyme-DNA covalent complex was the same as in the plasmid-nicking assay, except that 1000–2000 cpm of ^{32}P-labeled DNA replaced the supercoiled plasmid. The DNA was isolated by ethanol precipitation and analyzed on 8% polyacrylamide/50% urea gels, as described (37). The pattern and amount of DNA cleavage was determined by analysis of an autoradiogram of the gel.

Results

***In vivo* development of resistance to CPT.** Mice bearing intraperitoneal P388 leukemia cells were treated (intraperitoneally) with a single sublethal dose of CPT. After 10–14 days, the ascites fluid of the treated mice was transplanted into new recipients and the treatment process was repeated. This repeated drug treatment and transplantation resulted in the progressive development of resistance to CPT (Fig. 1).

In mice bearing P388/S, a maximally tolerated dose of CPT produced 159 ± 33% increase in lifespan and 4.6 ± 1.3 log of cell killing; at least 3 log of cell killing were observed in all experiments, and CPT proved to be curative (>6.5 log cell kill) in 7 of 29 experiments. Upon exposure to CPT, partial resistance was evident after one transplant generation (cell kill was outside the 95% confidence limits for the parental cell line). However, in two experiments within the first 20 transplant generations, no resistance was observed. By 40 transplant generations of exposure to CPT, resistance was virtually complete, i.e., little (<1 log) or no cell kill was achieved by a maximally tolerated dose of CPT. After 59 transplant generations, a subline was transplanted and maintained in mice without further CPT treatment. This subline (P388/CPT$^-$) continued to demonstrate complete resistance after 40 subsequent transplant generations in untreated mice, indicating that resistance was phenotypically stable. The other resistant subline, P388/CPT$^+$, was maintained in mice treated with CPT.

The growth kinetics of the P388/S and P388/CPT sublines were determined *in vivo* from parallel measurements in five experiments between generations 63 and 83. The doubling times were 19.5 ± 0.6 hr for P388/S, 20.8 ± 3.4 hr for P388/CPT$^+$, and 15.7 ± 4.0 hr for P388/CPT$^-$.

The sensitivity of P388/S and P388/CPT$^+$ *in vivo* to a spectrum of established antineoplastic agents is shown in Table 1. In two experiments, CPT at the optimal dose increased the lifespan of mice bearing P388/S by 156% and 170% and reduced the tumor cell burden by 5.3 and 6.7 log, respectively, whereas in mice bearing P388/CPT$^+$ cells CPT had little effect on tumor cell burden. There was no cross-resistance to cisplatin, mitomycin C, taxol, dactinomycin, or the topoisomerase II inhibitors amsacrine, ellipticine, teniposide, doxorubicin, and mitoxantrone.

Adaptation of the resistant line into cell culture and CPT cytotoxicity. For *in vivo* studies, clonally derived cell lines from P388/S and P388/CPT$^+$ (P388/S^c and P388/CPT^c) were obtained by growth and selection in soft agar. Doubling times for the resulting suspension cultures were 16.2 hr for P388/S^c and 17.6 hr for P388/CPT^c. The cultured sublines were treated continuously with varying concentrations of CPT for 7 days to determine the concentration required for inhibition of colony formation. The IC_{50} for CPT was 25 ± 10 nM for the wild-type P388 cells and 193 ± 23 nM for the CPT-resistant P388 cells. The resistance was found to be stable *in vitro* and, in subsequent cell passages in the presence of increasing concentrations of CPT, the cloned P388/CPT cell line became increasingly resistant to the drug.

Measurement of CPT-induced DNA lesions. To determine whether there was any difference between P388/S^c and P388/CPT^c cells in the biochemical effects of CPT, we measured the CPT-induced DNA single-strand breaks using alkaline elution. CPT produced fewer protein-associated DNA single-strand breaks in P388/CPT^c cells than in P388/S^c parental cells (Fig. 2). On the other hand, 2 μM teniposide, a DNA topoisomerase II inhibitor, produced approximately the same amount of DNA strand breakage (1000 rad equivalents) in both sublines, suggesting that the differential effect of CPT was specific.

Purification and CPT sensitivity of topoisomerase I from CPT-resistant and parental P388 lines. To investigate whether there was any qualitative alteration in the topoisomerase I from the CPT-resistant P388 tumor cells, we purified the enzyme from both P388/S and P388/CPT$^-$ tumor cells. Topoisomerase I activity from both cell lines eluted from the phenyl-Sepharose column at approximately 0.75 M ammonium sulfate and from the hydroxylapatite column at approximately 0.5 M potassium phosphate, suggesting that there are not large structural differences in the enzymes from either source. In addition, the purified enzymes from both the parental and CPT-resistant lines had a molecular mass of 100 kDa (Fig. 3)

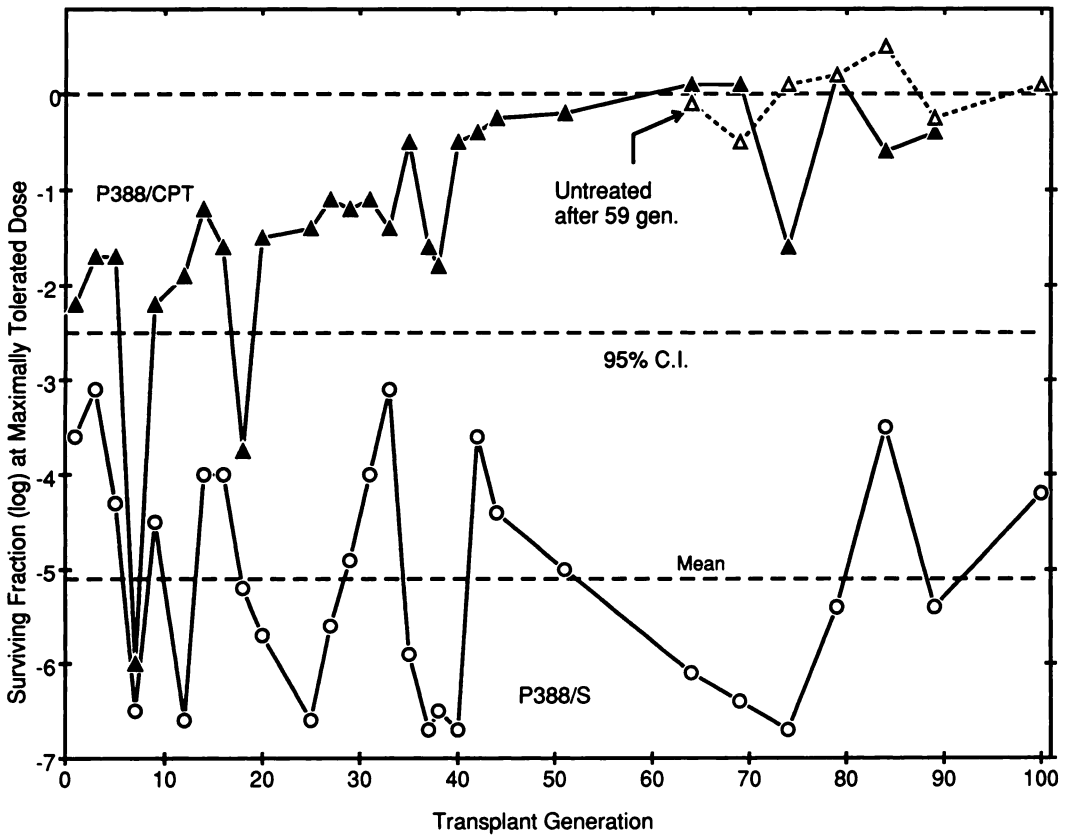

Fig. 1. Development and stability of resistance to CPT in a subline of P388 lymphocytic leukemia. The CPT-resistant line (P388/CPT$^+$) (▲) was serially transplanted in mice, as described in Experimental Procedures. After 59 transplant generations, a subline was serially transplanted without CPT treatment (P388/CPT$^-$) (△). Each point represents the response to a maximally tolerated dose of CPT administered intraperitoneally on days 1 and 5, in groups of five or six mice bearing 10^6 cells of wild-type lymphocytic leukemia (P388/S) (○) or one of the resistant sublines. *C.I.*, confidence interval.

TABLE 1

Cross-resistance of camptothecin-resistant P388 leukemia to antitumor agents

Drug	ILS at optimum dose[a]			Net cell kill[b]		
	P388/S	P388/CPT$^+$	P388/CPT$^-$	P388/S	P388/CPT$^+$	P388/CPT$^-$
		%				
Amsacrine	156	117 (1/5)		−5.4	−3.3	
	150		200 (1/5)	−4.4		−6.6
Cisplatin	200		>250 (3/5)	−6.4		−6.6
Dactinomycin	210	154 (1/5)		−6.7	−4.9	
Doxorubicin	>250 (4/5)	>250 (3/5)		−6.5	−6.8	
	>250		209 (2/5)	−6.4		−6.6
Ellipticine	130		100	−3.6		−4.3
Mitomycin C	140	236 (2/5)		−6.7	−6.4	
Mitoxantrone	>250 (3/5)	>250 (5/5)		−6.5	−6.8	
	130		>350 (3/5)	−3.6		−6.6
Taxol	80	91		−3.4	−2.2	
Teniposide	>250 (4/5)	>250 (5/5)		−6.5	−6.8	
	>250 (4/5)	217		−6.5	−6.8	
Camptothecin	170	82		−6.7	−1.8	
	156 (1/5)	25		−5.3	+0.3	

[a] Greatest increase in lifespan (ILS) relative to the untreated controls among three to five dose levels administered intraperitoneally on days 1 and 5. Fractions in parenthesis are long term, tumor-free survivors.

[b] Change in tumor cell burden (net cell kill in log) at the end of therapy (day 5).

and had similar specific activities. Analysis of these fractions indicated that topoisomerase I immunoreactivity was associated with the 100-kDa polypeptide (data not shown).

The two purified topoisomerase I proteins were characterized with respect to their CPT sensitivity and DNA cleavage specificity. Supercoiled DNA and the purified enzyme were mixed with drug to form covalent enzyme-DNA complexes. The complexes were trapped with SDS, treated with proteinase K to remove covalently bound enzyme, and electrophoresed. The amount of nicked DNA (form II) in the gel is a quantitative measurement of DNA-topoisomerase I intermediates stabilized by CPT (Fig. 4). There was no significant difference between the two enzymes with respect to CPT sensitivity. Furthermore, the concentration of CPT that resulted in 50% cleavage of the plasmid DNA using topoisomerase I from the P388/CPT$^-$ line (0.6 μM) was similar to that observed using topoisomerase I purified from calf thymus (7, 25). The enzymes were also similar with respect to their sensitivity to SK&F 104864, a water-soluble derivative of CPT (13).

The sequence specificity of DNA cleavage by the purified enzymes was determined using singly end-labeled DNA fragments and denaturing polyacrylamide gel electrophoresis. In

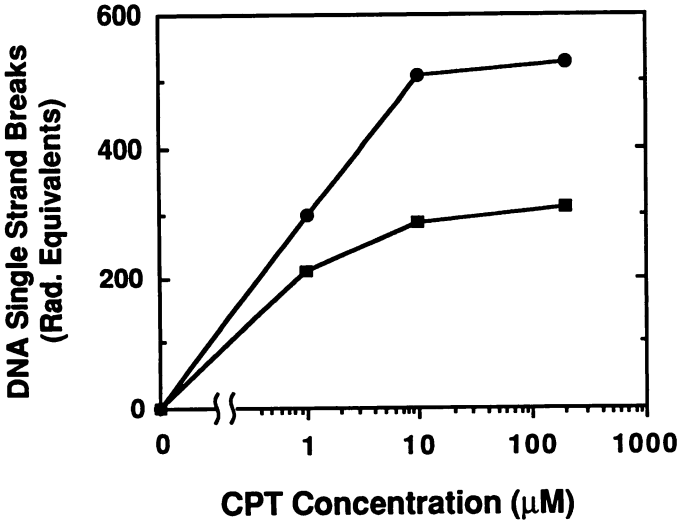

Fig. 2. CPT-induced DNA single-strand breaks in wild-type and CPT-resistant sublines of P388. P388/S^c (■) and P388/CPT^c (●) cells were labeled with [*methyl*-^{14}C]thymidine overnight and treated with various concentrations of CPT. DNA single-strand breakage was assayed by alkaline elution.

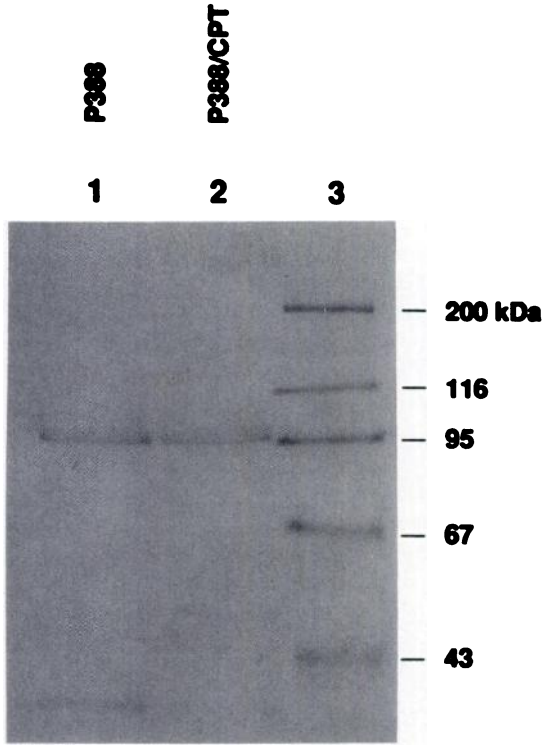

Fig. 3. SDS-polyacrylamide gel analysis of the purified topoisomerase I. The most highly purified fractions of the enzyme preparations were electrophoresed on a 10% polyacrylamide gel. *Lane 1*, purified enzyme from P388/S; *lane 2*, purified enzyme from P388/CPT$^-$; *lane 3*, 43–200-kDa molecular mass markers. The gel was stained with Coomassie blue.

the absence of drug, neither enzyme produced a significant amount of DNA cleavage (Fig. 5, *lanes 4* and *5*). In the presence of enzyme plus CPT (Fig. 5, *lanes 6* and *7*) or enzyme plus the CPT derivative SK&F 104864 (Fig. 5, *lanes 8* and *9*), several sites of DNA cleavage were produced. The enzyme from P388/CPT$^-$ cells produced a pattern of DNA cleavage products similar to that produced by the enzyme from the parental cells (compare Fig. 5, *lane 6* with *lane 7* and *lane 8* with *lane 9*). The

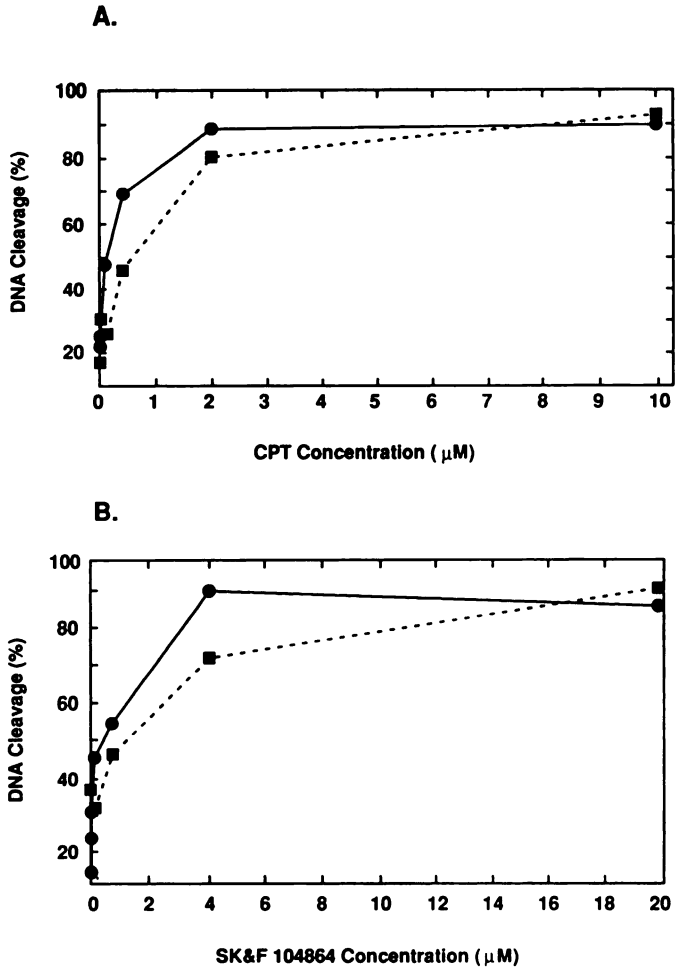

Fig. 4. Sensitivity of the topoisomerase I from P388/S and P388/CPT$^-$ to CPT and SK&F 104864. Supercoiled DNA and purified enzyme from P388/S cells (●) or P388/CPT$^-$ cells (■) were mixed in the presence of indicated concentrations of drugs. The resultant nicked plasmid was analyzed by agarose gel electrophoresis and quantitated by ethidium bromide staining, followed by densitometric tracing of the photographic negative of the stained gel. A, DNA cleavage in the presence of CPT; B, DNA cleavage in the presence of a water-soluble CPT analog, SK&F 104864.

DNA cleavage pattern produced in the presence of SK&F 104864 was very similar but not identical to the pattern produced in the presence of CPT.

Measurement of topoisomerase I and II activity. Nuclear extracts were prepared from P388/CPT$^+$, P388/CPT$^-$, and P388/S cells. The topoisomerase I and topoisomerase II activities in the extracts were assayed and specific activities were determined by dividing the measured activity by the amount of protein in the extracts (Table 2). Approximately 4-fold more topoisomerase I activity was found in the nuclear extracts from the parental cells (P388/S) than in the extracts from the resistant cells. To ensure that this difference reflected the total cellular topoisomerase I activity, extracts from whole-cell preparations were prepared and topoisomerase I specific activity was measured as in the nuclear extracts. A 4-fold difference was also observed between topoisomerase I activity extracted from the parental cells and that from the CPT-resistant cells (Table 2). In contrast to topoisomerase I activity, topoisomerase II specific activity in nuclear extracts from

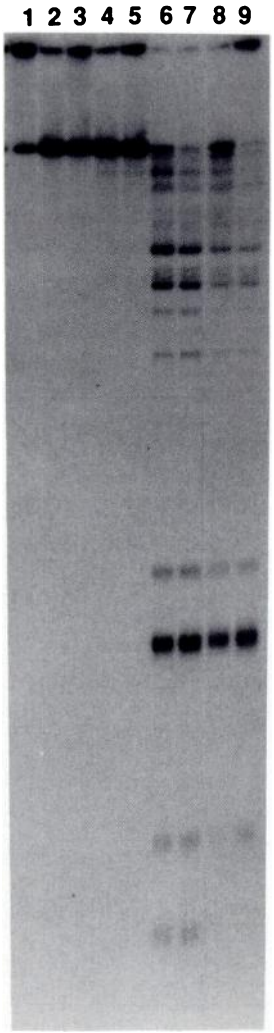

Fig. 5. DNA cleavage specificity of the purified topoisomerase I enzymes. The DNA cleavage assay is as described in Experimental Procedures. The DNA substrate was a 1049-base pair fragment from pBR322 labeled at one 3′ end. *Lane 1*, no addition; *lane 2*, 10 μM CPT with no enzyme; *lane 3*, 20 μM SK&F 104864 with no enzyme; *lane 4*, topoisomerase I from P388/S with no drugs; *lane 5*, topoisomerase I from P388/CPT$^-$ with no drugs; *lane 6*, topoisomerase I from P388/S with 10 μM CPT; *lane 7*, topoisomerase I from P388/CPT$^-$ with 10 μM CPT; *lane 8*, topoisomerase I from P388/S with 20 μM SK&F 104864; *lane 9*, topoisomerase I from P388/CPT$^-$ with 20 μM SK&F 104864.

P388/CPT cells was higher than in extracts from P388/S cells (Table 2).

Measurement of topoisomerase I immunoreactivity and mRNA. To determine whether differences in topoisomerase I activity and the differences in CPT-inducible DNA breaks between the sublines corresponded to differences in topoisomerase I protein content, cell lysates were analyzed by immunoblotting. Cells were harvested, washed, and immediately lysed in buffer containing SDS. The protein concentrations of the cell lysates were measured, and lysates containing equivalent amounts of protein were electrophoresed in SDS-polyacrylamide gels, transferred to nitrocellulose membrane, and probed with topoisomerase I antiserum (Fig. 6A). There was less topoisomerase I immunoreactivity in P388/CPT$^+$ cells (Fig. 6A, *lane 2*) than in parental cells (Fig. 6A, *lane 1*), which is consistent with the *in vivo* cell killing results and the enzyme activity measurements. The level of topoisomerase I immunoreactivity was lower still in the untreated resistant cells (P388/CPT$^-$) (Fig. 6A, *lane 3*). Densitometric analysis of autoradiograms from repeated experiments demonstrated there was 2–3-fold less topoisomerase I immunoreactivity in the two resistant lines than the parental line (Table 2).

In all cases, a broad band at approximately 100 kDa was recognized by the scleroderma antiserum (Fig. 6A), consistent with the size of the purified enzyme. This result suggests that there was no large alteration in the *in vivo* form of topoisomerase I protein associated with the resistant phenotype. In a separate series of experiments, we measured the immunoreactivity of topoisomerase II and found similar content of this enzyme in P388/CPT and P388/S cells (Table 2).

To determine whether sensitive and resistant cells contained different levels of topoisomerase I mRNA, total cellular RNA was prepared from the cells and subjected to Northern blotting analysis. A human topoisomerase I cDNA clone was used as a probe. Quantitatively similar reductions in the signal for the resistant cells were observed in the Northern blot experiment and in the immunoblot experiment (Fig. 6B). When the same Northern blot was probed with a human actin cDNA probe, no difference in the level of actin mRNA was found (data not shown), suggesting that similar amounts of RNA were loaded in each lane. Densitometric analysis indicated that the parental line contains approximately 3 times as much topoisomerase I mRNA as either of the resistant lines (Table 2). No abnormally sized topoisomerase I mRNA was detected by the probe, consistent with the observation that the enzyme in the resistant cells was not altered.

Rearrangement of the topoisomerase I gene in the mutant cell lines. To investigate whether there is any difference between the topoisomerase I gene of P388/S and P388/CPT cells, DNA was extracted from these cells and examined by Southern blot analysis. The topoisomerase I gene of P388/CPT cells was found to have been rearranged, as indicated by the appearance of an additional band in the Southern blot of DNA after restriction enzyme digestion (Fig. 7) (22). Importantly, all of the topoisomerase I-specific DNA fragments that were present in the parental cells were also present in the resistant cells, indicating that the DNA rearrangement did not occur in both alleles (22).

The autoradiograms were quantified by densitometry and the DNA contents of the different samples were normalized based on the intensity of unrearranged DNA fragments. One DNA fragment in the resistant cells was found to be diminished approximately 2-fold compared with the same DNA fragment of the parental cell (data not shown). The appearance of a new restriction fragment concomitant with a reduction of an existing DNA fragment further argues that the gene rearrangement occurred in one allele.

The observed DNA rearrangement in the topoisomerase I gene is most likely associated with the CPT resistance. The gene was rearranged in CPT-resistant cells but not in other sublines of P388, including those resistant to amsacrine, doxorubicin, or mitoxantrone (Fig. 7). Furthermore, Southern blot analysis of P388/CPT DNA with cDNA probes of topoisomerase II, β-actin, and MDR genes showed no evidence of either gene rearrangement or amplification (22).[1]

To determine whether the observed DNA rearrangement was

[1] Unpublished results.

TABLE 2

Topoisomerase activity, immunoreactivity, and mRNA in extracts from P388 cells

The quantities of topoisomerase immunoreactivity and mRNA were determined from densitometry of Fig. 5 and similar experiments and are expressed as fractions of the quantities in extracts from P388/S cells.

Tumor line	Topoisomerase I				Topoisomerase II	
	Specific activity		Relative immunoreactivity[b]	Relative mRNA level	Specific activity	Relative immunoreactivity
	Nuclear extract[a]	Whole-cell extract				
	(units/mg) × 10^{-4}				units/mg × 10^{-5}	
P388/S	8.0 ± 2.7	4.5	1.0	1.0	1.5	1.0
P388/CPT$^+$	1.9 ± 0.4	1.2	0.47 ± 0.11	0.29	3.9	1.0
P388/CPT$^-$	1.9 ± 0.4	1.0	0.29 ± 0.04	0.25	ND[c]	0.9

[a] Data are expressed as the mean ± standard deviation of two experiments.
[b] Three experiments.
[c] ND, not determined.

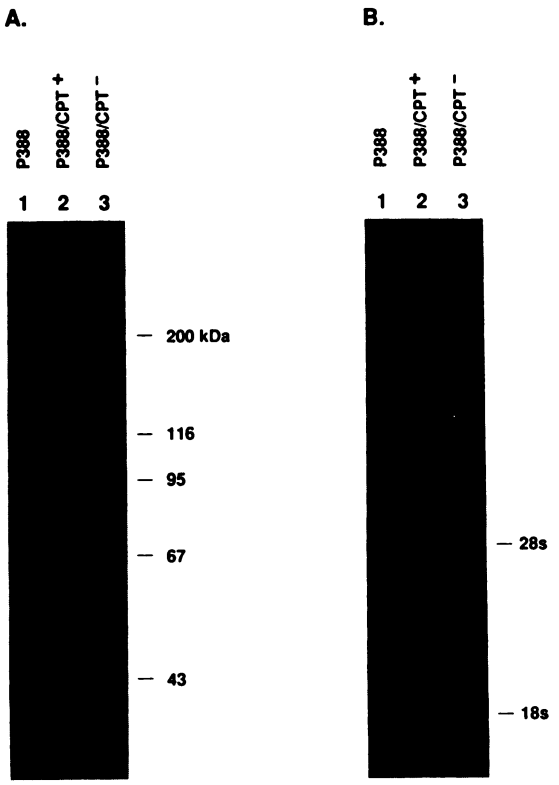

Fig. 6. Levels of topoisomerase I immunoreactivity and mRNA in P388/S, P388/CPT$^+$, and P388/CPT$^-$ cells. A, Topoisomerase I immunoreactivity. Seventy micrograms of total cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and Western blotting. The primary probe was human scleroderma antiserum, which reacts with topoisomerase I (23), and the secondary probe was ^{125}I-Protein A. The sizes and positions of the molecular mass markers are indicated on the *right*. B, Northern blotting analysis of topoisomerase I mRNA. Total cellular RNA (30 μg) was loaded in each lane. The probe was from a human topoisomerase I cDNA clone. rRNA markers are identified on the *right*. *Lanes 1*, P388/S; *lanes 2*, P388/CPT$^+$; *lanes 3*, P388/CPT$^-$.

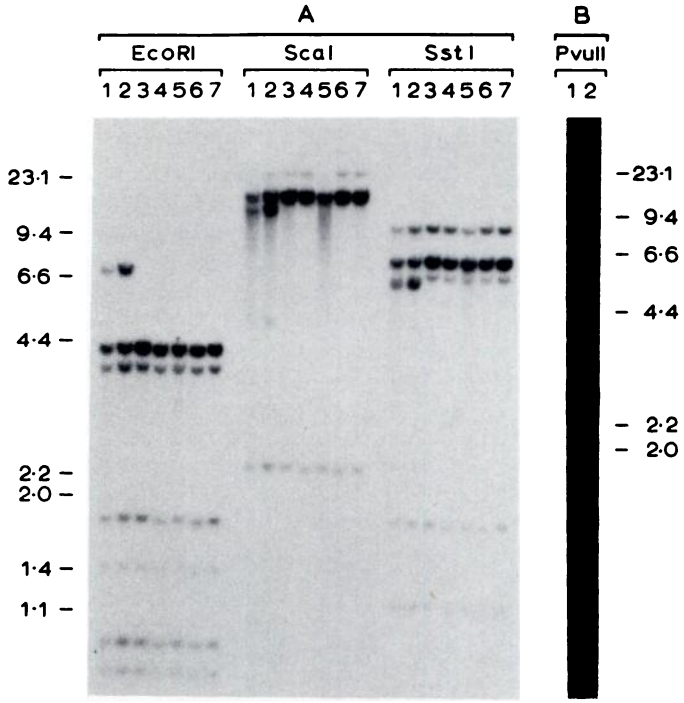

Fig. 7. Analysis of topoisomerase I gene in various drug-resistant sublines of P388. Cellular DNA (3 μg) was digested with restriction enzymes, fractionated on a 1% agarose gel, blotted onto a nylon membrane, and hybridized with a ^{32}P-labeled topoisomerase I 3′ probe. The positions and sizes (kb) of DNA molecular mass markers are shown in the *margins*. A, DNA from various sublines of P388 was digested with *Eco*RI, *Sca*I, or *Sst*I. The decreased amount of radioactivity in *lane 1* was due to loading error. The cell lines were: *lane 1*, P388/CPT$^-$; *lane 2*, P388/CPT$^+$; *lane 3*, P388/S; *lane 4*, A20 (an amsacrine-resistant subline of P388); *lane 5* PA (a doxorubicin-resistant subline of P388); *lane 6*, PF (a mitoxantrone-resistant subline of P388); *lane 7*, PK (an ellipticine-resistant subline of P388). B, DNA from CHO cell lines digested with *Pvu*II. Lane 1, CPT-resistant CHO cells (16); *lane 2*, wild-type (parental) CHO cells.

unique to P388/CPT sublines, we analyzed the topoisomerase I gene of a CPT-resistant CHO cell line (16). These CPT-resistant CHO cells contain a topoisomerase I that is resistant to inhibition by CPT *in vitro*; they also contain a reduced quantity of topoisomerase I relative to wild-type CHO cells (16). The CPT-resistant CHO cells, like the P388/CPT cells, contained a rearranged topoisomerase I gene (Fig. 6B), which presumably accounts for the quantitative effect on topoisomerase I cellular content.

Time course of resistance, as measured by topoisomerase I gene rearrangement and change in topoisomerase activities. Resistance to CPT was developed over a period of approximately 2 years. Because DNA rearrangement is a qualitative feature that can be readily assayed, we measured DNA rearrangements in the topoisomerase I gene in samples of P388/CPT$^+$ cells that had been collected and cryopreserved during the development of the resistant subline (see Experimental Procedures). DNA was extracted from cells correspond-

ing to various transplant generations and was subjected to Southern blotting analysis. Two separate probes were used; they were derived from 5′ and 3′ portions of a human topoisomerase I cDNA (see Experimental Procedures). Gene rearrangement was detected using each probe (Fig. 8). The earliest DNA rearrangement in the topoisomerase I gene was observed using the 5′ cDNA probe at transplant generation 18 (Fig. 8A). At later transplant generations, the intensity of the new (rearranged) DNA fragment slightly increased relative to other DNA fragments in the same sample (Fig. 8; *5′ probe, lanes 2–6*). Gene rearrangement that was detected by the 3′ cDNA probe was observed only in the fully resistant cells (Fig. 8, *3′ probe, lane 6*). The fact that each probe identified a different rearrangement (Fig. 8, *5′ probe* and *EcoRI, 3′ probe*) indicates that two rearrangements occurred at different portions of the topoisomerase I gene and at different stages of the development of resistance. We do not know whether the gene rearrangements detected with the 5′ and 3′ fragments of the cDNA probe are on the same or different alleles.

Methylation of DNA had been shown to down-regulate gene expression (38). Methylation of the topoisomerase I gene was investigated by comparing the restriction pattern of DNA cleaved with *Hpa*II (which does not cleave methylated cytosines in the nucleotide sequence 5′-CCGG-3′) with that of *Msp*I (isoschizomer of *Hpa*II that cleaves both methylated and nonmethylated cytosine residues). The *Msp*I restriction patterns of the topoisomerase I gene from cells at different generations after CPT treatment were identical. However, the *Hpa*II restriction pattern of the topoisomerase I gene from CPT-resistant cells was different from the pattern from parental P388 cells (Fig. 8). DNA from the parental line contained distinct restriction fragments of 1.2, 4.6, and 8.4 kb in size; after transplantation of the cell line *in vivo* in the presence of CPT, the 1.2- and 4.6-kb fragments diminished, whereas the 8.4-kb fragment increased. The reduction in intensity of smaller DNA fragments and increase in a larger DNA fragment suggest that the methylation state of topoisomerase I gene sequences increased during the development of CPT resistance. The DNA of cells from generation 98 (P388/CPT$^+$), which are maximally resistant to CPT, produced very few discernible fragments upon *Hpa*II digestion (Fig. 8, *HpaII*, lane 6), indicating that the topoisomerase I gene was extensively methylated. The disappearance of the 8.4-kb fragment in this case was most likely due to the extensive methylation of the *Hpa*II sites in the topoisomerase I gene.

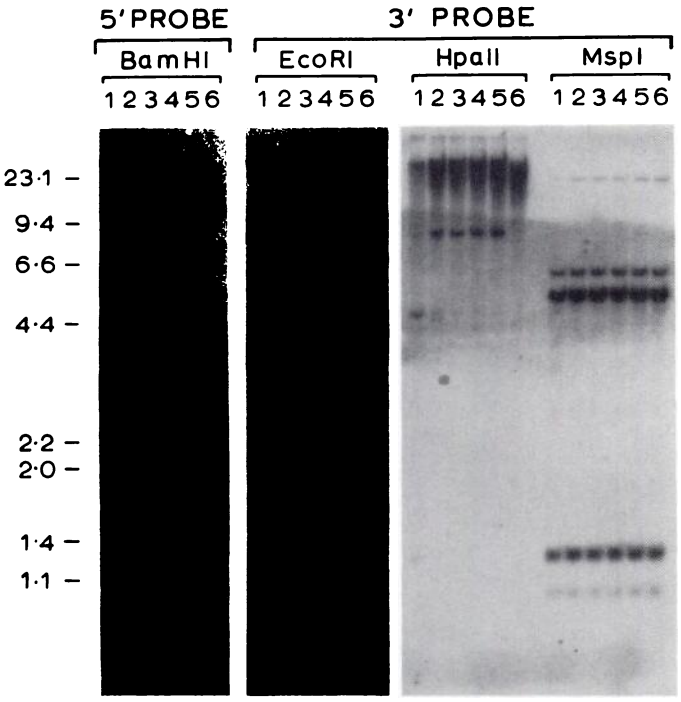

Fig. 8. Progressive DNA rearrangement and methylation of topoisomerase I gene during the development of CPT resistance *in vivo*. Cells representing different transplant generations were harvested and DNA was prepared for Southern blot analysis. *Left*, DNA was digested with *Bam*HI and hybridized to a topoisomerase I 5′ probe. *Right*, DNA was digested with *Eco*RI, *Hpa*II, or *Msp*I and hybridized to a topoisomerase I 3′ probe. The positions and sizes (kb) of DNA molecular mass markers are shown in the *margin*. *Lane 1*, P388/S; *lanes 2–6*, transplant generations 18, 30, 37, 49, and 98, respectively, of P388/CPT$^+$.

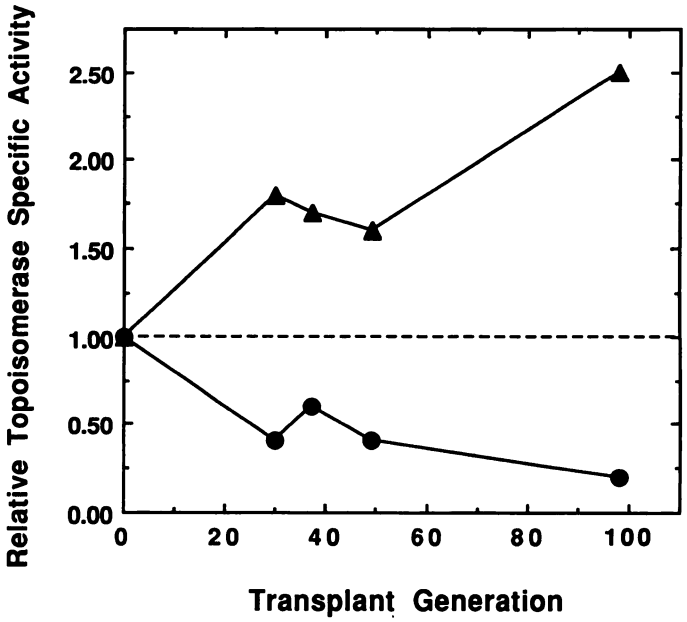

Fig. 9. Time course of alteration of topoisomerase specific activities during the development of CPT resistance *in vivo*. CPT-resistant cells were reestablished *in vivo* from cryopreserved specimens; cells were obtained from ascites and nuclear extracts were prepared. Topoisomerase I (●) or II (▲) activity was measured as described in Experimental Procedures. Data are expressed as relative specific activity (units/mg of protein), with the specific activity of the nuclear extract from the P388/S cell line set at 1.00.

It is likely that gene rearrangement and hypermethylation each reduced the level of topoisomerase I mRNA, leading to a reduced cellular content of topoisomerase I and, thus, CPT-inducible DNA lesions. Comparison of the levels of topoisomerase activities in cells at various transplant generations along the resistance course (Fig. 9) showed that, as the length of exposure to CPT (i.e., generation number) increased, the extractable topoisomerase I specific activity decreased. Some loss of extractable topoisomerase I activity was detected after 18 generations, consistent with rearrangement of the gene as detected by the 5′ probe. This decrease was accompanied by an increase in the specific activity of extractable topoisomerase II.

Discussion

We have developed, characterized, and established in culture a P388 subline that is resistant to CPT. The mechanism of

resistance of this cell line is distinguishable from those of other CPT-resistant cell lines (15, 16) in that a quantitative change in topoisomerase I levels appears to be solely responsible for CPT resistance. No differences were observed between topoisomerase I isolated from resistant cells and parental cells; in particular, both enzymes were equally sensitive to CPT (Figs. 2–4). The finding that CPT-resistant cells contained less topoisomerase I activity and protein is correlated with the reduced numbers of protein-associated DNA strand breaks upon treatment of P388/CPT cells with CPT.

Topoisomerase I mRNA and immunoreactivity decreased to similar extents as the cells became resistant to CPT (Table 2), suggesting that the reduction of topoisomerase I expression does not involve translation. A reduced level of topoisomerase I mRNA could have resulted from either diminished transcription or, alternatively, diminished stability of topoisomerase I mRNA. Either of these could result from changes in the structure of the topoisomerase I gene.

We have demonstrated a series of DNA rearrangements in the topoisomerase I gene of the resistant cell line (Figs. 6 and 7) (22). This kind of irreversible alteration in the gene is consistent with the stability of the resistant phenotype, even in the absence of drug exposure for 40 transplant generations *in vivo* (approximately 1 year). At least two rearrangements were detected, one using the 5′ portion of the cDNA probe and another using the 3′ portion. At present we do not know whether the two rearranged sequences are located on the same allele. In addition to DNA rearrangement, the topoisomerase I gene in CPT-resistant cells appears to be hypermethylated (Fig. 8). Hypermethylation is associated with inactive genes (38) and likely contributed to CPT resistance by down-regulating topoisomerase I expression. This is consistent with the finding that CPT-resistant cells contained lower amounts of topoisomerase I mRNA. Furthermore, as the length of time of exposure to CPT increased, there was an increase in DNA hypermethylation, concomitant with a decrease in extractable topoisomerase I activity. Because DNA methylation patterns are clonally inherited (38), this mechanism of resistance would likely be phenotypically stable.

Resistance was selected by repeated exposure of cells to sublethal concentrations of CPT. This method of resistance induction differs from that used for another CPT-resistant cell line (16) in which resistance was generated by exposure to a mutagen followed by selection at a high concentration of CPT. In that case, it is likely that a point mutation in the topoisomerase I gene resulted in a resistant enzyme. In the present study, the P388 cells became resistant to CPT *in vivo* more gradually, most likely by a mechanism of down-regulation of steady state mRNA. A disruption of one of the topoisomerase I alleles is likely to be a more frequent genetic event than the creation of a drug-resistant enzyme. Our results, however, do not preclude the possibility that recombinational events occurred in both alleles, producing one nonfunctional allele and one functional allele that contain stable genetic changes that reduce enzyme expression. Additionally, CPT has the capability of inducing recombinational events (20, 39) and may have contributed to the DNA rearrangement in addition to exerting selection pressure.

It has been shown in the lower eukaryotes *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* that topoisomerase I gene deletion mutants are viable and totally resistant to CPT (20, 21, 40, 41). In mammalian cells, mutants totally lacking topoisomerase I have not been found. In this study, the resistant P388 line maintained a low but stable level of topoisomerase I after 100 generations of transplantation. Furthermore, in a culture-adapted P388 cell line selected for growth in 45 μM CPT (>2000-fold resistant compared with parental cells), further depletion of topoisomerase I immunoreactivity was evident but a low level of enzyme activity was still detectable.[1] The failure of the P388 cells to become completely deficient in topoisomerase I suggests that, unlike the situation in yeast, some topoisomerase I activity is essential for mammalian cells.

Topoisomerase I down-regulation is accompanied by an increase in cellular activity of topoisomerase II (Table 2 and Fig. 9), suggesting that topoisomerase II activity may functionally compensate for the loss of topoisomerase I. Because only topoisomerase II activity in the nuclear extract increased and not immunoreactivity in whole-cell extracts, topoisomerase II may be activated by posttranslational modifications in the CPT-resistant cells. Alternatively, these findings may reflect increased extractability of topoisomerase II into nuclear extracts from P388/CPT cells. *In vivo*, the CPT-resistant P388 cells were found to be sensitive to several known topoisomerase II inhibitors, as well as DNA-binding agents and antimitotic agents (Table 1). Conversely, CPT and analogs such as SK&F 104864 retain activity in tumor sublines that display multidrug resistance to the aforementioned drugs (17–19). The cell line described herein and other CPT-resistant cell lines (16) do not show a typical multidrug resistance pattern. Such a lack of cross-resistance to commonly used anticancer agents is a desirable characteristic because clinical resistance, if it occurs in a similar fashion, could be overcome by combining non-cross-resistant drugs. Furthermore, concomitant increases in topoisomerase II activity in CPT-resistant cells suggest that resistance to CPT *in vivo* may be prevented or circumvented by co-treatment with an inhibitor of topoisomerase II. Preliminary experiments *in vivo* have suggested that treatment of mice bearing systemic leukemias with CPT in combination with teniposide resulted in therapeutic synergism.[1] Finally, the correlation between topoisomerase I gene methylation and CPT resistance suggests that agents that demethylate DNA (e.g., 5-azacytidine) may be useful in restoring CPT sensitivity to such resistant cells (42).

Acknowledgments

We thank Dr. Gerd Maul for providing scleroderma antisera and Dr. Leroy Liu for plasmid λkTOP1-D2 containing topoisomerase I sequences.

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