Advances in Brief

Identification of a Point Mutation in the Topoisomerase II Gene from a Human Leukemia Cell Line Containing an Amsacrine-resistant Form of Topoisomerase II

Michael Hinds, Karl Deisseroth, Janice Mayes, Elizabeth Altschuler, Ruud Jansen, Fred D. Ledley, and Leonard A. Zwelling

Abstract

HL-60/AMSA is a human leukemia cell line that is 50- to 100-fold more resistant to the cytotoxic actions of the topoisomerase II-reactive intercalator amsacrine than is its drug-sensitive HL-60 parent line. Previously, we have shown that the topoisomerase II from HL-60/AMSA is also resistant to inhibition by amsacrine and other intercalating agents. We therefore sought the molecular basis for the resistance of the topoisomerase II of HL-60/AMSA and, by inference, of the HL-60/AMSA line itself. We report the cloning and sequencing of the topoisomerase II genes from both the sensitive and resistant leukemia cell lines using polymerase chain reaction technology. We have identified a single base change associated with the drug-resistant form of topoisomerase II. This mutation is present in both cloned HL-60/AMSA complementary DNA and extracted HL-60/AMSA genomic DNA. A rapid assay for this mutation in clinical samples has been developed and applied to the DNA of cells from both normal volunteers and leukemia patients. Thus far, the HL-60/AMSA genotype has not been identified in the cells from any individual, suggesting that this genotype is indeed a mutation and not an allelic form of topoisomerase II. The novel assay developed will allow a rapid search for the prevalence of this mutation in clinical samples from patients with leukemia who have relapsed following intercalator therapy.

Introduction

Forms of the nuclear enzyme topoisomerase II that have decreased susceptibilities to the inhibitory actions of anticancer drugs that target this enzyme have been isolated from drug-resistant cell lines (1–4). These altered enzyme forms were thought to be at least in part responsible for the resistance of the cells to the drugs. However, proof that these drug-resistant forms of topoisomerase II cause the cellular resistance requires molecular studies in which the pharmacological phenotype of cells transfected with a resistant or sensitive form of this gene can be assessed. We report the cloning and sequencing of the cDNA3 for the topoisomerase II from a resistant human leukemia cell line (HL-60/AMSA) containing a drug-resistant form of topoisomerase II (4). This cDNA contains a mutation that may be the cause of the drug resistance of the enzyme for which it codes. Additionally, the sequence change produced by this specific mutation leads to a change in restriction enzyme digestion patterns that allows rapid detection of the mutation in genomic DNA from cell lines and from clinical samples.

Materials and Methods

Total RNA was isolated from HL-60 and HL-60/AMSA cells (see Ref. 5; grown as described in Refs. 4 and 6) by the method of Chirgwin et al. (7). The complete cDNA for topoisomerase II was generated from both cell lines in four sections using avian myeloblastosis reverse transcriptase (Promega Biotech, Madison, WI) followed by amplification (35 cycles of PCR) using Taq polymerase (Promega Biotech, Madison, WI) and the oligonucleotides (Genosys Biotechnologies, Inc., The Woodlands, TX) as indicated in Fig. 1 and its legend. Double-stranded DNA was isolated from 1% agarose gels with GeneClean S/K\(^+\), and transformed into Epicurean XL-1 Blue (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Six independent transformants were identified from LB plates containing 50 μg/ml of ampicillin except for the 3' end wherein only 4 colonies from HL-60 and only 5 from HL-60/AMSA were isolated.

Dideoxyn sequencing was carried out with Sequenase version 2.0 (United States Biochemical, Cleveland, OH). Reactions were primed from the T\(_5\), T\(_6\), m13-40 (United States Biochemical), or appropriate synthetic oligonucleotides (Genosys, The Woodlands, TX) as described in Fig. 1 and its legend. The sequence obtained from a single clone was compared with that from a pool of the remaining clones to identify errors generated during reverse transcription or PCR. The consensus sequence was then compared with that of the human topoisomerase II gene in GENBANK (8). All references to nucleotide position numbers in the topoisomerase II cDNA are according to GENBANK positions.

To identify whether the mutation described in the cDNA was also present in the genomic DNA of the cell lines or in DNA extracted from the cells of normal volunteers or patients with acute leukemia, we amplified the genomic sequence containing the area in which the mutation was found. This amplified DNA was isolated on agarose and digested to completion with restriction enzymes that can detect the wild-type or mutated sequence (see below). The products of these digests were then electrophoresed on 10% polyacrylamide gels to identify the molecular weight of the restriction fragments generated.

Results

The topoisomerase II (p170 form (1, 9)) cDNA from both HL-60 and HL-60/AMSA was cloned in four segments (see Fig. 1) [restriction analysis of cDNA's revealed we had not cloned the p180 form (9)]. The first segment (PCR1) was from the unique KpnI site at position 3470 to position 4683. The bases in GENBANK positions 4683 to 4680 were altered to GTGC (5' to 3') in the 3' PCR oligonucleotide (P\(_6\)) in Fig. 1 to create a Sall restriction site. The second segment (PCR2) was from the unique Kpnl site at position 2409 to the PstI site. The third segment (PCR3) was from the unique HindIII site at position 1518 to the Kpnl site. The final segment (PCR4) was from an artificially created Apal site at position 21 to the HindIII site. The Apal site was introduced by changing bases 17 and 18 to G in the 5' PCR oligonucleotide (Fig. 1) to create a non-specific restriction site. The products of these digests were then electrophoresed on 10% polyacrylamide gels to identify the molecular weight of the restriction fragments generated.

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2 To whom requests for reprints should be addressed, at Box 52, 1515 Holcombe Blvd., Houston, TX 77030.

3 The abbreviations used are: m-AMSA, amsacrine or 4'-[9-acridinyl]amino)methanesulfon-m-anisidide; cDNA, complementary DNA; PCR, polymerase chain reaction.

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Fig. 1. Cloning and sequencing strategy for human topoisomerase II cDNA from HL-60 and HL-60/AMSA. See "Materials and Methods" and "Results" for additional details. RT, oligonucleotides used to prime the reverse transcription reactions. P, oligonucleotides used to amplify cDNA segments. Arrows, direction of cDNA synthesis or amplification. Subscripts, PCR segment being synthesized. Smaller lines, oligonucleotides (see below) used for sequencing. Sequence primers for additional details. KI', oligonucleotides used to prime the reverse transcription reactions. /', oligonucleotides used to amplify cDNA segments. Arrows, direction of synthesis.

<table>
<thead>
<tr>
<th>PCR</th>
<th>RT oligo</th>
<th>5' PCR oligo</th>
<th>3' PCR oligo</th>
<th>Cloning sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>1676-1657</td>
<td>10-31*</td>
<td>1628-1609</td>
<td>ApaI, HindIII</td>
</tr>
<tr>
<td>3</td>
<td>2451-2432</td>
<td>1395-1414</td>
<td>2430-2411</td>
<td>HindIII, KpnI</td>
</tr>
<tr>
<td>2</td>
<td>3614-3595</td>
<td>2379-2398</td>
<td>3586-3567</td>
<td>KpnI, PstI</td>
</tr>
<tr>
<td>1</td>
<td>4709-4690</td>
<td>3389-3408</td>
<td>4683-4659*</td>
<td>PstI, SalI</td>
</tr>
</tbody>
</table>

* In PCR 5'-oligonucleotide, AT at positions 17 and 18 changed to GG to create ApaI site.

The sequencing oligonucleotides were as follows: 1, 467-483; 2, 970-986; 3, 1931-1947; 4, 2888-2904; 5, 3788-3804; 6, 4209-4225; a, 593-577; b, 1070-1054; c, 2018-2002; d, 2984-2968; e, 3930-3914; f, 4317-4301.

Discussion

Cloning and sequencing of the topoisomerase II cDNAs from intercalator-sensitive and intercalator-resistant human leukaemia lines revealed four important pieces of information: (a) a single base change in the cDNAs coding for topoisomerase II in the parent leukemia line (HL-60) versus the daughter line [HL-60/AMSA] is associated with a biochemically distinct form of topoisomerase II in HL-60/AMSA [resistant to intercalators (4)] and with a parallel resistance of the HL-60/AMSA cells themselves; (b) this change altered the restriction digestion pattern of the gene allowing the rapid detection of the change in clinical specimens and the identification of the change as being a mutation; (c) three bases were inserted in the 5' end of the gene that changed the predicted amino acid sequence of the topoisomerase II of both lines from that expected from the published sequence (8); (d) some additional differences between...
the published sequence and our sequence were found in the 3' end of the gene.

The mutation that results in a change in the amino acid sequence of topoisomerase II from arginine to lysine (amino acid position 486) is in a highly conserved region of the gene. All other topoisomerase II sequences as compared by Wyckoff et al. (10) contain an arginine at this position with the exception of Escherichia coli gyrase B, which contains a lysine-like HL-60/AMSA. Interestingly, E. coli is relatively resistant to the cytotoxic actions of amsacrine (11), while the bacteriophage T4 topoisomerase II (containing the arginine in this position) is sensitive to amsacrine (12). Yeast topoisomerase II, also containing arginine at this position (10), is sensitive to amsacrine (13). To emphasize the potential importance of this amino acid position to drug sensitivity or resistance, a change in this same position in gyrase B from the lysine to glutamic acid results in nalidixic acid resistance (14).

The data from the genomic DNA of normal volunteers and leukemia patients reaffirm the biochemical (4) and evolutionary (10-13) arguments that the HL-60/AMSA topoisomerase II genotype is not an allelic variant (15) but rather a true mutation. The PCR-based assay for the presence of the HL-60/AMSA mutation will make detection simple in tumors from patients that have been heavily treated with amsacrine or other topoisomerase II-reactive DNA intercalating agents. If this mutation can exist in the clinic, it might have significant impact on the interpretation of treatment failures in these patients as its presence may indicate likely treatment failure.

Other amino acid changes in the region around amino acid 486 of the topoisomerase II gene have been associated with changes in the sensitivity of cells to drugs. Yamagishi et al. (14) reported that an aspartic acid to asparagine transition at the gyrase position corresponding to HL-60 topoisomerase II position 462 was associated with nalidixic acid resistance. Suttle et al. (16) reported that an arginine to glutamine transition in position 449 of human leukemia cell topoisomerase II was associated with resistance to a variety of topoisomerase II-reactive drugs. What might the function of this region of topoisomerase II be?

This region is homologous to a region within E. coli DNA polymerase I (17). This region of the polymerase contains a flexible domain that is proposed to seal and unseal a four-sided DNA binding cleft through which DNA travels (18). If topoisomerase II molecules adopt a similar tertiary structure in this region, the modified amino acid in HL-60/AMSA topoisomerase II would occur in the hinge between an α-helix that forms one of the four DNA-binding domains and the flexible domain. Perhaps DNA with amsacrine intercalated cannot slide smoothly through at this position when the more bulky arginine (rather than the less bulky lysine) is present. This may affect topoisomerase II-mediated strand passage. Although this amino acid change appears to be conservative, a recent paper by Aukerman et al. (19) reported that the identical change in a highly conserved region of a maize transcription factor caused the loss of the specific recognition of promoters by the factor. Thus, an apparently conservative amino acid change can result in a large change in the function of a DNA-binding protein.

The insertions in the 5' end of the topoisomerase II cDNA sequence from both of our cell lines compared with the topoisomerase II sequence as published (8) led to a significant alteration in the amino acid sequence. Five of the six amino acids in our sequence precisely match the amino acids in Drosofila (10). At least three of six match the yeast sequence (10). It may be important to use the precise leukemia topoisomerase II gene in transfection studies into these leukemia cell lines to assure accurate transcription, translation, and posttranslational modification to test the ability of the genes to alter the pharmacological phenotype of recipient cells.

The additional differences between the sequences we detected and those already published reside in the 3' end of the gene, a

References


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