

Elevated Topoisomerase I Activity in Cervical Cancer as a Target for Chemoradiation Therapy

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Objective. The aim of this study was to determine whether the activity of topoisomerase I (topo I), the target of the anti-neoplastic drug camptothecin (CPT), is elevated in cervical cancer and whether CPT can radiosensitize cervical tumors.

Methods. The topo I activity of 11 normal cervix and 30 cervical carcinoma tumors was assayed by measuring the relaxation of supercoiled DNA. Subconfluent or postconfluent CaSki human cervical carcinoma cells were exposed to CPT (1–5000 ng/ml) and immediately X-irradiated (0–800 cGy). Cell survival was determined by clonogenic assay.

Results. Mean topo I activity in cervical cancer ($3.0 \pm 0.06 \text{ h}^{-1}$) was significantly greater than in normal cervix tissue ($0.29 \pm 0.06 \text{ h}^{-1}$). Stage 3 and 4 cervical carcinoma specimens displayed a trend of greater topo I activity ($5.88 \pm 3.7 \text{ h}^{-1}$) than stage 1 and 2 tumors ($2.57 \pm 0.47 \text{ h}^{-1}$). No correlation between topo I protein levels and catalytic activity was found. Combined treatment of subconfluent CaSki cells with CPT and ionizing radiation resulted in additive killing of cells. Combined treatment of postconfluent CaSki cells with low doses of radiation (200 and 400 cGy) and 1 or 10 ng/ml CPT for 2 or 48 h produced significant cytotoxicity compared to CPT or radiation alone, which were ineffective at these doses.

Conclusions. Topo I activity is elevated in cervical cancer compared to normal cervix. The radiosensitivity of noncycling cells within cervical tumors may be increased by simultaneous treatment with low doses of CPT or other topo I inhibitors. © 2000 Academic Press

Key Words: camptothecin; topoisomerase I; cervical carcinoma; radiation; chemoradiation.

INTRODUCTION

Cervical carcinoma is one of the most common lethal malignancies affecting women, despite the widespread use of cervical cytological screening programs. It is estimated that 12,800 new cases of invasive carcinoma of the cervix and 4600 deaths related to this disease will occur in the United States in 2000 [1]. In Taiwan, although many preinvasive lesions are

detected early and effectively treated, there are still over 2000 new cases of invasive cervical carcinoma and 1000 deaths from this disease every year [2]. These high incidence and mortality rates suggest that improved methods of detection and treatment of cervical carcinoma are needed.

Radiotherapy is the standard, definitive therapy for advanced disease with cure rates ranging from 18 to 75% depending on the extent of disease [3, 4]. Typically, multiple rounds of radiation are given. Although increasing the dose of radiation improves control of disease, the dose that can be delivered is limited by late complications [5]. Methods to reduce the radiation dose with the same or better therapeutic efficacy could improve patient prognosis and quality of life. Concurrent drug and radiation treatment (chemoradiation therapy) is therefore under active investigation for many cancers. Chemoradiation therapy for advanced or recurrent cervical carcinoma has demonstrated promising activity in clinical trials [6, 7].

Camptothecin (CPT)² and related analogs such as topotecan and CPT-11 are currently under investigation for cancer chemotherapy. Topotecan, a water-soluble derivative of CPT, has demonstrated activity against ovarian cancer in clinical trials [8] and is approved by the Food and Drug Administration for the salvage treatment of ovarian cancer [9]. Irinotecan (CPT-11), a CPT prodrug, exhibits activity against refractory cervical carcinoma [10]. The modest activity of CPT analogs against advanced gynecologic tumors, however, suggests that combination therapy will be required to realize the full benefits of these drugs. Recent work has suggested that topoisomerase I (topo I) inhibitors may potentiate the lethal effects of ionizing radiation [11–13]. Combination of CPT with radiotherapy may therefore be an attractive strategy to potentiate the efficacy of both radiotherapy and CPT.

Topo I is a nuclear enzyme that plays an important role in DNA replication and RNA transcription [14]. Topo I generates single-strand breaks in DNA to allow the relaxation of tor-

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² Abbreviations used: ADPRT, poly(ADP-ribose)-transferase (EC 2.4.2.30); CPT, camptothecin; IC₉₀, concentration of drug causing 90% cell death; PBS, phosphate-buffered saline (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄); topo I, topoisomerase I.

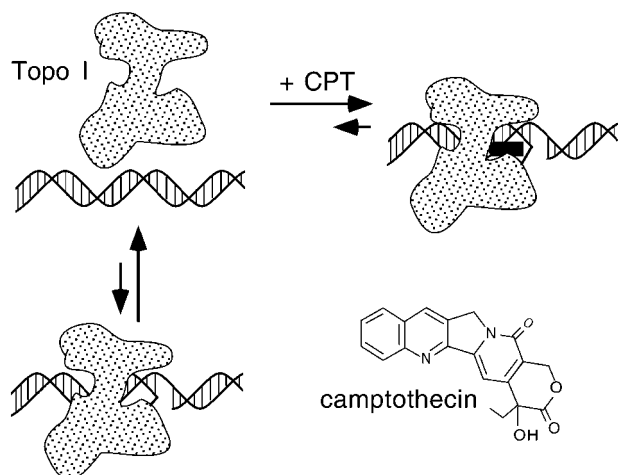


FIG. 1. Camptothecin forms a ternary complex with topo I and DNA. During DNA relaxation, topo I forms a covalent bond to the 3' phosphoryl end of the newly formed single-strand break in DNA. Camptothecin stabilizes the normally transient DNA-topo I complex, preventing religation of the broken DNA strand.

sional stresses generated during DNA transcription and replication [15]. The crystal structure of human topo I with duplex DNA indicates that topo I completely wraps around B-form DNA, allowing nucleophilic cleavage of one DNA strand and subsequent covalent attachment of the hydroxyl group of tyrosine 723 to the 3' phosphoryl end of the broken DNA strand [16, 17]. Torsional stress in supercoiled DNA is then relieved in a process termed "controlled rotation" before the DNA strand is religated and topo I is released [17]. Camptothecin, a natural plant alkaloid isolated from *Camptotheca acuminata* [18], stabilizes the normally transient complex formed between topo I and nicked DNA, preventing the release of topo I and religation of the broken DNA strand [19] (Fig. 1). Interaction of the stabilized tertiary complex and the replication fork is thought to result in double-strand DNA breaks and cell death [20].

We have previously shown that camptothecin can potentiate the effects of radioimmunoconjugate therapy in a rat hepatoma ascites model [21]. In the present study, we examined whether CPT could potentiate the lethal effects of ionizing radiation to human cervical carcinoma cells. Cellular sensitivity to CPT appears to correlate with topo I catalytic activity [22, 23]. We therefore also measured topo I activity in human cervical normal and tumor samples.

MATERIALS AND METHODS

Reagents and cells. Camptothecin and common biological reagents were purchased from Sigma Chemical Company (St. Louis, MO). Immunochemicals were purchased from Organon Teknika (Turnhout, Belgium). Camptothecin stock solutions were made in dimethylsulfoxide at 2.5 mg/ml, sterilized by

filtration, and stored at -70°C . *Spodoptera frugiperda* cells were purchased from Pharmingen (San Diego, CA) and cultured according to the supplier's instructions. The CaSki human cervical carcinoma cell line was kindly provided by Dr. R. A. Pattillo, Medical College of Wisconsin, Milwaukee. HS 1025 and HS 1023 cervical carcinoma and H2669 melanoma cell lines were provided by Dr. Hellstrom, University of Washington, Seattle. TSGH 8302 cervical carcinoma cells were obtained from the Cancer Research Laboratory, Department of Medical Research, Tri-Service General Hospital, Taipei, Taiwan. HA22T human hepatocellular carcinoma cells were provided by Dr. C. P. Hu, Veteran's General Hospital, Taipei, Taiwan. All other cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI 1640 medium supplemented with 2.98 g/L HEPES, 2 g/L NaHCO_3 , 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 5% heat-inactivated bovine serum (culture medium).

Tissues. Samples of cervical carcinoma and normal cervix were obtained from patients who underwent primary surgery at Tri-Service General Hospital, Taipei, Taiwan. Care was taken to ensure that normal and tumor components of samples were separated during tumor dissection. All specimens were immediately frozen in liquid nitrogen and stored at -135°C . The pathology of all tissues was microscopically confirmed by a pathologist.

Nuclear extracts. Cells or finely diced tissues samples were washed once each with ice-cold PBS and nuclear buffer (150 mM NaCl, 1 mM KH_2PO_4 , 5 mM MgCl_2 , 1 mM EGTA, 0.2 mM dithiothreitol, 10% (v/v) glycerol, and 0.1 mM PMSF, pH 6.4). Tissues or cells were suspended in nuclear buffer containing 0.3% Triton X-100 and disrupted in a mechanical dounce homogenizer on ice. Nuclei, collected by centrifugation at 1000g for 10 min, were washed once with nuclear buffer before the addition of nuclear buffer containing 0.25 M NaCl. Nuclei were gently rotated for 30 min at 4°C , centrifuged at 16,000g for 30 min to remove debris, and immediately assayed for topo I activity or stored at -135°C . At least two independent nuclear extracts were prepared for each sample.

Topo I relaxation assay. Supercoiled pBR322 plasmid was purified from bacterial cultures by alkaline lysis followed by equilibration centrifugation in a cesium chloride-ethidium bromide continuous gradient for 36 h. Aliquots of supercoiled DNA were stored in absolute ethanol at -80°C . To assay topo I activity, 1.0 μg supercoiled pBR322 was preheated to 37°C in 15 μL of reaction buffer (10 mM Tris-HCl, pH 7.5, 200 mM KCl, 10 mM MgCl_2 , 1 mM EDTA, 1 mg/ml BSA, and 1 mM dithiothreitol) before the addition of 5 μL nuclear extract diluted in reaction buffer. The reaction was terminated after 30 min by addition of 2 μL of 10% SDS. Two microliters of loading dye (40% sucrose, 0.025% bromophenol blue, and 50 mM EDTA) was added and samples were electrophoresed at 3 V/cm for 5 h in a 1% TPE agarose gel containing 0.03% SDS

to separate relaxed and supercoiled DNA. Gels were extensively washed with water to remove SDS and stained with ethidium bromide. Gel images were captured on an Eagle eye under UV illumination. The supercoiled DNA band was quantified on a Macintosh computer using the public domain NIH image program (developed at NIH and available at <http://rsb.info.nih.gov/nih-image/>). Topo I activity was calculated as the number of micrograms pBR322 relaxed per microgram of nuclear extract per hour and has units of h^{-1} . Serial dilutions of each extract were assayed and only bands containing between 10 and 90% relaxed pBR322 DNA were used to calculate topo I activity. Each extract was assayed two to three times and the mean topo I activity was calculated.

Production of recombinant human topo I. The 3645-base *EcoRI* cDNA fragment of human topo I present in a pUC9 plasmid (T1B, generously provided by Dr. Alastair Mackay, Department of Cell Biology and Anatomy, JHU Medical School, Baltimore, MD) was subcloned into the unique *EcoRI* site of the baculovirus transfer vector pVL1393 (PharMingen) under the control of the polyhedrin promoter. A single recombinant baculovirus, produced in Sf21 cells according to the supplier's instructions (PharMingen), was selected for production of human topo I. Sf21 cells were infected with virus at a MOI of 10 and cultured for 4 days at 27°C. Nuclear extracts were prepared from the cells and topo I was purified on Hitrap heparin and phenyl Sepharose CL-4B columns (Pharmacia) as described [24] to yield a single protein band on SDS-PAGE. Purified topo I was concentrated to about 200 $\mu\text{g}/\text{ml}$ and stored at -80°C . Recombinant topo I was active as assessed by its ability to relax supercoiled DNA.

Generation of anti-topo I antibodies. A female rabbit was sc injected with 150 μg of recombinant human topo I in complete Freund's adjuvant and boosted at 1-month intervals with 100 μg of topo I in incomplete adjuvant. Blood was collected from the marginal ear vein 2 weeks after a final boost. Serum was collected and stored at -80°C .

Immunoblots. Nuclear extracts were diluted in nucleus buffer to known concentrations and electrophoresed on a 3–12.5% gradient SDS-PAGE under reducing conditions. Proteins were transferred to nitrocellulose paper by passive diffusion as described [25]. Dot blots were prepared by spotting 10 μL of nuclear extract samples on nitrocellulose paper. Blots were blocked with 5% skim milk in PBS and sequentially probed with rabbit anti-topo I serum (1:2000) and horseradish-peroxidase-conjugated goat anti-rabbit Ig (1:5000). Antibody binding was visualized by ECL detection according to the manufacturer's instructions (Pierce, Rockford, IL).

Clonogenic assays. CPT cytotoxicity against subconfluent cells was determined in six-well plates by adding CPT for various periods to 150–15,000 cells in culture medium. The cells were then washed twice with PBS to remove free drug and cultured for 6 days in clonogenic medium (culture medium supplemented with 5% conditioned medium, prepared from

confluent cultures of the corresponding cell line). For postconfluent studies, cells were cultured in six-well plates until confluence was reached. The cells were then cultured for 1 week in medium containing 0.1% bovine serum. Three-fourths of the culture medium was refreshed daily. Under these conditions, greater than 95% of the cells were in the G_0/G_1 phase of the cell cycle as determined by propidium iodide staining of DNA followed by flow cytometer analysis. After cells were exposed to CPT for 2 or 48 h in medium containing 0.1% bovine serum, the cells were washed twice with PBS and 100 to 10,000 cells were cultured in six-well culture plates in clonogenic medium for 6 days.

For radiation studies, CaSki cells were irradiated in a Torrex 150D X-ray source (EG&G Astrophysics Research Corp., Long Beach, CA) at a rate of 160 cGy/min. Preconfluent CaSki cells were cultured in clonogenic medium for 6 days whereas postconfluent cells were plated in clonogenic medium at known cell concentrations 2 or 48 h after irradiation and cultured for 8 days. For combination studies, CPT was added immediately before X-ray irradiation. After 2 or 48 h, the cells were washed twice with PBS and incubated in clonogenic medium for 8 days. In some experiments, cells were exposed to 5 mM 3-aminobenzamide for 1 h before the addition of CPT and irradiation of the cells. Colonies were fixed and stained with 0.5% methylene blue in 50% ethanol/50% water (vol/vol) and visually counted under a microscope. Pre- and postconfluent CaSki cells had a plating efficiency of 40–50% under these conditions. Cell survival is expressed as the percentage of survival relative to control cells that were not exposed to CPT or radiation.

Isobolograms. Analysis of the effects of combination treatment in exponentially growing cells was performed by constructing isoeffect curves (isobolograms) as described by Steel and Peckham [26]. Mode I isoeffect curves were constructed by reading the doses of radiation or camptothecin from the respective single-agent dose–response curves, starting at zero dose of each agent, that added up to 20% cell survival. Mode II curves were formed in the same way except that the steepest portion of the radiation dose–response curve was used in the construction. Combination treatment results were plotted on the isobologram by interpolation of the combined dose–response curves at 20% cell survival. Combinations resulting in points falling in the region to the left of the isoeffect curves correspond to positive or supra-additive interactions between the combined agents, points to the right of the curves represent subadditive effects, and combined doses falling in the region between the mode I and mode II curves are attributed to additive interactions of the single agents [26]. Combination treatment of postconfluent cultures was analyzed by comparing the survival of cells treated with CPT and radiation with the survival of radiation-treated cells after subtracting the cell death caused by CPT alone.

TABLE 1
Topoisomerase I Activities in Normal Cervix and Cervical Tumor Tissues

Tissue	Topo I activity (h^{-1}) ^a	Number of samples
Normal cervix	0.29 ± 0.06	11
Cervical cancer	3.0 ± 0.62^b	30
Cervical cancer (stages 1–2)	2.57 ± 0.47	26
Cervical cancer (stages 3–4)	5.88 ± 3.7	4

^a Activity is expressed as micrograms of supercoiled DNA relaxed per microgram of nuclear extract in 1 h.

^b Significantly greater ($P \leq 0.0005$) than normal cervix.

Statistical analysis. Statistical significance of differences between mean values was calculated with the shareware program Schoolstat (White Ant Occasional Publishing, West Melbourne, Australia) using the independent *t* test for unequal variances.

RESULTS

Topoisomerase I Activity

The topo I activity of nuclear extracts prepared from normal and tumor specimens was assayed by measuring the relaxation of supercoiled DNA. Densitometer quantitation of supercoiled DNA band intensity after electrophoresis of DNA on agarose gels allowed an estimation of topo I activity. Under the conditions employed in the assay, the intensity of pBR322 DNA standards was linearly related to DNA concentration from 0 to 1 μg (data not shown). A range of concentrations was assayed for each nuclear extract. The addition of increasing amounts of nuclear extract produced increased relaxation of supercoiled pBR322 DNA to the fully relaxed form. Only band intensities corresponding to between 10 and 90% DNA relaxation were employed for calculation of topo I activity to prevent saturation of the assay and increase the assay precision. Table 1 summarizes the mean topo I activities measured in cervical specimens. The mean topo I activity in cervical carcinoma tumors was significantly ($P \leq 0.0005$) greater than in normal cervix tissue. Topo I activity was higher in advanced (stages 3 and 4) compared to early (stages 1 and 2) cervical carcinomas, but the difference was not significant. Topo I activity varied widely between individual samples of cervical tumors (Fig. 2). Twenty-two of 30 cervical carcinomas (73%) displayed topo I activities greater than three standard deviations above the mean values of topo I activity in normal cervical tissue.

To examine topo I protein levels, polyclonal antibodies were generated against recombinant human topo I. Anti-topo I anti-serum bound to topo I present in nuclear extracts prepared from CaSki cervical carcinoma cells (Fig. 3). The lack of additional bands present in the crude nuclear extracts on the immunoblot demonstrates that the anti-serum was specific for topo I. Figure 4 shows that topo I protein levels in nuclear extracts did not

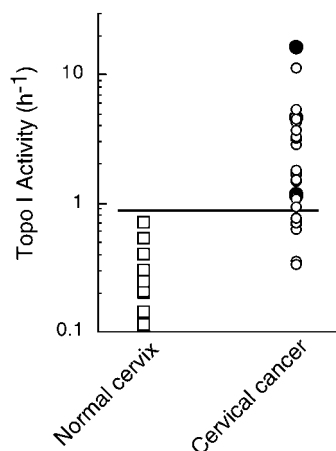


FIG. 2. Topo I activity in clinical specimens. Topo I activities in surgical specimens were assayed as described under Materials and Methods. Topo I activity corresponding to three standard deviations above the mean activity of normal cervix samples is indicated by a horizontal bar. Stage 1 and 2 cancers are shown as open symbols whereas stage 3 and 4 cancers are represented with solid symbols.

correlate with topo I activity. For example, a cervical carcinoma sample with a topo I activity of 11.4 h^{-1} contained less topo I protein than a normal cervix sample with a topo I activity of 0.3 h^{-1} (Fig. 4A, sample 64 versus sample 284). Similar results were also found for ovarian carcinoma 1211, which possessed less topo I protein than normal cervix samples 284 and 143, even though it displayed 5- to 10-fold higher topo I activity (Fig. 4A). Immunoblots were reproducible, as shown by similar levels of topo I protein detected in replicate samples of several samples (Fig. 4B).

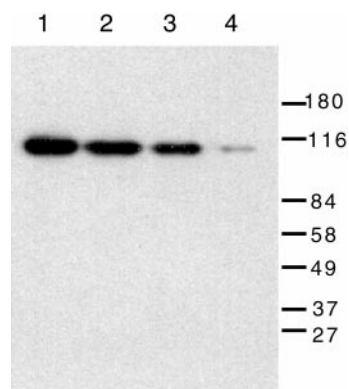


FIG. 3. Characterization of anti-topo I antibody specificity. Nuclear extract prepared from CaSki human cervical carcinoma cells was electrophoresed on a 3–12.5% gradient polyacrylamide gel, transferred to nitrocellulose paper, and immunoblotted with rabbit anti-topo I serum. The blot was incubated with horseradish-peroxidase-conjugated goat anti-rabbit IgG and specific bands were visualized by ECL detection. Lane 1, 20 μg extract; lane 2, 15 μg extract, lane 3, 10 μg extract, lane 4, 5 μg extract. Molecular mass in kilodaltons is indicated.

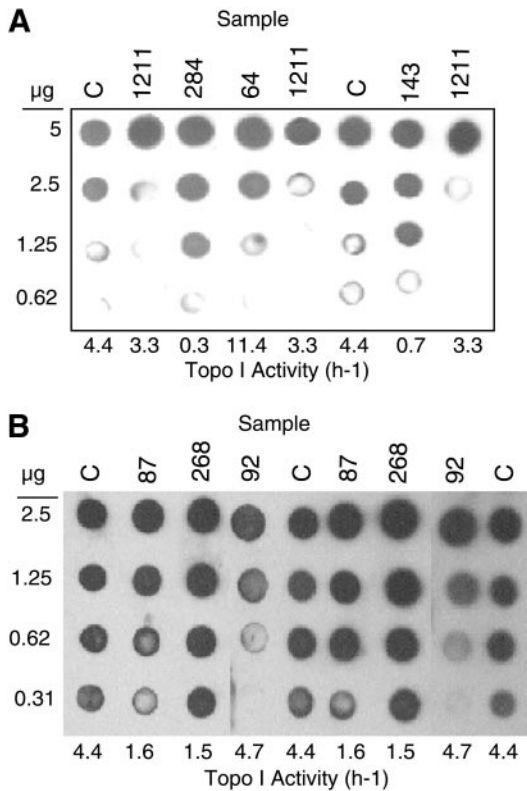


FIG. 4. Immunoblot of topo I protein levels in nuclear extracts. Nuclear extracts containing the indicated amounts of protein were spotted on nitrocellulose paper and probed with rabbit anti-topo I serum followed by horseradish-peroxidase-conjugated goat anti-rabbit IgG. Antibody binding was visualized by ECL detection. C, CaSki cells; 1211, ovarian adenocarcinoma specimen; 284, normal cervix specimen; 64, squamous cervical carcinoma specimen; 143, normal cervix specimen; 87, normal ovary specimen; 268, normal cervix specimen; 92, squamous cervical carcinoma specimen. The topo I activities of each nuclear extract are indicated below each lane.

Toxicity of Single Agents

Exponentially growing CaSki cells were killed by ionizing radiation in a dose-dependent manner with an IC_{90} value of 790 cGy (Fig. 5A). Postconfluent CaSki cells were more resistant to irradiation; confluent cells plated 2 or 48 h after irradiation displayed IC_{90} values of 1460 and 1580 cGy, respectively. The toxicity of CPT to CaSki cervical carcinoma cells was strongly dose and time dependent (Fig. 5B). The IC_{90} value for exponentially growing CaSki cells exposed to CPT for 2 h was 15.8 μ M, whereas 90% of cells were killed by 8.3 nM CPT after 48 h. Postconfluent CaSki cells were resistant to CPT with an IC_{90} value greater than 3.4 μ M after 48 h. Other subconfluent cervical carcinoma cell lines as well as HT29 colorectal carcinoma cells exhibited similar IC_{90} values as CaSki cells after 2 or 48 h of exposure to CPT (Table 2).

Combination CPT and Radiation Treatment

Combination treatment of exponentially growing CaSki cells with CPT and radiation was performed by adding CPT to

cells immediately before X-ray irradiation. Figure 6A shows that irradiation of CaSki cells in the presence of CPT for 48 h resulted in cytotoxicity that was within the envelope formed by type I and type II isoeffect curves on a 20% survival isobologram, indicating that combined CPT and irradiation produced additive killing of subconfluent CaSki cells. Similarly, irradiation and 2-h exposure to CPT also produced additive killing of CaSki cells (Fig. 6B). To examine whether inhibition of ADP-ribosylation could potentiate the combined effects of radiation and CPT, an ADPRT inhibitor was added 1 h before irradiation and CPT treatment of CaSki cells. In the presence of 5 mM 3-aminobenzamide, radiation and CPT caused supra-additive killing of CaSki cells (Fig. 6B). Doses of less than 10 mM 3-aminobenzamide were nontoxic to CaSki cells (results not shown).

In contrast to exponentially growing cells, the cytotoxic

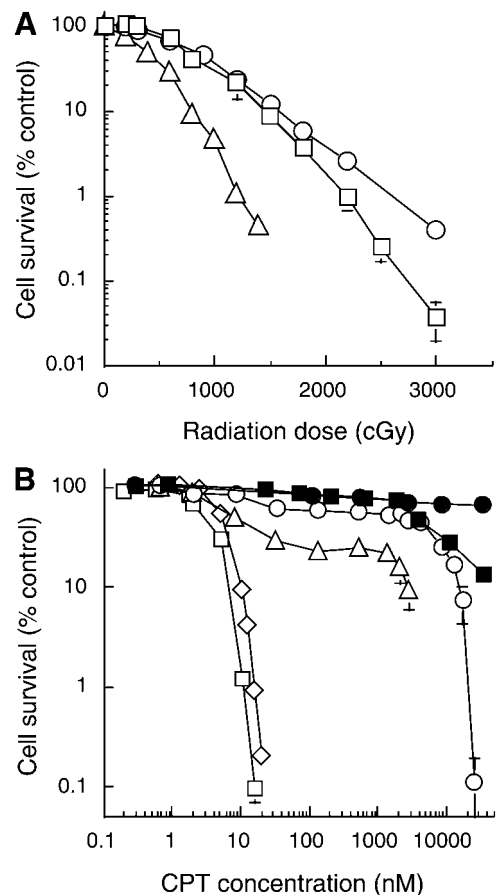


FIG. 5. Toxicity of single agents to CaSki cells. (A) Subconfluent CaSki cells (Δ) were irradiated and cultured in the same plate for 6 days whereas postconfluent CaSki cells were replated 2 (\square) or 48 h (\circ) after irradiation. Clonogenic survival was determined 6 days later. (B) Subconfluent CaSki cells were exposed to camptothecin for 2 (\circ), 8 (Δ), 24 (\diamond) or 48 (\square) h. Postconfluent CaSki cells were exposed to camptothecin for 2 (\bullet) or 48 (\blacksquare) h before the cells were replated in fresh medium. Clonogenic survival was determined 6 days later. Results are expressed as the percentage of cell survival compared to untreated cells and represent mean values of triplicate determinations. Bars, SE.

TABLE 2
Sensitivity of Human Tumor Cells to Camptothecin

Cell line	Tumor type	IC ₉₀	
		2 h (μM)	48 h (nM)
HT29	Colorectal	2.9	5.7
CaSki	Cervical	1.6	8.3
HS1023	Cervical	2.8	7.2
HS1025	Cervical	0.75	7.5
ME180	Cervical	3.7	12
SiHa	Cervical	2.4	6.3
TSGH 8302	Cervical	ND	8.0
HA22T	Hepatocellular	ND	18
H2669	Melanoma	ND	13
CaSki (postconfluent)	Cervical	>3.4	>34000

Note. The IC₉₀ values of preconfluent tumor cells exposed to CPT for 2 or 48 h are shown.

effect of ionizing radiation on postconfluent CaSki cells was significantly augmented by CPT. Figure 7A shows that combination treatment with 200 or 400 cGy and 1, 10, or 1000 ng/ml CPT for 2 h produced significantly greater cytotoxicity than the combined effect of irradiation and CPT given alone. Exposure to CPT for 2 h did not, however, significantly potentiate the cytotoxicity produced by irradiation of CaSki cells with 800 cGy. Combined treatment of CaSki cells with irradiation and CPT for 48 h produced significantly more cytotoxicity than the combined effect of the single agents at all radiation and CPT doses examined (Fig. 7B). For example, treatment of postconfluent CaSki cells with either 200 cGy or 10 ng/ml CPT for 2 h did not affect cell viability whereas combined treatment with 200 cGy and 10 ng/ml CPT killed 74% of the tumor cells.

DISCUSSION

Topo I is the sole cellular target of CPT and related analogs such as topotecan and CPT-11. CPT stabilizes the intermediate covalent complex formed between topo I and duplex DNA (Fig. 1), resulting in double-stranded DNA breaks and cell death. Catalytically active topo I must be present in cells for CPT-induced toxicity [22]. Moreover, the sensitivity of tumor cells to CPT is believed to be positively correlated with topo I activity [22, 23, 27]. We therefore examined the activity of topo I in normal and neoplastic cervical specimens to justify the application of CPT for radiation sensitization of this malignancy. Topo I activity was 10-fold higher in cervical tumors compared with their normal counterparts. Although mean topo I activity was significantly elevated in cervical tumors, the activity in individual tumors varied widely, ranging from 0.33 to 16.6 h⁻¹. Tumors displaying low topo I activity may be refractive to CPT. We found that 73% of cervical tumors possessed topo I activity greater than three standard deviations

above the mean topo I activity in normal cervix tissue. Although the absolute topo I activity required for cellular sensitivity to topo I has not been defined, these results suggest that a major portion of gynecologic tumors may be sensitive to CPT. Topo I activity has also been found to be higher in colon (5- to 35-fold) and prostate (2- to 20-fold) but not kidney tumors compared to matched normal counterparts [28]. Relatively high topo I activities were measured in human colon and cervical tumors compared to breast and lung tumors, although the catalytic activity of the corresponding normal tissues was not investigated [29]. Elevated topo I activity has also been reported for squamous cell carcinoma of the head and neck [30] as well as for malignant ovarian tumors [31]. These studies and our results indicate that a wide range of cancers display elevated topo I activities, suggesting that CPT may be effective for these diverse tumors.

An interesting finding of our study was a trend of increased topo I activity in the late stage in cervical carcinoma. The

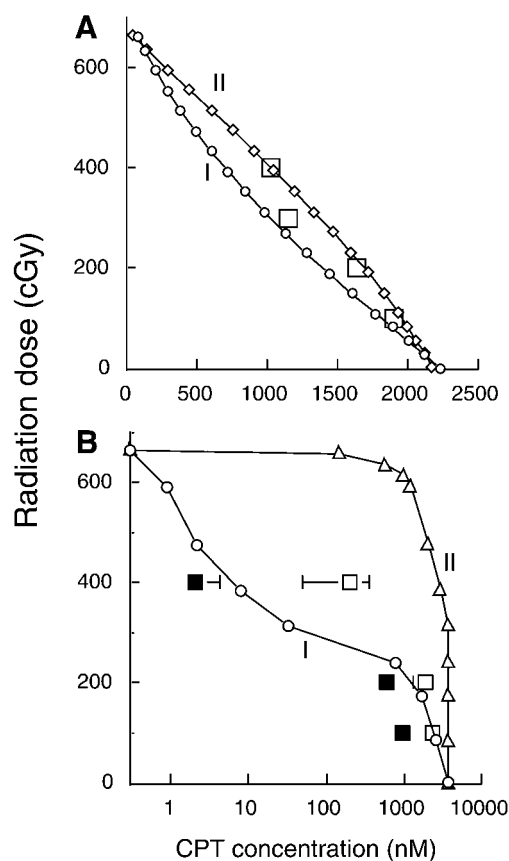


FIG. 6. Isobologram analysis of combination treatment of preconfluent CaSki cells with CPT and radiation. CPT was added to preconfluent CaSki cells for 2 (A) or 48 h (B) immediately before irradiation. Cell survival was determined 8 days later by clonogenic assay. The combined doses of radiation and CPT resulting in 20% survival of CaSki cells are shown (□). The combined doses of radiation and CPT producing 20% cell survival in cells pretreated for 1 h with 5 mM 3-aminobenzamide are also shown (■). Type I (I) and type II (II) isoeffect curves for 20% survival are indicated. Bars, SE.

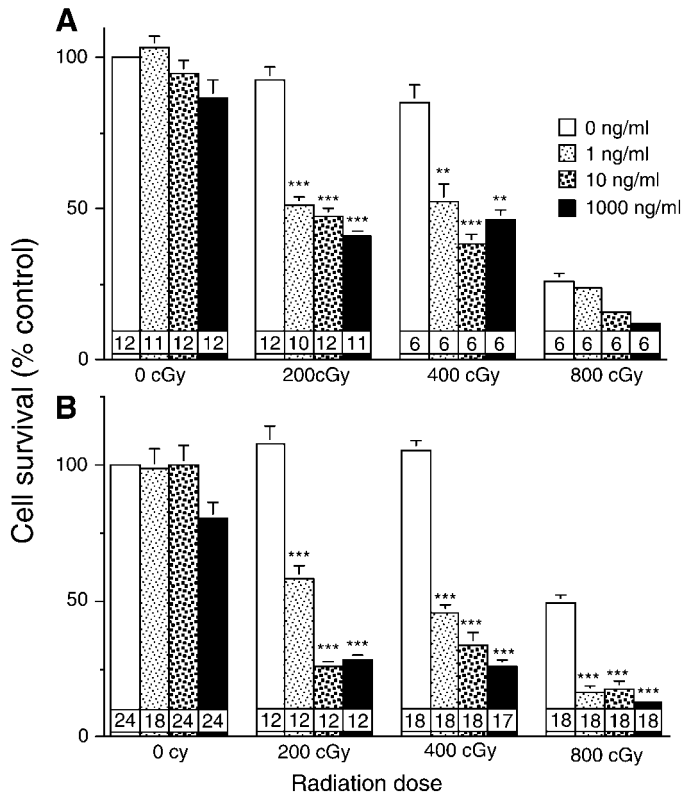


FIG. 7. Combination treatment of postconfluent CaSki cells with CPT and radiation. Postconfluent CaSki cells were exposed to the indicated concentrations of CPT for 2 (A) or 48 h (B) immediately before irradiation with the indicated doses. Cells were replated in fresh medium and cell survival was determined 8 days later by clonogenic assay. Results represent mean cell survival values as a percentage of the survival of untreated cells. Numbers at the base of each column show the number of replicates. Significant differences in the survival of cells treated with a combination of CPT and radiation and the sum of the surviving cells that were individually treated with radiation and CPT are indicated: ** $P \leq 0.005$; *** $P \leq 0.0005$. Bars, SE.

limited number of advanced cervical tumors examined, however, did not allow statistical significance to be achieved. A trend of increasing topo I protein levels in advanced colon carcinoma compared to early stage disease was reported [32]. However, no correlation was found between disease stage and topo I activity in colorectal and prostate cancers in another study [28]. Most studies have examined a modest number of tumor samples, making correlation of topo I activity with disease stage difficult. Our results suggest that CPT treatment may be more effective against advanced cervical tumors compared with early disease.

Comparison of topo I protein levels and enzymatic activity revealed that topo I activity did not correlate with topo I protein levels. A similar lack of a correlation between topo I protein levels and topo I activity has been reported for malignant ovarian tumors [31, 33]. In addition, cellular sensitivity to SN-38 was positively correlated with topo I activity but not to topo I mRNA expression in human colon cancer cell lines [27]. Sensitivity to SN-38 in a panel of human lung cancer cells also

did not correlate with topo I protein levels [34]. In contrast, a relationship between topo I protein levels and catalytic activity in colon and prostate tumors [28] and a trend of increased topo I protein with enzymatic activity in several tumor types [35] have been reported. It is unclear why conflicting results have been obtained for the relationship between topo I protein levels and catalytic activity but one explanation is differential post-translational modification of topo I protein. Topo I catalytic activity is increased by serine phosphorylation mediated by casein kinase type II [36] and protein kinase C [37], whereas topo I enzymatic activity is inhibited by poly(ADP-ribosylation) [38]. The catalytic activity and stability of topo I can also be increased by association with the tumor suppressor protein p53 [39]. It is tempting to speculate that the posttranslational modifications, and thus activity, of topo I may vary with tumor type or disease stage. Further research is required to ascertain these possible relationships.

CaSki human cervical carcinoma cells were employed to investigate whether CPT could potentiate the effects of ionizing radiation. CaSki cells exhibited sensitivity to CPT similar to that of several other cervical carcinoma cell lines and possessed a topo I activity (4.4 h^{-1}) similar to the mean topo I activity of cervical carcinoma tumors (3.0 h^{-1}), suggesting that these cells may be representative of cervical carcinomas. Subconfluent CaSki cells were relatively sensitive to ionizing radiation with an IC_{90} value of 790 cGy. Postconfluent cells, as expected, were more resistant to the lethal effects of radiation, especially at low radiation doses, as evidenced by the pronounced shoulder on the dose–response curve (Fig. 5A). Delaying the plating of postconfluent CaSki cells from 2 until 48 h after radiation treatment increased the radioresistance of the cells, reflecting repair of potentially lethal damage. Treatment of cells with CPT revealed both time-dependent and cell status cytotoxicity. Exposure of subconfluent CaSki cells to CPT for 24 h produced equivalent cytotoxicity at three orders of magnitude less concentration than a 2-h exposure (Fig. 5B). Postconfluent cells, in contrast, were refractive to CPT-induced cytotoxicity, even when exposed to drug for 48 h. Combined treatment with CPT and radiation was examined in both subconfluent and postconfluent cells as well as with 2- and 48-h CPT exposure due to the large differences in the radio- and drug sensitivity of subconfluent and postconfluent cells as well as the time-dependent cytotoxicity of CPT. The 2-h exposure was designed to simulate bolus administration of drug whereas the 48-h exposure may model continuous infusion of CPT. CPT was added to cells immediately before radiation exposure because previous studies [21, 40] showed that radiosensitization by CPT is only effective when cells are simultaneously exposed to drug and radiation.

Isobologram analysis was employed to examine the interaction between CPT and radiation treatment of CaSki cells. This analysis takes into account the nonlinear dose response of cells to CPT and radiation. Isobolograms, or isoeffect curves, show the expected killing from CPT and radiation assuming that the

effects are additive. Indeed, we found that combined treatment of preconfluent CaSki cells with CPT and ionizing radiation resulted in additive killing of cells regardless of whether the cells were treated with CPT for 2 or 48 h (Fig. 6). The less than synergistic killing of preconfluent CaSki cells treated with CPT and radiation may be related to CPT-induced apoptosis of the cells, which can decrease cellular sensitivity to radiation [41]. A previous study found that irradiation of mammalian cells induced rapid ADP-ribosylation of topo I, resulting in decreased catalytic activity [42]. Cells that are deficient in ADPRT are also hypersensitive to CPT [43]. We therefore tested whether inhibition of ADPRT with 3-aminobenzamide could increase the efficacy of chemoradiation therapy with CPT. Pretreatment of the cells with 3-aminobenzamide 1 h before the addition of CPT and irradiation of the cells produced supra-additive killing of CaSki cells (Fig. 6). This result implies that topo I catalytic activity is required for CPT-mediated radiosensitization of cells and suggests that methods to prevent down-regulation of topo I activity in tumors may increase the radiosensitization provided by CPT.

In contrast to actively dividing cells, CPT acted synergistically with radiation to kill postconfluent CaSki cells at all radiation doses and drug doses tested when the cells were exposed to CPT for 48 h and at lower radiation doses (200 and 400 cGy) when CPT was present for 2 h (Fig. 7). Potentiation of cell killing was observed even at a low concentration of CPT (1 ng/ml, 2.87 nM) that was not toxic by itself to postconfluent cells. This concentration of CPT or CPT analog is readily achievable in humans. For example, an early pharmacological study of the sodium salt of CPT found that CPT concentrations were greater than 1 $\mu\text{g/ml}$ at 2 h after administration of 2–10 mg CPT/kg and greater than 1 $\mu\text{g/ml}$ after 48 h [44]. Serum levels of irinotecan (CPT-11) were greater than 10 ng/ml at 24 h after infusion of 50 mg/m² of drug [45]. Thus CPT dramatically increases the radiosensitivity of noncycling cells at concentrations achievable in patients. In contrast to cells cultured *in vitro*, most solid tumors are composed of both cycling and noncycling cells. The growth fraction is typically 30–40% for cervical carcinoma tumors [46, 47]. Because noncycling cells are refractive to the cytotoxic effects of both CPT and radiation, combination treatment with CPT and radiation may be particularly useful for killing these cells and preventing repopulation of the tumor after radiation treatment.

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