Mechanisms of Resistance in a Human Cell Line Exposed to Sequential Topoisomerase Poisoning

Ahamed Saleem, Nageatte Ibrahim, Milan Patel, Xi-Guang Li, Elora Gupta, John Mendoza, Panayotis Pantazis, and Eric H. Rubin

ABSTRACT

Camptothecins are a new class of anticancer drugs that target DNA topoisomerase I; current efforts are directed toward elucidating optimal combinations of these drugs with other antineoplastic agents. A rationale for the use of sequential therapy involving the combination of camptothecins with topoisomerase II-targeting drugs, such as etoposide, has arisen from observations of increased topoisomerase II protein levels in cell lines resistant to camptothecin. In an effort to understand potential mechanisms of resistance to this strategy, we developed a U-937 cell subline, denoted RERC, that is capable of surviving exposure to sequential topoisomerase poisoning. The RERC cells are 200-fold resistant to camptothecin, 8-fold resistant to etoposide, and 10-fold hypersensitive to cisplatin compared to the parental U-937 cells. Biochemical analyses indicate that the resistant phenotype involves alterations in both topoisomerase I and topoisomerase IIα. Topoisomerase I catalytic activity in the resistant cells is similar to that of the parental line but is resistant to camptothecin. Moreover, the resistant cells express a single mRNA species of topoisomerase I that codes for a mutation in codon 533. In addition, topoisomerase IIα protein levels are decreased 10-fold in the resistant line, coincident with a two-fold decrease in the expression of topoisomerase IIα mRNA. Collectively, these results indicate that resistance to sequential topoisomerase poisoning may involve a reduction in total cellular topoisomerase activity.

INTRODUCTION

DNA topoisomerases are enzymes that regulate DNA structure and are involved in a variety of cellular processes, such as DNA replication, transcription, and recombination (1). Several potent antitumor agents derived wholly or partially from natural sources are known now to target DNA Top2 (2, 3). More recently, camptothecins, also derived from natural sources, have been shown to be effective antitumor agents that specifically target DNA Top1 (4–7). Topoisomerase-targeting drugs share a common mechanism of action in which they “poison” the enzyme by trapping it in a covalent complex with DNA, resulting in persistent DNA strand breaks that result in cell death (4).

Studies of cells resistant to either Top1 or Top2 poisons have elucidated cellular mechanisms of resistance; this information may be useful in efforts to improve the antitumor activity of these drugs (reviewed in Refs. 7–9). In resistant cell lines, the targeted topoisomerase is often down-regulated or mutated, with a reciprocal increase in the activity and/or level of a nontargeted topoisomerase (10–21). Studies performed with specimens obtained from patients treated with camptothecin analogues indicate that down-regulation of Top1 may be associated with up-regulation of Top2 in tumor tissue or peripheral blood cells (22–24). These findings predict that sequential administration of a Top1 and a Top2 poison may result in enhanced or even synergistic antitumor activity compared to single drug administration, a prediction that has been validated in animal models (25–27). There is thus a strong rationale for the clinical use of sequential topoisomerase poisoning, and such trials have been initiated by our group and by others. We questioned whether or not mammalian tumor cells grown in culture would be capable of surviving such a strategy, and if so, what mechanisms would be used. The results of this work are presented here.

MATERIALS AND METHODS

Drugs. 9NC was prepared semisynthetically at the Stehlin Foundation from commercially available camptothecin. The final product was greater than 96% pure and is used for both preclinical and clinical studies. 9NC stock solutions were prepared as 100-mM concentrations in polyethylene glycol 400. Etoposide was obtained from Sigma Chemical Co. and was prepared as a 10 mM stock solution in DMSO.

Cell Culture and Flow Cytometry. U-937 monoblastic leukemia cells (American Type Culture Collection, Rockville, MD), and resistant sublines were grown in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. U-937 cells resistant to etoposide were selected for by growth in increasing concentrations of this drug, with the cells exposed to each drug concentration for 3 weeks. After growth in media containing 2 μM etoposide for 2 months, the cells were selected for resistance to 9NC using a similar strategy. The resultant clonally resistant cells were exposed to 2-μM concentrations of etoposide and 9NC for 3 months; at this time, the number of viable cells in culture was consistently above 95% as assessed by trypsin blue dye exclusion, and the generation times and cell cycle histograms of cells tested at different intervals were stable. The resultant cell subline was designated RERC (for “resistant to etoposide and resistant to camptothecin”). Flow cytometric analyses were performed using propidium-iodide staining as described (28).

Cytotoxicity Assays. Drug cytotoxicity assays were performed as described using the tetrazolium-based compound MTT (17). Experiments were performed in triplicate. Drug concentrations associated with 50% inhibition of growth (IC50) were obtained by a curve-fitting analysis of the percentage of absorbance versus drug concentration, using PC-NONLIN (version 4.0). The data were fitted to the sigmoidal inhibitory effect model as described by the following equation: 

$$E = E_{max} \times \left(1 - \frac{C}{K+EC_{50}}\right),$$

where $E$ is the percentage of absorbance, $C$ is the drug concentration, and $y$ is the curve shape parameter. Mean U-937 and RERC IC50 values obtained from replicate experiments were compared using a Mann-Whitney test.

Topoisomerase Activity Assays. Nuclear extracts were prepared using 0.5 M salt extraction as described (17). Top1 enzyme activity was measured by a DNA relaxation assay using supercoiled plasmid DNA and agarose electrophoresis (17). Top2 enzyme activity was measured using kinetoplast DNA from Crithidia fasciculata (Topogen, Inc., Columbus, OH). Top2 reaction mixtures contained various amounts of nuclear extract and in some cases etoposide, and 50 mM Tris-HCl (pH 8.0), 10 mM MgCl2, 0.5 mM ATP, 0.5 mM DTT, and 30 μg/ml BSA. Top2 enzyme reactions were performed at 37°C for 30 min, and the products were analyzed by electrophoresis in a 1% agarose gel containing 0.5 μg/ml ethidium bromide using TAE buffer (40 mM Tris-HCl, pH 7.2, 2 mM sodium acetate, 1 mM EDTA).

Immunoblotting. Whole-cell extracts were prepared using a buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1% SDS as described.
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(29). Nuclear or whole-cell extracts were subjected to electrophoresis in 7.5% SDS-polyacrylamide gels and then transferred to nitrocellulose filters. Immunoblotting was performed using polyclonal antibodies to Top1 antibody, Top2α (Topogen, Inc.), or Top2β (30). The blots were developed using an enhanced chemiluminescence technique (ECL detection system; Amersham Corp.) and Kodak X-OMAT XR film. Photographic negatives were scanned, and protein bands were quantitated with Bio-Rad Molecular Imager System GS-525.

Top1 cDNA Sequencing. Total RNA was isolated from cells using guanidine isothiocyanate/cesium chloride gradient centrifugation (31). Reverse transcription and PCR-based cDNA amplification were performed as described (17). Briefly, oligonucleotide primers of 28–34 bp were synthesized to amplify regions of 500-1000 bp of the human Top1 cDNA corresponding to regions known to be “hot spots” for mutations conferring camptothecin resistance (Table 1; Ref. 9). The resultant PCR products were purified and sequenced using 32P-labeled deoxyribonucleotides and thermal cycling with the Thermosequenase enzyme (Amersham Life Sciences). Thermal cycling was performed at 95, 60, and 72°C using 30, 30, and 60 s intervals, respectively, for 30 cycles. The products were analyzed in a 6% polyacrylamide gel containing 8.3 M urea and TBE (0.1 M Tris-borate and 2 mM EDTA [pH 8.3]). After electrophoresis, the gel was dried on filter paper and exposed for 16–48 h to Kodak film using an intensifying screen.

RNA Isolation and Northern Blot Hybridization. RNA was isolated as described above, separated by electrophoresis in 1% agarose/2 M formaldehyde gels, and transferred to nitrocellulose filters. 32P-labeled DNA probes for Top2α and Top2β were generated using PCR with [32P]dCTP and isozyme-specific primers (Table 1). The primers were designed based on the divergent nucleotide sequences in the carboxyl regions of the Top2 isozymes. Plasmids containing either Top2α (YEpWOB6; Ref. 32) or -β (YEpTop2B, provided kindly by Dr. Leroy Liu) coding sequences were used as templates for the PCR reactions. Hybridizations were performed as described (31). The filters were washed and exposed to Kodak X-OMAT XR film using an intensifying screen. Autoradiograms were scanned and analyzed as described above.

RESULTS

Phenotype of Resistant Cells Obtained by Sequential Exposure to Etoposide and 9NC. U-937 human monoblastic leukemia cells resistant to etoposide were selected for by growth in increasing concentrations of this drug. The resistant cells were then exposed to increasing concentrations of 9NC. A resultant subline was isolated containing either Top2α (YEpWOB6; Ref. 32) or -β (YEphTOP2B, provided kindly by Dr. Leroy Liu) coding sequences were used as templates for the PCR reactions. Hybridizations were performed as described (31). The filters were washed and exposed to Kodak X-OMAT XR film using an intensifying screen. Autoradiograms were scanned and analyzed as described above.

Table 1 Primers used for amplification of topoisomerase I and II cDNAs

<table>
<thead>
<tr>
<th>Amplified cDNA size (bp)</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top1 “hot spot” codons</td>
<td></td>
</tr>
<tr>
<td>361, 363</td>
<td>611</td>
</tr>
<tr>
<td>503, 533</td>
<td>635</td>
</tr>
<tr>
<td>722, 729</td>
<td>544</td>
</tr>
<tr>
<td>Top2 isozyme/region*</td>
<td></td>
</tr>
<tr>
<td>a/3218-4577</td>
<td>359</td>
</tr>
<tr>
<td>β/4443-4775</td>
<td>332</td>
</tr>
<tr>
<td>a/3218-4577</td>
<td></td>
</tr>
<tr>
<td>β/4443-4775</td>
<td></td>
</tr>
</tbody>
</table>

* Numbering of bases according to Refs. 58 and 59.
Table 2: Estimation of IC_{50} values for U-937 and RERC cells

<table>
<thead>
<tr>
<th></th>
<th>U-937</th>
<th>RERC</th>
<th>Relative resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Camptothecin</td>
<td>0.05 ± 0.006</td>
<td>10.9 ± 8.7</td>
<td>218^abc</td>
</tr>
<tr>
<td>Etoposide</td>
<td>1.2 ± 0.07</td>
<td>10.1 ± 0.50</td>
<td>8.4^ab</td>
</tr>
<tr>
<td>Etoposide + verapamil</td>
<td>0.56 ± 0.09</td>
<td>3.25 ± 2.58</td>
<td>5.8^a</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>2.02 ± 1.2</td>
<td>0.191 ± 0.12</td>
<td>0.09^b</td>
</tr>
<tr>
<td>Methyl methanesulfonate</td>
<td>1.76 ± 0.38</td>
<td>1.62 ± 0.25</td>
<td>0.9^c</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>3.92 ± 2.72</td>
<td>1.10 ± 1.01</td>
<td>0.3^c</td>
</tr>
</tbody>
</table>

^a Data represent means ± SD of at least three replicates.  
^b Mean of RERC cells divided by mean of U-937 cells.  
^c Difference between means is statistically significant (P < 0.05 using a Mann-Whitney test).

DISCUSSION

Previous studies indicate that cellular resistance to a Top1 poison is often associated with hypersensitivity to a Top2 poison and vice versa. These results and others have led to clinical trials of sequential topoisomerase poisoning. Preliminary results from such trials indicate that tumor regression is short-lived after therapy, suggesting the existence of resistant tumor clones (24). Although mechanisms of drug resistance operative in vivo may be quite different from those observed in cultured cells, to gain insight into possible clinical mech-
Fig. 2. TopI activity and protein are similar in U-937 and RERC cells. A. TopI plasmid relaxation activity. Supercoiled plasmid DNA (SC) was incubated with nuclear extract for 30 min at 37°C. Relaxed DNA was identified by agarose gel electrophoresis. First lane, incubation of the plasmid without nuclear extract. From left to right in the U-937 and RERC groups, the amounts of nuclear extract were 180, 60, 30, 15, and 7.5 ng. B. TopI immunoblotting. The indicated amounts of nuclear extract were subjected to immunoblotting using a polyclonal antibody to TopI. Arrow, position of the intact M, 100,000 form of TopI. C. Effects of camptothecin on TopI-induced plasmid nicking. Supercoiled plasmid DNA was incubated with 180 ng of nuclear extract and camptothecin (CPT) for 30 min at 37°C. Nicked DNA (nickd) was identified by agarose gel electrophoresis performed in the presence of 0.5 μg/ml ethidium bromide. First lane, incubation of the plasmid without nuclear extract. From left to right in the U-937 and RERC groups, the concentrations of camptothecin were 0, 12.5, 25, 62.5, 125, and 250 μM.

Mechanisms of resistance to sequential topoisomerase poisoning, we have generated a cell line, denoted RERC, that is resistant to both camptothecin and etoposide.

Biochemical analyses indicate that the RERC cells contain alterations in both Top1 and Top2 proteins relative to the parental U-937 cells. Top1 activity in the resistant cells is resistant to camptothecin, likely the result of the expression of a single mRNA species coding for asparagine rather than aspartate at amino acid 533. Substitution of

Fig. 3. Detection of a Top1 mutation at codon 533 in the RERC cells. Reverse transcription and PCR were used to isolate Top1 cDNAs from U-937 and RERC cells as described in the “Materials and Methods” section. The products were sequenced directly using dideoxynucleotides. The guanine-to-adenine base substitution in the RERC cells is indicated.
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Fig. 4. Reduction of Top2 activity and protein in the RERC cells. A. Top2 decatenating activity. Kinetoplast DNA (kDNA) was incubated with nuclear extract for 30 min at 37°C. Decatenated minicircles (minicircles) were identified by agarose gel electrophoresis. First lane, incubation of the kinetoplast DNA without nuclear extract. From left to right in the U-937 and RERC groups, the amounts of nuclear extract were 400, 300, 200, and 100 ng. B. Top2 immunoblotting. The indicated amounts of nuclear extract were subjected to immunoblotting using antibodies recognizing the α and β isozymes. Arrows, positions of the bands representing the Mr 170,000 α protein and Mr 180,000 β protein. C. Effects of etoposide on Top2 decatenating activity. Kinetoplast DNA was incubated with 400 ng of U-937 nuclear extract and 800 ng of RERC nuclear extract in the presence of etoposide. First lane, incubation of the kinetoplast DNA without nuclear extract. From left to right in the U-937 and RERC groups, the concentrations of etoposide were 1200, 600, 300, and 100 μM.

this amino acid has been implicated previously in camptothecin resistance in the CPT-K5 cell line, although in this case, glycine was substituted for the aspartate at this position (33). In both cases, the substitution appears to have little effect on the ability of the protein to relax supercoiled DNA. Taken together with the observation that deletion of Top1 has not been found in any camptothecin-resistant mammalian cell line, these findings suggest that in contrast to yeast (34, 35), mammalian cells require Top1 activity for growth. This conjecture is supported by recent studies with murine embryonic stem cells, in which cells carrying homozygous disrupted Top1 alleles appeared incapable of growth (36).

The RERC cells also contain a reduction in total and nuclear Top2 levels compared to the parental cells. This reduction involves the Top2α isozyme and is at least in part due to diminished expression of Top2α mRNA. Because our results indicate a 2-fold reduction in Top2α mRNA levels, it is intriguing to speculate that in the RERC cells one of two Top2α alleles has been rearranged, leading to loss of transcripts from this allele. Such a phenomenon has been described previously in P388 cells selected for resistance to etoposide (18). Our results are also consistent with several prior reports demonstrating alterations in Top2α in cell lines resistant to drugs, such as etoposide and mitoxantrone (37–44). On the other hand, alterations in Top2β have been detected in other cell lines resistant to Top2-targeting drugs (45, 46). Therefore, the relative importance of each isozyme as a drug target appears variable and to depend on the cell line studied.

Taken together, our studies indicate that resistance to topoisomerase poisons may involve a reduction in the sum of Top1 and Top2 activities. Although it is possible that the recently described human topoisomerase III (47) or another topoisomerase is up-regulated in a compensatory manner in the RERC cells, its activity is not detectable in our topoisomerase assays. Our findings thus refute the hypothesis that the sum of Top1 and Top2 activities must be constant for mammalian cells to remain viable. However, when large numbers of RERC cells (2 × 10^7) are inoculated into nude mice, they fail to induce tumors within 4 months, whereas similar inoculations of parental cells yield visible tumors within 8–10 days of inoculation (data not shown). Therefore, although there are several conceivable explanations for the observed loss of tumorigenicity in the RERC cells, it is possible that a certain level of topoisomerase activity is required for tumorigenicity but not for viability.

Although we are not aware of other cell lines selected sequentially for resistance to Top1- and Top2-targeting drugs, other cell lines have been found to exhibit a dual resistance phenotype (20, 48, 49). In each case, these cell lines were selected for resistance to Top2-targeting drugs and were found to be cross-resistant to camptothecins. Interestingly, in none of these cases was the camptothecin resistance associ-
synergistic cytotoxicity that has been observed with the combination of cisplatin and topoisomerase poisons in preclinical models (52–57). Although alternative explanations, such as altered cell cycle distribution, may be proposed to explain the hypersensitivity of the RERC cells to cisplatin and vinblastine, the magnitude of the increased sensitivity to cisplatin supports the continued clinical investigation of the combination of cisplatin with dual or sequential topoisomerase poisoning.

ACKNOWLEDGMENTS

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REFERENCES

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