Evidence for a Mutant Allele of the Gene for DNA Topoisomerase II in Adriamycin-resistant P388 Murine Leukemia Cells

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ABSTRACT

Previous studies have shown that DNA topoisomerase II enzyme activity and protein levels are reduced in cloned lines of Adriamycin-resistant P388 leukemia cells relative to drug-sensitive cells (Defie et al., Cancer Res., 49: 58-62, 1989). The molecular basis of the reduced topoisomerase II levels in these resistant cells has been investigated. Northern blot analysis of total cellular RNA from drug-sensitive and -resistant cells using a 1.8-kilobase human topoisomerase II complementary DNA revealed the presence of two mRNA species: a 6.6-kilobase transcript that was strongly expressed in drug-sensitive cells but reduced 7- to 8-fold in resistant cells; and a 5.5-kilobase transcript detected only in drug-resistant cells. Southern blot analysis of genomic DNA digested with BamHI, SalI, or PvuII and probed with the 1.8-kilobase complementary DNA for human topoisomerase II showed that, in Adriamycin-resistant cells, there were two different alleles for topoisomerase II, one identical to the native allele but with a lower gene copy number than that found in sensitive cells, and a second allele containing a mutation present only in resistant cells. These findings suggest that the reduced levels of topo II protein in drug-resistant cells may be due to reduced amounts of the native 6.6-kilobase mRNA. The unique 5.5-kilobase mRNA in resistant cells may represent a shortened transcript of the mutated topoisomerase II allele.

INTRODUCTION

Mammalian DNA topoisomerases are nuclear enzymes that alter the topological state of DNA via mechanisms that involve concerted breakage-rejoining of single- (type I enzyme) or double-stranded DNA (type II enzyme). A substantial body of direct and indirect evidence indicates that the topological interconversions of DNA mediated by topoisomerases are important in almost every aspect of DNA metabolism, including replication, transcription, recombination, repair, and chromosomal condensation (for reviews, see Refs. 1 to 6).

Recently, the type II enzyme has been implicated in the acquisition of drug resistance by various cultured cell lines. Topoisomerase II activity has been reported to be reduced in cells resistant to epipodophyllotoxins (7-9), amscarines (10-12), ADR1 (13, 14), and ellipticines (15, 16). The relationship between topoisomerase I and drug resistance is not as firmly established as that for topoisomerase II, but there are reports that the drug camptothecin exerts its cytotoxic effect via topoisomerase I-mediated DNA breakage (17-19). Others have suggested that resistance of Chinese hamster cells to 4′-(9-acridylaminomethanesulfon-m-аниsidide and VP-16 is due to topoisomerase I modulation of topoisomerase II activity (20).

The cDNAs for both DNA topoisomerase I and II have been cloned and sequenced. In humans, topo I and topo II genes have been mapped to chromosomes 20 and 17, respectively (21-23), and both are reported to be single copy genes in human cells and other eukaryotes (21-27). The regulation of expression of these genes is, however, still largely unknown.

Even though there have been several reports about reduced topoisomerase II activity in drug-resistant cells, few investigators have actually provided any insight into the molecular basis of the reduced enzyme activity. Some reports (7, 8) have proposed a qualitative change of the enzyme in drug-resistant cells, while others (11, 14) have suggested reduced quantity of the enzyme in resistant cells. In our studies of the multifactorial basis of resistance of P388 cells to ADR (28, 29), we have shown recently (14) that decreased levels of topoisomerase II activity, which may represent quantitative reduction of the enzyme, are an additional characteristic of ADR-resistant cells.

In this study, we provide evidence that, in drug-resistant cells, a mutation in one of the alleles for topo II may be a major factor in the reduced levels of the native topo II mRNA and gene product.

MATERIALS AND METHODS

Cell Lines and Cultures. Cloned cell lines of ADR-sensitive (P388/ 4) and -resistant leukemia (P388/ADR/3 or clone 3 and P388/ADR/7 or clone 7 cells) have been described previously (28). Clone 3 and clone 7 cells are 5- and 10-fold more resistant to ADR than the drug-sensitive cells. All three cell lines are maintained in RPMI medium supplemented with 15% fetal bovine serum (GIBCO, Grand Island, NY) and grow exponentially with a doubling time of 11 to 12 h.

Chemicals. All chemicals were of reagent grade and were purchased from commercial sources. [3P]dCTP was obtained from ICN Biochemicals, Irvine, CA.

cDNA Probes. The 1.8-kilobase human topoisomerase II cDNA (AhTOP2-Z2) in plasmid pCIS, the 5.6-kilobase full-length human topoisomerase II cDNA (pBS-hTOP2), and the 0.7-kilobase human topoisomerase I cDNA in plasmid pGEM-4DI were kindly provided by Dr. Leroy Liu (The Johns Hopkins University School of Medicine). The cDNA inserts were removed from the plasmids by digestion with EcoRI followed by agarose gel electrophoresis and electroelution.

RNA Extraction and Northern Blot Analysis. Total cellular RNA was extracted from exponentially growing cells by the guanidinium isothiocyanate method (30). One hundred μg of total RNA from each cell line were fractionated in a 1% formaldehyde-agarose gel and transferred onto nitrocellulose filters (Bio-Rad) in 10x SSC (1x SSC is 0.15 M NaCl-0.015 M sodium citrate). The filters were prehybridized for 5 to 10 min at 65°C in the buffer system of Church and Gilbert (31). Hybridization was performed overnight in the same buffer containing randomly primed radiolabeled probes of either topoisomerase II or topoisomerase I cDNA. After hybridization, the filters were washed in buffer systems reported previously (31). Autoradiograms were obtained at -70°C on Kodak XAR film with DuPont Cronex Lightning-Plus screens.

DNA Extraction and Southern Blot Analysis. Genomic DNA was extracted from cultured cells with phenol and chloroform as described by Davis et al. (32). After digestion with several enzymes (Boehringer-Mannheim, Mannheim, Federal Republic of Germany), equivalent amounts of DNA were electrophoresed in 0.8% agarose gel and blotted overnight onto nitrocellulose filters. Prehybridization, hybridization, and wash conditions were as described above.

RESULTS

Northern Blot Analysis of Total RNA from Adriamycin-sensitive and -resistant Cells. Total cellular RNA isolated from
ADR-sensitive and -resistant cells was investigated by Northern blot analysis using the 1.8-kilobase human topoisomerase II cDNA insert of plasmid pCIS. The cDNA, which represents the 3’-end of the human topoisomerase II gene, hybridized to two mRNA species (Fig. 1a). A transcript of approximately 6.6 kilobases, which apparently corresponds to the 6.2-kilobase message reported by others (23), was expressed in all three cell lines. Hybridization of the same blot with a control β-actin cDNA probe indicated equal loading of RNA in all three lanes (Fig. 1c). Densitometric scanning was used to compare the intensity of the 6.6-kilobase message relative to that obtained with β-actin; this ratio (mean ± SE, 4 determinations) was 1.58 ± 0.17 in ADR-sensitive cells, 0.20 ± 0.03 in resistant clone 3 cells, and 0.22 ± 0.02 in resistant clone 7 cells. Hence, there was approximately a 7- to 8-fold reduction in intensity of the 6.6-kilobase message in resistant cells, and in each case the difference was highly significant (P < 0.001). (b) A shorter transcript of approximately 5.5 kilobases was strongly expressed in ADR-resistant cells of clones 3 and 7 but was not present in sensitive cells.

The presence of DNA topoisomerase I mRNA was also detected using the 0.7-kilobase topoisomerase I cDNA insert of plasmid pGEM-4-D1. A single mRNA species of 5.1 kilobases hybridized with the topoisomerase I cDNA probe, and the level of expression was comparable in all three cell lines (Fig. 1b).

Southern Blot Analysis of Restriction Fragments of Genomic DNA from Adriamycin-sensitive and -resistant Cells. Genomic DNA from ADR-sensitive and -resistant cell lines was digested with BamHI, StuI, or PvuII and probed with the 1.8-kilobase top II cDNA (Fig. 2). For all three restriction enzymes, the Southern blot identified a DNA fragment apparently derived from the native allele for the topo II gene (as shown by the arrow, Fig. 2). The copy number of the native allele was reduced in drug-resistant cells. Furthermore, restriction fragments unique to the resistant cells were also observed (depicted by arrowhead, Fig. 2). Similar results were obtained using the full length cDNA for topoisomerase II (pBS-hTOP2). Southern blots of genomic DNA digested with EcoRI, HindIII, PstI, BglII, MspI, KpnI, or XbaI did not show any differences among the three cell lines (results not shown).

**DISCUSSION**

We have previously reported that DNA topoisomerase II catalytic and drug-mediated cleavage activity was significantly reduced in nuclear extracts from ADR-resistant P388 leukemia cells (14). As well, Western blot analysis of nuclear extracts from drug-sensitive and -resistant cells revealed reduced levels of topoisomerase II protein in the resistant cells (7–16). Even though several other studies have reported reduced topo II enzyme activity and/or protein content in resistant cells (7–16), none has provided the molecular mechanism to account for these reductions. In this study, we provide evidence for a molecular genetic basis for the reduced levels of topo II in ADR-resistant cells.

Northern blot analysis demonstrated a 6.6-kilobase transcript present in all three cell lines. The level of this transcript was reduced 7- to 8-fold in drug-resistant cells relative to sensitive cells (Fig. 1a), which is consistent with our previous report of a decreased amount of immunoreactive topo II protein in resistant cells detected by Western blots (14). The 6.6-kilobase transcript probably corresponds to the single mRNA species of approximately 6.2 kilobases described in HeLa cells using the same topo II cDNA probe (23) and may represent the native, full length transcript for topo II. Thus, it appears that the decreased content of topo II in ADR-resistant P388 cells is, at least in part, accounted for by reduced levels of the native mRNA. No previous report has shown that reduced steady-state levels of topo II mRNA account for the reduction in the catalytic and drug-mediated cleavage activity of topo II in ADR-resistant cells.

DNA topoisomerase II catalytic activity is reduced in drug-resistant cells (7-16). Even though several other studies have reported reduced topo II enzyme activity and/or protein content in resistant cells (7–16), none has provided the molecular mechanism to account for these reductions. In this study, we provide evidence for a molecular genetic basis for the reduced levels of topo II in ADR-resistant cells.
enzyme content in drug-resistant cells. On the other hand, one report has shown that topo II mRNA and protein were increased in a nitrogen mustard-resistant Raji cell line (33).

The second mRNA species of 5.5 kilobases in drug-resistant cells with no counterpart in the sensitive cells (Fig. 1a) is a novel observation. The origin of this transcript is not yet known. It is unlikely that it represents a second topo II gene, since Southern blot analysis of DNA digested with several restriction enzymes does not show any evidence of a second gene or differences in gene copy number among the three cell lines (results not shown). In addition, it has been shown in HeLa cells and many eukaryotes that topo II is encoded by a single gene (21–27). Whether or not the 5.5-kilobase transcript encodes for functional topo II protein is not known. Western blots do not detect any protein, in either nuclear extracts (14) or cytosolic extracts (results not shown), that corresponds with the presence of the 5.5-kilobase transcript. The inability to detect a protein product for the 5.5-kilobase transcript suggests several possibilities. (a) The 5.5-kilobase mRNA may not be translated. (b) The protein product, if translated, may be unstable and quickly degraded in the cell. (c) The protein may be immunologically unreactive with the antibody used in the Western blots.

In order to determine whether the pattern of mRNA expressed in ADR-sensitive and -resistant cells has a molecular genetic basis, we performed Southern blot analysis on genomic DNA from the three cell lines. By Southern analysis, we have shown RFLP in ADR-sensitive and -resistant P388 cells using the topo II cDNA probe and DNA digested with BamHI, Stul, or PvuII. With each enzyme, a DNA fragment present in all three cell lines was reduced in intensity in resistant cells. Another DNA fragment (two fragments in the case of BamHI) was also found to be present only in resistant cells. These observations suggest allelic heterozygosity for the topo II gene in ADR-resistant cells. Presumably the normal allele is represented by the DNA fragment present in all three cell lines but reduced in drug-resistant cells. The second allele present only in resistant cells contains an indeterminate mutation resulting in generation of shortened restriction fragments. The 6.6-kilobase mRNA detected on Northern blots is most likely a transcript of the normal allele, whereas the 5.5-kilobase mRNA present only in resistant cells is probably transcribed from the mutant allele. Thus the reduction in the level of topo II protein in ADR-resistant P388 cells seems to have a genetic basis. We postulate from these studies that the RFLPs found with BamHI, Stul, and PvuII, together with the Northern analysis showing the shorter 5.5-kilobase transcript unique to resistant cells, suggest that, in resistant cells, there are two different alleles for topo II, one identical to that found in sensitive cells, presumably the native allele, and a second shorter allele that probably results from a mutation. This mutation may contribute to the reduction of the native mRNA and gene product in drug-resistant cells.

Although enzyme activity studies had previously shown stepwise decrements in both catalytic and drug-induced cleavage activities in resistant cells of clones 3 and 7 (14), the results of the Southern, Northern, and Western blots showed comparable reductions in the two resistant cell lines. This may indicate that, in addition to a quantitative reduction in the level of topo II in drug-resistant cells, qualitative differences might exist between the enzyme of resistant cells of clones 3 and 7. Alternatively, differences in other cellular factors that affect enzyme activity might exist between the two cell lines. Also, the discrepancy between the level of reduction in topo II mRNA on the Northern blot compared with the reduction in the native gene copy number on Southern blots indicates that other factors, such as differences in transcription and stability of the native topo II transcript, may be involved in regulating the level of topo II in ADR-resistant P388 leukemia cells.

This study is the first to provide a molecular genetic mechanism for the reduced levels of topo II associated with drug resistance. The nature of the allelic mutation proposed is not yet fully understood. Point mutation(s) does not seem to be a likely mechanism, since the RFLP is seen with a number of restriction enzymes. Other possible mutational changes under investigation are rearrangement, deletion, or translocation.

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REFERENCES


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