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ORIGINAL ARTICLE

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Sensitivity to camptothecin of human breast carcinoma and normal endothelial cells

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Abstract *Purpose*: To assess parameters that might determine resistance to the topoisomerase I inhibitor, camptothecin (CPT), the sensitivities of three established human breast cancer cell lines (ER^-) and of normal bovine endothelial cells to CPT in the free form and incorporated into liposomes (LCPT), were contrasted with topoisomerase I (topo I) content and activity, and with cell cycle response to CPT treatment. *Methods*: Drug sensitivities were determined using the tetrazolium dye assay and by ^{3}H-thymidine incorporation. Topo I levels were determined by Western blot analysis, and catalytic activity was determined with a plasmid relaxation assay, using nuclear protein from each cell line. CPT stabilization of cleavable complexes in nuclear extracts was determined using a labeled oligonucleotide with a specific topo I cleavage site. Cell cycle response to CPT was determined by flow cytometric analysis of propidium iodide-stained nuclei. *Results*: CPT was extremely potent against MDA-MB-157 cells with an IC_{50} value of 7 n*M* compared with IC_{50} values of 150 n*M* for GI 101A and 250 n*M* for MDA-MB-231 cells. In contrast, CPT inhibited the incorporation of ^{3}H-thymidine at very low doses in GI 101A and MDA-MB-231 cells with IC_{50} values of 9 n*M* and 5 n*M*, respectively; while MDA-MB-157 cells did not stop incorporating ^{3}H-thymidine until very high doses (500 n*M*) of CPT were used. When incorporated into multilamellar liposomes (LCPT), CPT retained its potency, with IC_{50} values similar to that of the free drug. No correlation was found between CPT-induced cytotoxicity and any of the topo I parameters determined. Cell cycle analysis, however, showed an accumulation of cells in G_2/M phase after 24 h treatment with low doses (5 n*M*) of CPT in only GI 101A and MDA-MB-231 cells with no arrest in normal endothelial or MDA-MB-157 cells. At higher doses (50 n*M*), however, a dramatic accumulation of cells in the S phase was observed in MDA-MB-157, MDA-MB-231 and GI 101A cells. In contrast, a G_2/M phase block was seen with the normal bovine endothelial cells using the higher doses of CPT. *Conclusions*: The results suggest that cell cycle regulation plays an important role in determining the effect of CPT on malignant and normal cells. The possible mechanisms explaining the sensitivities of the two cellular compartments to the action of CPT are discussed.

Key words Camptothecin · DNA topoisomerase I · Cell cycle · Breast cancer · Endothelial cells

Introduction

Camptothecin (CPT), the prototype topoisomerase I (topo I) poison, is the principal active component of the extract derived from the bark of the Chinese tree *Camptotheca acuminata* [1]. Early clinical trials with CPT showed that this plant alkaloid had antitumor activity against a variety of solid tumors [1, 2]. Further development of the drug, however, was discontinued owing to unpredictable and severe host toxicities, especially hemorrhagic cystitis and myelosuppression [3, 4]. The failure of these early clinical trials apparently was the result of the drug's rapid conversion at basic pH from the active lactone form into an inactive carboxylic acid form [5]. The equilibrium between the open and

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closed forms appears to be heavily in favor of the open form and the presence of serum albumin may actually facilitate the existence of the open form [6]. Our laboratory hypothesized, however, that if the drug could be protected from rapid conversion to the open form by incorporation into lipid-based vesicles (liposomes) before parenteral injection, it might retain its biological activity and clinical efficacy [7]. Further experiments have shown that indeed a single intramuscular injection of liposomal camptothecin (LCPT) maintains its antitumor activity against malignant breast carcinoma xenografted into nude mice [8].

A clear understanding of the mechanisms of action of CPT in normal cells as well as tumor cells is a critical precursor for predicting and modulating the activity of topo I-directed drugs in any preclinical setting. In the cells, CPT specifically and reversibly stabilizes the formation of cleavable complexes, a DNA–topo I enzyme intermediate, by inhibiting their religation. The cytotoxicity of CPT is believed to be a consequence of DNA breaks as a result of a collision between moving replication forks and CPT-stabilized cleavable DNA–topo I complexes [9–11]. However, many studies have shown that other cellular events subsequent to DNA break formation are essential for CPT cytotoxicity. These include cell cycle progression and DNA repair processes [12, 13]. Other cellular determinants of drug activity remain to be elucidated.

The aims of this study were to explore the effects of CPT on three estrogen receptor negative human breast cancer cell lines (ER^-) with a wide inherent range of sensitivity to the drug with respect to growth potential/viability of proliferating cultures, topo I trapping and cell cycle progression. Two bovine endothelial cells (BVEC, BLEC) with known differences in CPT sensitivity compared to tumor cells [14] were selected for the study to aid in the identification of cellular events that correlate with cytotoxicity. The results presented here suggest that the response of cells (tumor or endothelial) to the DNA-damaging effect of CPT and the cells' ability to regulate the cell cycle may be the determining factors in resistance to the drug. Parts of this study have been presented previously as an abstract [15].

Materials and methods

Chemicals and supplies

CPT (NSC 94600) was obtained from the Drug Development Branch, National Cancer Institute, NIH (Bethesda, MD) dissolved in dimethyl sulfoxide (DMSO) at 4 m*M*, aliquoted, and stored at −70 °C. Further dilutions were made in culture medium just before use. The final concentration of DMSO in culture did not exceed 0.1% (v/v) which is nontoxic to cells. Cholesterol (CHOL) and thiazolyl blue (MTT) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Dipalmitoylphosphatidylcholine (DPPC), phosphatidylinositol (PI) and sphingomyelin were obtained from Avanti Polar Lipids (Birmingham, Ala.). [Methyl-^{3}H]-thymidine (6.7 Ci/mmol) was purchased from DuPont/NEN (Boston, Mass.). All other chemicals were reagent grade.

Cell and culture conditions

Human breast MDA-MB-157 cells (ATCC HTB 24; American Type Culture Collection, Rockville, Md.), MDA-MB-231 cells (provided by Dr. B.C. Giovanella of the Stahelin Foundation for Cancer Research, Houston, Tex.) and GI 101A cells (provided by Dr. Josephine Hurst of the Goodwen Institute for Cancer Research, Plantation, Fl.) were maintained in RPMI-1640 medium (Gibco, Grand Island, N.Y.) supplemented with 10% bovine calf serum (Hyclone, Logan, Utah) at 37 °C in a humidified atmosphere containing 5% CO_2. Bovine lymph and venular endothelial cells were provided by Dr. S. Wasi of Ontario, Canada, and were maintained in our laboratory in DMEM medium (Gibco) supplemented with 1 m*M* sodium pyruvate (Gibco), and 20% bovine calf serum (Hyclone) at 37 °C in a humidified atmosphere containing 5% CO_2.

Liposome preparation

Multilamellar liposome-incorporated CPT (LCPT) was prepared as previously described [7, 8]. The preparation used in the present study comprised DPPC, sphingomyelin, CHOL, PI and CPT at a molar ratio of 2.4:6.6:1.0:0.05:1.0. The incorporation efficiency was 80% as determined using an HPLC assay for measuring CPT concentration. The multilamellar vesicles produced were sterilized by passage through a 0.22 μm filter.

Cytotoxicity assays

The growth inhibition effects of CPT and LCPT were evaluated by the MTT colorimetric method on quadruplicate assays after continuous exposure of the cells to various concentrations of CPT, LCPT or control vehicle for 24 h and incubation in drug-free medium for two or three doubling times as described previously [16]. The IC_{50} (drug concentration resulting in 50% inhibition of MTT dye formation, compared to control) was determined directly from semilogarithmic concentration-response curves.

Antiproliferation assays

The antiproliferative activities of CPT were measured by the loss of ^{3}H-thymidine incorporation into cellular DNA as described previously [17]. Briefly, monolayer cultures were treated with various concentrations of CPT for 24 h, then rinsed twice with warm phosphate-buffered saline (PBS) and radiolabeled with 1 μCi ^{3}H-thymidine for 4 h. The culture medium was removed and the cells were fixed for 1 h with acetic acid/methanol (1:3 v/v). Cells were rinsed twice with 80% methanol and finally digested for 1 h with 0.1 *M* NaOH and radioactivity measured in a TRI-CARB 2500 TR liquid scintillation analyzer. The studies were carried out on quadruplicate samples and the results are expressed as the percentage of initial values relative to the control counts.

Cell cycle analysis by flow cytometry

Cells in 100-mm tissue culture dishes were treated with 5 n*M* CPT, 50 n*M* CPT or vehicle for controls for 24 h. At selected timepoints, cells were washed with cold PBS, pH 7.4, harvested by centrifugation and incubated on ice in 70% ethanol. Following fixation, cells were washed twice with ice-cold PBS and incubated overnight at 4 °C in PBS containing 50 μg/ml PI, 100 μg/ml RNase A and 0.1% Triton X-100. Samples were analyzed with a FACScan flow cytometer (Becton Dickinson), whereby the fractions of cells in G_0/G_1, S and G_2/M were calculated using the SFIT analysis model of CELLFit software provided by the manufacturer.

Isolation of nuclear extracts

Nuclear extracts were prepared according to a protocol supplied by TopoGEN (Columbus, Ohio). Briefly, cells (approximately 10^7

cells, growing in log phase) were scraped in ice-cold TEMP buffer (10 m*M* TRIS-HCl, pH 7.5; 1 m*M* EDTA; 4 m*M* $MgCl_2$; 0.5 m*M* phenylmethylsulfonyl fluoride), and centrifuged. The cell pellet was suspended in ice-cold TEMP and homogenized in a tight-fitting homogenizer and nuclei were pelleted by centrifugation. The nuclear pellet was suspended in ice-cold TEP (TEMP without $MgCl_2$). An equal volume of 1 *M* NaCl was added, and the mixture vortexed and incubated on ice for 30–60 min. The precipitate was removed by ultracentrifugation at 100 000 *g* for 60 min at 4 °C and the supernatant containing the topo I activity was collected. After adding glycerol to 10%, aliquots were stored at −70 °C.

Topo I enzymatic assays

Topo I relaxation assays were measured using a kit supplied by TopoGEN and accomplished according to the manufacturer's instructions. Briefly, 1 µl of nuclear extract (of various protein concentrations) was incubated for 30 min at 37 °C with 1 µl supercoiled pHOT1 plasmid DNA in assay buffer (10 m*M* TRIS-HCl, pH 7.9; 1 m*M* EDTA; 0.15 m*M* NaCl; 0.1% bovine serum albumin; 0.1 m*M* spermidine and 5% glycerol) to a total volume of 20 µl. The reactions were terminated by the addition of 5 µl loading buffer (5% sarkosyl, 0.125% bromophenol blue and 25% glycerol). The supercoiled and relaxed topoisomerases were separated by electrophoresis on 1% agarose gels. After electrophoresis the gels were stained with ethidium bromide (0.5 µg/ml), destained in water, and photographed and the relaxation of supercoiled plasmid was visually estimated by inspection of the photographs.

Western blotting

Nuclear extract protein (3 µg) was separated by SDS-PAGE using a 10% gel and transferred electrophoretically onto nitrocellulose membranes. The nitrocellulose blots were blocked in 5% nonfat dehydrated milk for 1 h at 37 °C followed by a 2-h incubation with a human polyclonal antihuman topo I antibody collected from the serum of human scleroderma patients (TopoGEN). Washed blots were then incubated with horseradish peroxidase-conjugated goat antihuman antibody for 1 h at room temperature. After three washes with PBS containing 0.1% (v/v) Tween20, reactive proteins were visualized with ECL chemiluminescence (Amersham, Arlington Heights, Ill.).

DNA oligonucleotide cleavage assay

The topo I-mediated cleavage of a labeled oligonucleotide was measured according to the method of Tanizawa et al. [18]. Briefly, the double-stranded oligonucleotide (33-mer) provided by Dr. Yves Pommier of the National Cancer Institute (Bethesda, Md.) was labeled at the 3′-terminus of one strand using α-[^{32}P]-cordycepin with terminal transferase (DuPont NEN, Wilmington, Mass.). The ^{32}P-labeled oligo (3×10^5 cpm) containing a strong topo I cleavage site was incubated with nuclear extract (12 µg nuclear extract protein/reaction mixture) at 37 °C for 60 min in the presence or absence of CPT. The reaction was stopped by the addition of 1% SDS to stabilize topo I-induced cleavage, and the DNA was denatured by adding loading buffer (90% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol and 10 m*M* EDTA, pH 8.0). The oligo was separated into single-strand DNA fragments on a denaturing gel (16% polyacrylamide containing 7 *M* urea) at 52 °C. Gels were autoradiographed for 24–36 h at −20 °C and the cleaved (19-mer) and uncleaved (33-mer) products were quantified by densitometric scans (Scanalytics) of the resulting X-ray films. The percent cleavage was indicated by the ratio of the cleaved product to the total loaded sample.

Results

Cytotoxicity and antiproliferative activity of CPT

Survival curves for MDA-MB-157, MDA-MB-231 and GI 101A cells after treatment with CPT (1–2000 n*M*) for 24 h are shown in Fig. 1A, and Table 1 lists the average IC_{50} values for CPT in these cell lines. As shown in Fig. 1A, MDA-MB-157 cells were more sensitive to the cytotoxic action of CPT with an IC_{50} value of 7 n*M*, as compared with GI 101A and MDA-MB-231 cells with

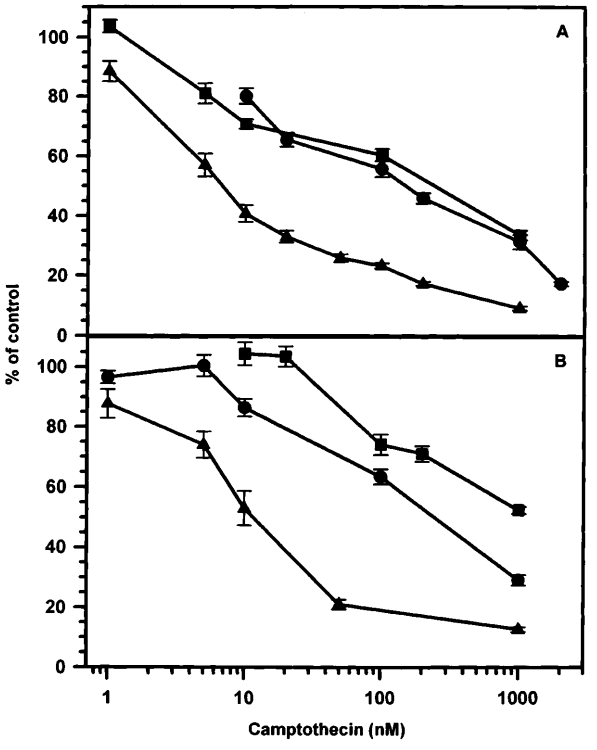

Fig. 1A,B Dose-response curves of MDA-MB-157 (▲), GI 101A (■) and MDA-MB-231 (●) cells following treatment with various concentrations of CPT **(A)** or of LCPT **(B)** for 24 h, as determined by the thiazolyl blue assay. The points are the means of quadruplicate determinations ± SE

Table 1 Relationships between doubling time and sensitivity to CPT of the studied breast cancer cells

Cell line	Doubling time (h)	IC_{50} (n*M*)[a]	IC_{50} (n*M*)[b]
MDA 157	36	500	7
MDA 231	24	5	250
GI 101 A	48	9	150

[a]IC_{50} for ^{3}H-thymidine uptake assay as described in Materials and methods
[b]IC_{50} for MTT assay as described in Materials and methods

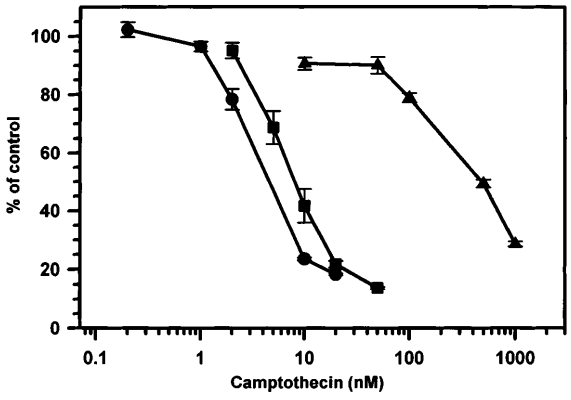

Fig. 2 Inhibition of MDA-MB-157 (▲), GI 101A (■) and MDA-MB-231 (●) cell proliferation by CPT. Cells were seeded in 24-well plates and incubated in the absence or presence of CPT (0.1–1000 n*M*) for 24 h, and evaluated for determination of IC_{50} by ^{3}H-thymidine incorporation assay as described in Materials and methods. The points are means of quadruplicate determinations ± SE

IC_{50} values of 150 n*M* and 250 n*M*, respectively. In contrast to the cytotoxic profile of the drug on these three different cell lines, CPT was not able to inhibit the incorporation of ^{3}H-thymidine into the DNA of MDA-MB-157 cells until extremely high doses (500 n*M*) were used, as shown in Fig. 2. However, MDA-MB-231 and GI 101A cells stopped incorporating ^{3}H-thymidine at much lower doses, with IC_{50} values of 5 n*M* and 9 n*M*, respectively, suggesting that these cells were much more sensitive to the antiproliferative actions of CPT than MDA-MB-157 cells. It is important to note that the cytotoxicity was measured two doubling times following CPT treatment, while the antiproliferative activity was measured immediately following drug treatment. In the LCPT form, CPT was slightly less cytotoxic than in the "free" form when cells were treated for 24 h (Fig. 1B), but the cytotoxicity was nearly the same after 72 h exposure time (data not shown). This observation agreed with our previous reports that the cytotoxic effect of the liposomal drugs on transformed cells is basically time-dependent [17]. Although it has been shown previously that CPT is particularly toxic to S-phase cells [13, 19, 20], in our current study the sensitivity of the cell lines to 24-h CPT treatment was not well correlated with the doubling time of the cells (Table 1). The most sensitive cell line, MDA-MB-157, has an intermediate doubling time of 36 h, whereas the more resistant cell lines have doubling times of 24 h and 48 h for MDA-MB-231 and GI 101 A, respectively.

While it is true that the GI 101A cells have the longest doubling time and had the greatest resistance to 24-h treatment with LCPT, the MDA-MB-231 cells were nearly as resistant and have the shortest doubling time. Furthermore, the MDA-MB-157 cells were much more sensitive, and yet have an intermediate doubling time. Thus, it seems unlikely that there is a true correlation between the cell doubling time and LCPT activity.

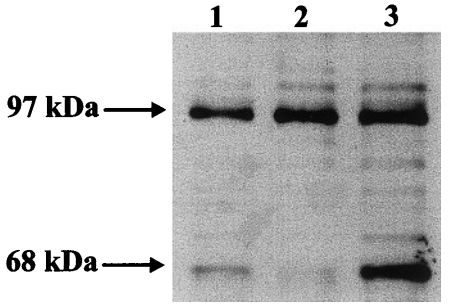

Fig. 3 Topo I western blotting of nuclear extracts prepared from breast cancer cells. Nuclear extract protein (3 μg) was electrophoresed under denaturing conditions, blotted onto nitrocellulose, and probed with a human polyclonal antihuman topo I antibody as described in Materials and methods. Detection was by enhanced chemiluminescence (*Lane 1* MDA-MB-157, *lane 2* MDA-MB-231, *lane 3* GI 101A cells)

Topoisomerase I protein levels and activity

A representative immunoblot of the three cell lines using a human polyclonal antihuman topo I antibody is shown in Fig. 3. Two bands were identified by immunoblotting, the intact M_r 97 000 topo I protein and a M_r 68 000 topo I proteolytic fragment, only in GI 101A cells, as shown previously [12]. Densitometric analysis of both the 68 kDa and 97 kDa bands showed the resistant lines (GI 101A and MDA-MB-231) to contain greater levels of topo I protein compared with MDA-MB-157 cells. No changes in the level of topo I protein were observed in response to 24-h treatment with 50 n*M* CPT (data not shown), indicating that CPT treatment did not cause downregulation of topo I protein levels. Similar observations were also noted when different breast cancer cells were used [14]. The Western blot results agreed with the levels of topo I activity measured by the ability of nuclear extract to relax supercoiled DNA, as shown in Fig. 4. The resistant GI 101A and MDA-MB-231 lines showed about a twofold greater topo I-mediated DNA relaxing activity than the MDA-MB-157 cell line. Approximately

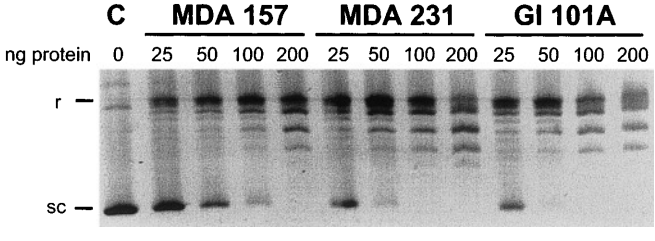

Fig. 4 Measurement of topo I catalytic activity in nuclear extracts of breast cancer cells. The indicated amounts of nuclear extract protein prepared from MDA-MB-157, MDA-MB-231, and GI 101A cells were analyzed for DNA relaxing activity as described in Materials and methods. Increased topo I activity is seen as a loss of the supercoiled substrate DNA (*sc*) to the relaxed DNA (*r*) when the plasmid was incubated with increasing amounts of nuclear extract

100 ng nuclear protein from MDA-MB-157 cells was required to completely deplete the supercoiled plasmid band, while just 50 ng was sufficient for both GI 101A and MDA-MB-231 cells. Although there was a good correlation between topo I enzyme activity and protein level in each cell line, no correlation was found between these parameters and sensitivity to CPT (Fig. 1).

CPT-induced cleavage of DNA oligonucleotides

Nuclear extracts from MDA-MB-157, MDA-MB-231 and GI 101A cells were assayed for DNA cleavage in the presence of CPT (1–100 μ*M*) using a radiolabeled oligonucleotide containing a strong topo I cleavage site, and the results are shown in Fig. 5A. The extent of CPT-stabilized cleavable complex formation for each cell line was well correlated with the other topo I parameters determined. For example, higher intensity cleavage was noted in reactions containing GI 101A extracts than in extracts from other lines (Fig. 5B) and at 10 μ*M* CPT

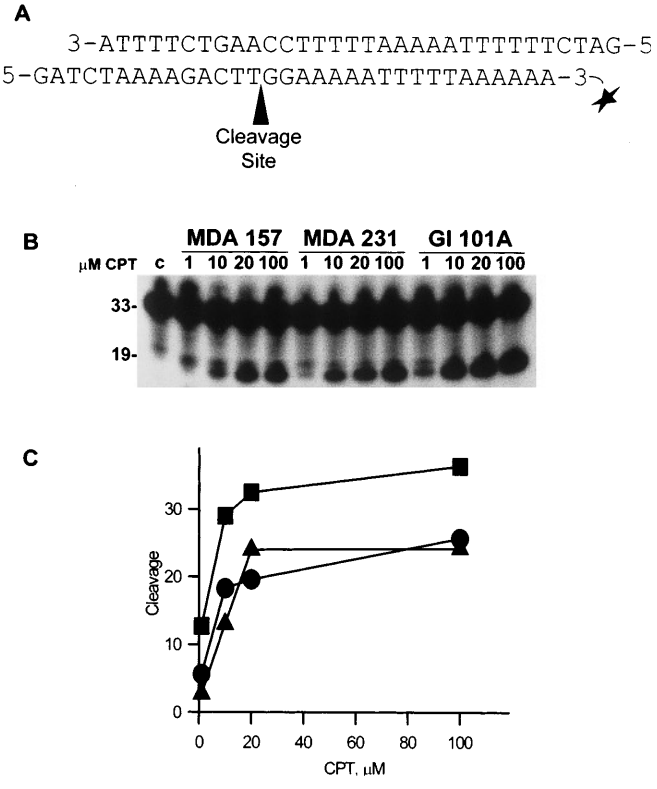

Fig. 5A–C CPT-dependent DNA oligonucleotide cleavage by nuclear extracts from breast cancer cells. The DNA oligonucleotide (33-mer) was labeled with α-[^{32}P] cordycepin at the 3′ end of one strand, incubated with nuclear extract in the presence or absence of CPT, and the cleavage products separated by electrophoresis as described in Materials and methods. **A** cleavage site for topo I (*arrowhead*). **B** Autoradiographic analysis of polyacrylamide gels of nuclear extracts following incubation with the indicated μ*M* CPT. **C** Ratios of the cleaved product to total loaded sample of nuclear extract from MDA-MB-157 (▲), GI 101A (■), and MDA-MB-231(●) cells

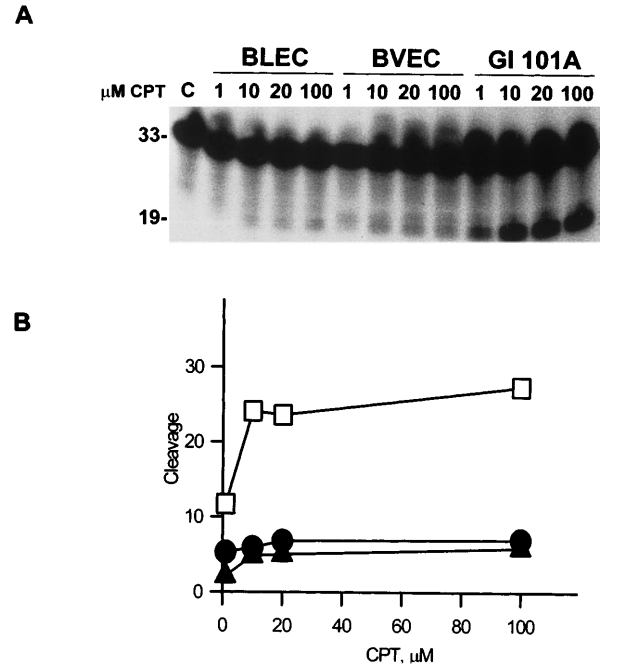

Fig. 6A,B CPT-dependent DNA oligonucleotide cleavage by nuclear extracts from normal endothelial cells and GI 101A human breast cancer cells. The procedure was similar to that described for Fig. 5, except that labeled oligonucleotides were incubated with nuclear extracts from normal bovine endothelial cells and GI 101A tumor cells. **A** Autoradiographic analysis of polyacrylamide gels of nuclear extracts following incubation with and without CPT. **B** Ratios of the cleaved product to total loaded sample of nuclear extract from BLEC (▲), BVEC (●) and GI 101A (□) cells

the level of cleavage was about twofold higher than that noted in the reactions containing MDA-MB-157 nuclear extract (Fig. 5C). No significant changes in the cleavage products were detectable with the use of much higher concentrations (100 μ*M*) of CPT. In contrast, lower intensity cleavage was noted in reactions containing nuclear extracts from BVEC and BLEC than in reactions containing GI 101A extracts (Fig. 6). The topo I enzyme in extracts obtained from the normal endothelial cells was highly resistant to CPT-induced cleavage even when much higher CPT concentrations were used. Similar observations have been noted when SDS-KCl coprecipitation assays have been used [14].

Cell cycle analysis of human malignancies and normal bovine endothelial cells

The results from flow cytometric analyses performed on the three human malignant cell lines and on normal bovine endothelial cell lines are shown in Fig. 7. The data have been normalized to control values to correct for possible variations in the distribution of cell population over time. Treatment of cells with low doses of CPT (5 n*M*) for 24 h resulted in the accumulation of the resistant MDA-MB-231 and GI 101A cells in G_2/M phase from 6 to 24 h following the initiation of the

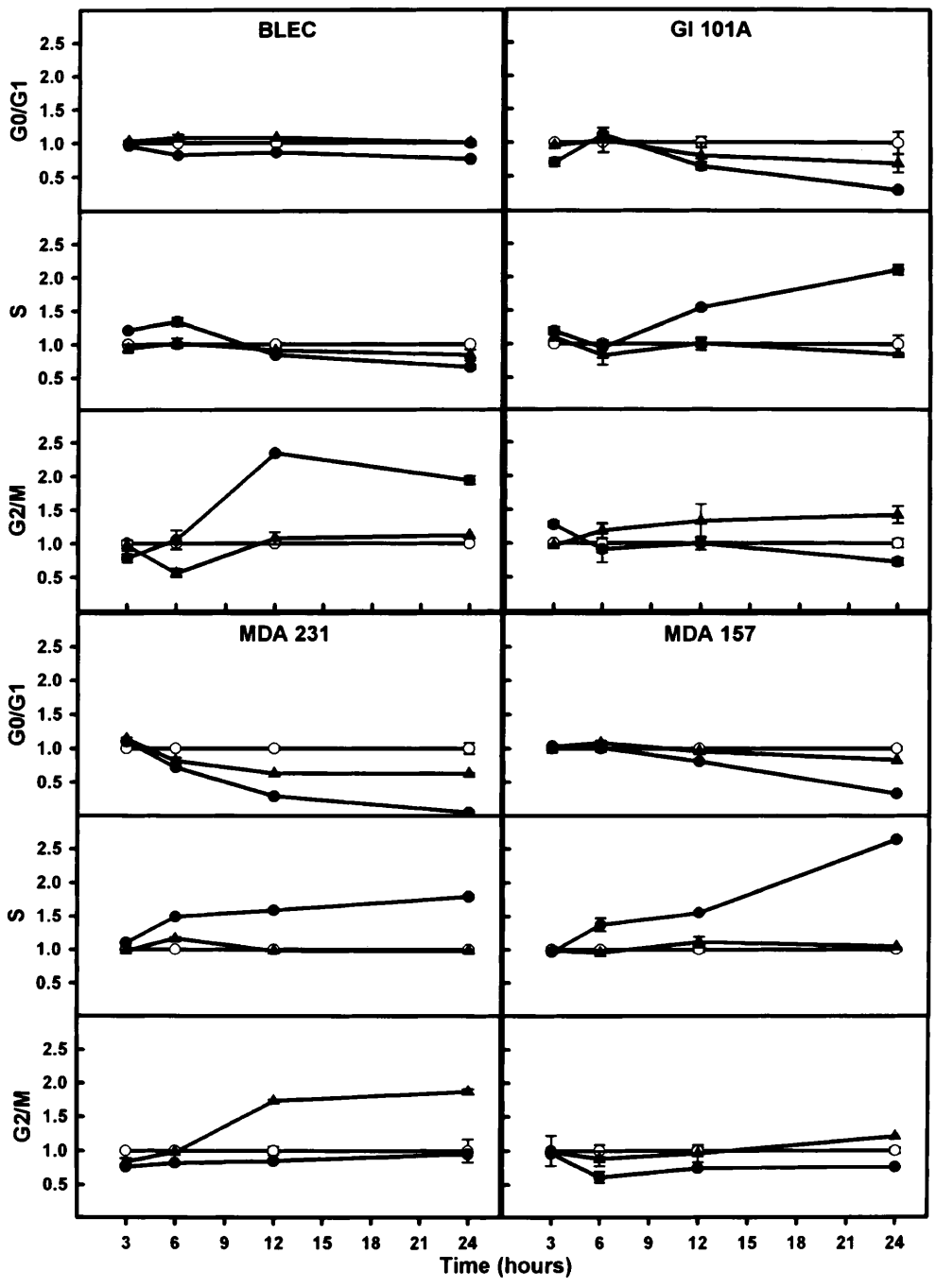

Fig. 7 Cell cycle analysis of normal bovine lymphatic endothelial and breast cancer cells following incubation with CPT for 24 h. Exponentially growing cultures of normal endothelial and malignant cells were incubated with various concentrations of CPT and analyzed for DNA content with a Becton-Dickinson fluorescence-activated cell analyzer using SFIT analysis programs provided by the manufacturer, as described in Materials and methods. The cultures received no treatment (○) or were treated with 5 n*M* (▲) or 50 n*M* (●) CPT for the indicated times. The results were normalized to control values of cells at each time-point and represent the mean of two or three determinations ± SE

treatment. At higher doses (50 n*M*), however, these cells accumulated in S phase in about the same time frame. In contrast, the sensitive MDA-MB-157 cell line showed no G_2/M phase arrest at the low dose, but accumulated in S phase with the 50 n*M* CPT treatment. Although, 5 n*M* CPT was a lethal dose to the MDA-MB-157 cells, as shown in Fig. 1A, no apparent accumulation of the cells in the S phase at this dose was detected. The bovine endothelial cells showed little cell cycle arrest at the low dose (5 n*M*), and accumulated slightly in G_2/M phase at the higher dose (50 n*M*). There was no S phase accumulation seen in the endothelial cells.

The results of the topo I-related experiments and the cell cycle analysis are summarized in Table 2. The cell cycle response of venular endothelial cells was essentially the same as that of the lymph endothelial cells and is included in the summary but not in Fig. 7.

Discussion

Topo I is an abundant nuclear enzyme that is receiving increasing attention as the target for CPT antineoplastic agents. The enzyme is present in all somatic cells, where

Table 2 Summary of topo I-related parameters and cell cycle response to CPT (*ND* not determined)

Cell line	Topo I protein[a]	Topo I activity[b]	Oligo cleavage[c]	Cell cycle response to CPT[d]					
				5 n*M* CPT			50 n*M* CPT		
				G_0/G1	S	G_2/M	G_0/G_1	S	G_2/M
MDA 157	+ +	+ +	+ +	↔	↔	↔	↓	↑	↔
MDA 231	+ + +	+ + +	+ + +	↓	↔	↑	↓↓	↑↑	↔
GI 101 A	+ + + +	+ + +	+ + +	↓	↔	↑	↓	↑	↔
BLEC	ND	ND	±	↔	↔	↔	↓	↔	↑
BVEC	ND	ND	+	↔[e]	↔	↔	↓	↔	↑

[a]Determined by western blot as described in Materials and methods
[b]Determined by plasmid relaxation assay as described in Materials and methods
[c]Determined by DNA oligonucleotide cleavage assay as described in Materials and methods
[d]Determined after 24 h CPT treatment relative to controls; ↔ no change, ↑ increased percentage, ↓ decreased percentage
[e]BVEC data not shown in Fig. 7

it plays a major role in relieving the torsional strain that accumulates as a consequence of replication and transcription (reviewed in reference 21). Although there is no sequence similarity between the prokaryotic and eukaryotic topo I genes [22], there is a strong homology among the eukaryotic enzymes, including yeast and humans [23–25]. Unlike topo II (a DNA unwinding enzyme involved in replication), topo I transcript and protein levels do not change as cells progress through their cell cycle, although enzyme activity increases slightly as cells enter S phase [26]. Higher levels of topo I have been reported in neoplastic than in normal cells [27–29] suggesting that this enzyme could provide an attractive target for selective cytotoxic activity in tumor cells. Our recent studies [14] indicate that a human mammary carcinoma cell line is about 200-fold more sensitive to CPT cytotoxic activity than normal endothelial cells when tested in vitro. Furthermore, LCPT displays effective antitumor activity with minimal host toxicity in a nude mouse model xenografted with the same human mammary carcinoma cells [8]. Thus in this study, the sensitivity to CPT was further investigated in three different ER^- human breast carcinoma cell lines not selected in vitro for drug resistance, and their response to CPT treatment was compared with that of normal bovine endothelial cells. The role of the endothelial cell compartment in tumor vasculature is known to be vital for the establishment, growth and metastasis of solid tumors such as breast cancer [30–32]. It is therefore of interest to consider the sensitivity of these normal cells compared with neoplastic cells to topo I inhibitors.

The present study indicates that neither topo I protein levels nor its catalytic activity in the presence or absence of CPT were indicative of the cytotoxic activity of CPT on the three breast cell lines studied. The ability of mammary tumor cells and/or endothelial cells to trigger cell cycle arrest appeared to be the main factor in determining their sensitivity to CPT. Thus, the DNA damage induced by CPT on these cells appeared to be converted in a series of events that led to cell cycle arrest or cell kill. There was no good correlation with the sensitivity of the cells to CPT cytotoxic activity and its antiproliferative effects. The most sensitive cell line, MDA-MB-157, did not stop incorporating radiolabeled thymidine until treated for 24 h with very high doses (500 n*M*) of CPT. In the same time, the calculated IC_{50} value (7 n*M*) as measured by the MTT dye assay was about 70 times less. In contrast, the more resistant MDA-MB-231 and GI 101A cell lines appeared to halt the synthesis of DNA at a very low dose of CPT, as indicated in Fig. 2. These results indicate that low doses of CPT (< 10 n*M*) induce inhibition of cell proliferation and DNA synthesis without affecting the metabolic activity and viability of the cells. It is possible that these cells are able to pause their DNA synthesis and reverse the cleavable complexes formed by CPT and topo I/DNA before the collision of a replication fork causes a double-strand break at the lesion site. The cleavable complexes produced by CPT have previously been shown to be rapidly reversible upon the removal of CPT [33, 34]. Consistent with this proposal, O'Conner et al. [35] have also observed that inhibition of DNA synthesis in human colon carcinoma HT-29 cells treated with CPT is much more protracted (up to 6 h) than CPT-induced single-strand break reversal (15 min). By pausing the synthesis of DNA, the resistant cells are able to avoid the lethal effects of CPT.

The incorporation of CPT into liposomes did not seem to greatly alter the activity of the drug when cells were treated for 24 h in culture, as indicated in Fig. 1B. The small loss in potency observed with the treatment of GI 101A cells was attenuated in comparison with the "free" drug when cells were treated for 72 h, with either form (data not shown). This is basically due to the sustained release nature of lipophilic drugs incorporated into liposomes [17].

Immunoblot analysis of the topo I protein in the nuclear extract of the three cell lines (Fig. 3) showed the presence of the native 97 kDa protein in addition to the proteolytic 68 kDa fragment only in the GI 101A cells. However, the topo I protein levels in the sensitive MDA-MB-157 cell line were much lower than those of the more resistant MDA-MB-231 and GI 101A cell lines, indicating no correlation between the sensitivity of tumor cells to topo I inhibitors and their topo I protein

levels. In this sense, our results agree with those of Perego et al. [36] who also found that the level of topo I expression is not the only critical determinant of cell sensitivity to CPT in unselected human cancer cell lines of different tumor types. Nevertheless, a good correlation was found between the level of topo I immunoprotein and topo I enzymatic activity of the cells, as shown in Fig. 4. These results are in agreement with those of Husain et al. [29] and Bronstein et al. [37] who also found that tumors with increased topo I activity levels contain increased amounts of topo I immunoprotein.

Topo I enzymatic activity also correlated well with the dose-dependent CPT-induced cleavage of DNA oligonucleotides (Fig. 5), although the degree of cytotoxic response seen in these cells (Fig. 1A) was the inverse of what would be expected from the results of our oligonucleotide cleavage assays, suggesting that critical factors besides topo I catalytic activity and CPT-induced DNA cleavage are more important for tumor sensitivity to CPT-like drugs. Cell cycle regulation of tumor cells, for example, could play a key role in triggering cell death by topo inhibitors as well as other damaging agents. Recent studies by Dubrez et al. [38] have shown that the ability of human leukemic cells to regulate cell cycle progression and to trigger cell death (apoptosis) is more indicative of their sensitivity to topo poisons than cleavable complexes induced by these drugs. The cell cycle phases that are most sensitive to topo inhibitors have been shown to be S and G_2 phases while these agents are less cytotoxic in resting cells [13, 39]. In the present study, the cell proliferation status and accumulation of the cells in the G_2 phase of the cell cycle, appeared to be essential determinants of the cells' sensitivity to CPT. Treatment of cells for 24 h with sublethal doses of CPT (5 n*M*) caused an accumulation of the resistant cells (MDA-MB-231 and GI 101A) and the endothelial cells in the G_2 phase of the cell cycle.

The G_2 phase provides a safety gap, allowing the cells to ensure that DNA replication is completed before entering into mitosis [40]. Delay in progression through the cell cycle allows repair of damage before cells enter the S or M phase, when damage would be perpetuated and would result in genomic instability and loss of cell function. Thus the G_2 arrest in these cells is consistent with the recent report by Pantazis et al. [41] who showed that treatment with 9-nitrocamptothecin leads to accumulation of nontumorigenic breast epithelial cells in G_2 phase and the death of a small proportion of cells by apoptosis. Thus resistant cells and normal endothelial cells treated with sublethal doses of CPT undergo G_2 arrest simply to avoid CPT lethal damage. In contrast, the sensitive MDA-MB-157 cells were not arrested in G_2 when treated with the same doses of CPT (Fig. 7). It is possible that these cells are deficient in their ability to recognize the damage to their DNA and do not trigger any repair as a consequence. The lack of G_2 arrest may simply be a visible effect of impaired DNA damage recognition.

The major protein regulating the G_2/M checkpoint is $p34^{cdc2}$ (reviewed in reference 42). Prior to the mitotic phase, the cdc25 phosphatase dephosphorylates and activates $p34^{cdc2}$. Recent studies have indicated that CPT can inhibit the activation of $p34^{cdc2}$ protein in HeLa cells without affecting the regulatory function of the cdc25 protein [43]. Thus it is possible that other gene products may play a critical role in G_2 arrest induced by sublethal doses of CPT in normal and tumor cells. This possibility as well as the therapeutic significance of G_2 arrest in tumor cells compared to normal cells, are possible areas for future investigation.

In summary, the present study indicates that neither topo I levels nor CPT-stabilized cleavable complexes are indicative of the cytotoxic activity of CPT in three human breast carcinoma cells compared to the normal endothelial cells. Instead, several events following the initial damage induced by CPT determine possible sensitivity of cells to the drug action. Specifically, it seems that the ability to cease DNA synthesis and to arrest at the G_2/M phase checkpoint are critical for the survival of the cell following CPT treatment.

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