Novel missense mutation of the DNA topoisomerase I gene in SN-38-resistant DLD-1 cells

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Abstract

Irinotecan hydrochloride, a camptothecin derivative, is one of the most effective drugs for colorectal cancer, and SN-38 is its main active metabolite. Development of resistance is a major obstacle to the clinical application of this drug. We established an SN-38-resistant subline from DLD-1 human colon cancer cells by continuous exposure to SN-38 and studied the mechanisms of resistance. The resistant subline (designated as DLDSNR6) had 10- to 100-fold higher resistance to camptothecin derivatives but showed no cross-resistance to doxorubicin, mitomycin C, and etoposide. DLDSNR6 cells carried a missense mutation in one allele of the DNA topoisomerase I gene that substituted glycine for serine at amino acid residue 365 accompanied by loss of the latter part of the remaining wild-type allele. Topoisomerase I expression was equal in DLDSNR6 and DLD-1 cells, but the nuclear extract of DLDSNR6 cells showed lower topoisomerase I catalytic activity. Moreover, exposure to camptothecin caused less accumulation of topoisomerase I-DNA complexes in DLDSNR6 cells than in DLD-1 cells. These findings suggest that the mutation interfered with both the catalytic activity of topoisomerase I and the stability of the ternary complex between topoisomerase I, DNA, and SN-38. This SN-38-resistant DLDSNR6 cell line may be useful for understanding the mechanisms of topoisomerase I function and drug-enzyme interactions. [Mol Cancer Ther 2006;5(3):502–8]

Introduction

Camptothecin derivatives have been widely used for cancer chemotherapy over the last two decades. Irinotecan hydrochloride is a promising camptothecin derivative that shows a broad spectrum of activity against advanced lung, ovarian, and colorectal carcinoma as well as hematologic malignancies (1–3). SN-38 is the active metabolite of irinotecan and shows 100- to 1,000-fold greater cytotoxicity than the parent compound (4). Although irinotecan hydrochloride achieves a response rate of ~30% in patients with various cancers, drug resistance is still a critical problem and the mechanisms of resistance still remain to be determined.

DNA topoisomerase I is an essential enzyme in higher eukaryotes and is the prime target of camptothecin derivatives. Topoisomerase I relaxes supercoiled DNA to allow the processes of replication, transcription, and recombination to occur by reversibly nicking one strand and forming transient covalent DNA cleavage complexes (5). While bound to DNA, topoisomerase I guides the relaxation of coiled strands, after which it religates the nicked strand and dissociates from the DNA. Camptothecin analogues stabilize covalent topoisomerase I-DNA complexes and thus cause DNA strand breaks, which lead to the apoptosis of drug-treated cells. Preclinical studies have shown that resistance to camptothecin analogues might result from inadequate accumulation of the drug in tumor cells, quantitative and qualitative changes of topoisomerase I, or changes of the response to the topoisomerase I-DNA interaction (6).

Investigation of the X-ray crystal structure of human topoisomerase I has provided insights into the structure-activity relationships of this enzyme (7, 8). Recently, several structural models have been proposed, which suggest that camptothecin binds at or near the active site and inhibits the DNA religation activity of topoisomerase I (9). Several mutations of topoisomerase I have been identified that confer tumor cell resistance to camptothecin derivatives (10), and the structural models allow such resistance-related mutations to be understood (9). However, these structural models can only explain the in vitro inhibition of religation by camptothecin analogues, so the mechanism of in vivo inhibition is still unclear.

In the present study, we established an SN-38-resistant human colon cancer cell line and identified a novel missense mutation of the topoisomerase I gene. We studied the characteristics of the resistant cells and evaluated the role of mutant topoisomerase I. Our findings may prove useful for improving the structural models of drug-enzyme interactions and for developing new drugs that target topoisomerase I.

Materials and Methods

Drugs and Antibodies

SN-38 was kindly provided by Yakult Co. Ltd. (Tokyo, Japan), topotecan was purchased from LKT Laboratories (St. Paul, MN), and other drugs were purchased from Sigma (St. Louis, MO). SN-38, camptothecin, and topotecan
were resuspended with MeSO as stock solutions and stored at –20°C. Rabbit anti–topoisomerase I antibody was purchased from TopoGEN (Columbus, OH), and mouse anti-DNA topoisomerase II antibody was purchased from MBL (Nagoya, Japan). Rabbit anti–histone H4 antibody and mouse anti-α-tubulin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell Culture and Cytotoxicity Assay**

The DLD-1 human colon cancer cell line was kindly provided by the Cell Resource Center for Biomedical Research Institute of Tohoku University (Sendai, Japan) and was cultured at 37°C in RPMI 1640 supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (HyClone, Logan, UT) under an atmosphere containing 5% CO2. SN-38-resistant DLD-1 cells were selected by continuous exposure to stepwise increasing SN-38 concentrations up to 10 nmol/L for 12 weeks. These cells were cloned by the limiting dilution technique to obtain an SN-38-resistant cell line (DLDSNR6 cells). We routinely checked for the absence of Mycoplasma (Invitrogen, Carlsbad, CA). The full-length topoisomerase I gene was amplified by PCR with primers described previously (12) and sequencing primers of the plasmid vector (M13 and M13 reverse primers). Genomic DNA was prepared from DLD-1 and DLDSNR6 cells using Isogen reagent, and exon 12 of the topoisomerase I gene was amplified by PCR with primers located in the adjacent intron sequences. Parts of introns 8 and 14 were also amplified from genomic DNA (primer sequences and annealing temperatures are available on request). These PCR products were sequenced directly using the PCR primers and BigDye Terminator Version 3.0 Cycle Sequencing kit (Applied Biosystems, Foster City, CA).

**Flow Cytometry**

To analyze the effect of SN-38 on the cell cycle, DLD-1 and DLDSNR6 cells were incubated either without SN-38 or with 10 to 100 nmol/L SN-38 for 48 to 120 hours, and the DNA content was measured. At the indicated time, cells were harvested, fixed in 70% precooled ethanol, and incubated in PBS containing 10 μg/mL propidium iodide and 10 μg/mL RNase A for 1 hour at room temperature. The fluorescence (excitation at 488 nm and emission at 585/42 nm) of 20,000 cells from each sample was measured with a FACSCalibur (Becton Dickinson, San Jose, CA) and the results were displayed as histograms.

**Preparation of Protein Samples and Immunoblotting**

Crude cell extracts were prepared as described previously (11). To extract nuclear proteins, DLD-1 and DLDSNR6 cells were lysed in cell lysis buffer (20 mmol/L HEPES, 20% glycerol, 10 mmol/L NaCl, 1.5 mmol/L MgCl2, 0.2 mmol/L EDTA, 1 mmol/L DTT, 0.1% NP40, 1 mmol/L phenylmethylsulfonyl fluoride) on ice for 15 minutes. Nuclei were collected by centrifugation at 800 × g for 5 minutes and then lysed in nuclear lysis buffer (with the same composition as cell lysis buffer, except for 500 mmol/L NaCl) on ice for 30 minutes. The samples were subsequently centrifuged at 15,000 × g for 15 minutes and the supernatant were stored at –80°C. The protein concentration of each sample was measured by the Bradford method. Protein samples were separated by 7.5% SDS-PAGE and immunoblotting was done using topoisomerase I, topoisomerase II, or histone H4 antibodies.

**Detection of Topoisomerase I Mutation**

Total RNA was extracted from DLD-1 cells and SN-38-resistant DLDSNR6 cells by the acid guanidinium thiocyanate-phenol-chloroform extraction method (Isogen, Nippon Gene, Tokyo, Japan), and reverse transcription was done using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA). The full-length topoisomerase I gene was amplified by PCR with primers: 5'-CAT-GAGTGGGACACCTCCAAACAGATTTCC-3' and 5'-GCTGACAAATTCCCATCCTGCGCCCTTTT3'. The resulting PCR fragments were inserted into the PCR-II-TOPO TA Cloning vector (Invitrogen), and full-length topoisomerase I cDNA was sequenced using primers described previously (12) and sequencing primers of the plasmid vector (M13 and M13 reverse primers). Genomic DNA was prepared from DLD-1 and DLDSNR6 cells using Isogen reagent, and exon 12 of the topoisomerase I gene was amplified by PCR with primers located in the adjacent intron sequences. Parts of introns 8 and 14 were also amplified from genomic DNA (primer sequences and annealing temperatures are available on request). These PCR products were sequenced directly using the PCR primers and BigDye Terminator Version 3.0 Cycle Sequencing kit (Applied Biosystems, Foster City, CA).

**Plasmid Relaxation Assay**

The topoisomerase I catalytic activity of nuclear extracts from DLD-1 and DLDSNR6 cells was assayed by measuring relaxation of the supercoiled pHOT1 plasmid (TopoGEN). This plasmid contains a topoisomerase I cleavage site that is derived from the *Tetrahymena* ribosomal gene repeat (13). Supercoiled pHOT1 plasmid (0.25 μg) was incubated with 13 to 200 ng nuclear extract in 10 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, and 1 mmol/L EDTA at 37°C for 30 minutes (final volume, 20 μL). Then, the reaction was terminated by adding 5 μL of 0.05% SDS and the samples were loaded onto 1% agarose gel. After electrophoresis, the gel was stained with Tris-borate EDTA buffer [89 mmol/L Tris-borate, 2 mmol/L EDTA (pH 8.0)] containing 0.5 μg/mL ethidium bromide and visualization was done by transillumination with UV light. Experiments were done in triplicate and the relative fluorescence of supercoiled DNA in each sample was determined with ImageJ software.2

**Band Depletion Assay**

Exponentially growing DLD-1 and DLDSNR6 cells were harvested and 10⁶ cells were incubated for 30 minutes in 1 mL RPMI 1640 containing 0 to 20 μmol/L camptothecin. After drug treatment, the cells were lysed with lysis buffer containing 6 mol/L guanidine hydrochloride, and these lysates were dialyzed against 4 mol/L urea and then against 0.1% SDS (14). The samples were mixed with 0.25 volume of 5× SDS sample buffer [125 mmol/L Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol] and incubated at 65°C for 20 minutes. Then, aliquots

2 http://rsb.info.nih.gov/ij/
containing $10^5$ cells were separated by SDS-PAGE and immunoblotting was done using topoisomerase I antibody to detect free topoisomerase I (100 kDa), with $\alpha$-tubulin antibody as the loading control. The relative intensity of immunoblot signals (topoisomerase I/$\alpha$-tubulin) was determined with ImageJ software.

Results

Cross-Resistance of DLDSNR6 Cells to Camptothecin Derivatives

A resistant cell line, DLDSNR6, was derived from the DLD-1 human colon cancer cell line by continuous exposure to SN-38. We initially examined DLDSNR6 cells for resistance to SN-38 and other camptothecin derivatives by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulphonyl)-2H-tetrazolium, inner salt assay, which showed that these cells exhibited resistance to SN-38, camptothecin, and topotecan with a 10- to 100-fold increase of IC\textsubscript{50}s (Fig. 1), whereas there was no cross-resistance to doxorubicin and mitomycin C (data not shown). DLDSNR6 cells were hypersensitive to etoposide (IC\textsubscript{50}, 28.8 $\mu$mol/L for DLD-1 cells and 4.43 $\mu$mol/L for DLDSNR6 cells).

Cell Cycle Analysis of SN-38-Treated Cells

We analyzed the cell cycle distribution of DLD-1 and DLDSNR6 cells to determine how SN-38 affected cell cycle progression (Fig. 2). When parental DLD-1 cells were treated with 10 nmol/L SN-38 for 48 hours, accumulation in G\textsubscript{2} phase was observed. Treatment with higher SN-38 concentrations (40–100 nmol/L) for 48 hours caused accumulation of DLD-1 cells in late S to G\textsubscript{2} phase. A significant increase of apoptotic cells (recognized as the sub-G\textsubscript{1} population) was observed after DLD-1 cell were exposed for 120 hours to 10 nmol/L SN-38. On the other hand, DLDSNR6 cells only showed slight accumulation in G\textsubscript{2} phase after exposure to SN-38 for 48 hours, and few apoptotic cells were observed after exposure to 10 nmol/L SN-38 for 120 hours. These data suggested that SN-38 failed to cause DNA damage followed by G\textsubscript{2} arrest and apoptotic death in DLDSNR6 cells.

Topoisomerase I Expression in Resistant DLDSNR6 Cells

A decrease of topoisomerase I protein expression is a common mechanism by which cancer cells develop resistance to camptothecin (15–17). Therefore, we examined topoisomerase I expression in the DLD-1 and DLDSNR6 cell lines by immunoblotting, which showed that topoisomerase I protein levels were equal in DLD-1 and DLDSNR6 cells (Fig. 3). We also examined topoisomerase II expression because DLDSNR6 cells were more sensitive to the topoisomerase II inhibitor etoposide than DLD-1 cells (data not shown), but topoisomerase II protein levels were also equal in both cell lines.

Topoisomerase I Mutation in DLDSNR6 Cells

Topoisomerase I mutations that confer resistance to camptothecin derivatives have been detected in several studies of mammalian cells (12, 18, 19). We sequenced the topoisomerase I gene by reverse transcription-PCR analysis. Amplified fragments of the full-length coding region of topoisomerase I from DLD-1 and DLDSNR6 cells were subcloned into the PCR-II vector and each of 10 individual clones was sequenced. DLDSNR6 cells carried a topoisomerase I point mutation (GGC-to-AGC at codon 365), which resulted in alteration of glycine to serine (G365S; Fig. 4A). No wild-type allele was observed in DLDSNR6 cells, whereas DLD-1 cells only had the wild-type allele. Genomic DNA from each cell line was also amplified and sequenced, but the wild-type allele was not detected in DLDSNR6 cells (Fig. 4B; exon 12). Direct DNA sequence analysis revealed that DLD-1 cells carried three single nucleotide polymorphisms with heterozygosity. All these single nucleotide polymorphisms were identified previously.\(^4\) Two (SNP6072269 and SNP6124315) of the three single nucleotide polymorphisms were in intron 8 and one (SNP1980456) was in intron 14 (Fig. 4B). SNP6124315, which is in the latter part of exon 8, and SNP1980456 showed loss of heterozygosity in DLDSNR6 cells.

Topoisomerase I Catalytic Activity of Nuclear Extracts

Next, we studied the activity of G365S-topoisomerase I. The catalytic activity of topoisomerase I in each cell line was determined by adding nuclear extracts to a reaction mixture containing pHOT1 plasmid as the substrate. To prevent other enzymes from acting on the substrate DNA, EDTA was added to the reaction mixture, but it did not contain ATP. Under such experimental conditions, nicking of supercoiled pHOT1 was due to topoisomerase I activity. Although topoisomerase I expression was equal in both cell lines (Fig. 3), the topoisomerase I activity of DLDSNR6 cells was lower than that of DLD-1 cells. Almost twice the amount of nuclear extract from DLDSNR6 cells was required to obtain the same relaxation as that produced by extract form DLD-1 cells (Fig. 5).

Formation of Topoisomerase I-DNA Complexes and Band Depletion Assay

Topoisomerase I forms transient covalent complexes with DNA and treatment with camptothecin analogues increases the number of such complexes (20). If cells undergo rapid denaturation after treatment with camptothecin, the covalent complexes are trapped irreversibly. Topoisomerase I that is covalently bound to DNA migrates more slowly than free topoisomerase I on SDS-PAGE, so a decrease of free topoisomerase I on immuno blotting indicates an increase of topoisomerase I-DNA covalent binding (21). Accordingly, we examined the formation of topoisomerase I-DNA complexes in DLD-1 and DLDSNR6 cells. The free topoisomerase I band was markedly diminished by treatment of DLD-1 cells with 5 μmol/L camptothecin (Fig. 6). In contrast, the free topoisomerase I band showed little decrease at even higher concentrations of camptothecin in the case of DLDSNR6 cells.

Discussion

In this study, we established an SN-38-resistant subline from the DLD-1 human colon cancer cell line and identified a novel missense mutation in the topoisomerase I gene that involved substitution of glycine for serine at codon 365. DLDSNR6 cells exhibited a 10- to 100-fold increase of resistance to camptothecin analogues compared with parental DLD-1 cells.

DNA-damaging agents like camptothecin analogues activate DNA damage checkpoint kinases (ATM and ATR) in mammalian cells, and these kinases phosphorylate Chk1 and Chk2. The Chk kinases inactivate cdc25, blocking the activation of cdc2 and cell cycle transition into M phase. Exposure to low concentrations of SN-38 causes arrest in G2 phase for both wild-type p53 and mutant p53 cells (22). In DLDSNR6 cells, however, SN-38 only had a slight influence on cell cycle progression and on the induction of apoptosis. This suggested that accumulation of DNA damage, which influences cell cycle check point regulation, was reduced in DLDSNR6 cells. It is also compatible with the high resistance of these cells in the cytotoxicity assay.

Inadequate drug accumulation in tumor cells, reduced expression of topoisomerase I, and mutation of topoisomerase I are the possible mechanisms of resistance to camptothecin derivatives (6). Several studies have shown
previously that ATP-binding cassette proteins are involved in drug efflux and the resistance of mammalian cells to camptothecin analogues (23, 24). The rhodamine 123 efflux assay can predict the activity of ATP-binding cassette proteins, such as P-glycoprotein and MRP, but we found that the pattern of efflux from DLDSNR6 cells was almost the same as that from DLD-1 cells (data not shown). Moreover, DLDSNR6 cells did not show cross-resistance to drugs transported by P-glycoprotein, such as doxorubicin and etoposide. These findings suggested that the mechanism of resistance might be related to some alteration of topoisomerase I.

DLDSNR6 cells carried the G365S mutation, but no other mutation was identified in the topoisomerase I gene. Neither the wild-type topoisomerase I mRNA nor the genomic DNA was detected. Based on the known crystal structure of topoisomerase I, this mutation site would be located close to the catalytic Tyr^222^ residue (7). Topoisomerase I is composed of four domains (NH2-terminal, core, linker, and COOH-terminal domain), and the core domain (residues 215–635) contains all of the active site residues, except the catalytic tyrosine. The core domain is divided into three subdomains that form two distinct lobes, and these lobes clamp around the DNA when topoisomerase I is in action (7). Most of the reported topoisomerase I mutations that confer resistance to camptothecin are located sterically close to the catalytic tyrosine, and some of the drug-resistant mutations cluster in the cap region (10, 12).

Some previous studies showed that amino acid residues 361 to 364 of human topoisomerase I are involved in both enzyme catalysis and camptothecin resistance, and mutations of this region result in cellular resistance to camptothecin derivatives (10, 25).

Conserved in eukaryotes and has homology to sequences in other nucleic acid-binding proteins (25). According to structural binding model, lactone forms of camptothecin derivatives can interact with the Arg^364^ residue (7).

We investigated the effect of the G365S mutation in human topoisomerase I with respect to catalytic enzyme activity and drug sensitivity. The decrease of topoisomerase I catalytic activity and the reduced accumulation of...
topoisomerase I-DNA complexes when DLDSNR6 cells were exposed to camptothecin indicated that the G36SS mutation altered the function of this enzyme. The change in function of this mutant enzyme was presumably due to close proximity of the mutation site, Arg364 that interacts with camptothecin analogues, and catalytic Tyr233. However, further studies are needed to thoroughly evaluate the structure-function relationships of this mutant enzyme.

The DLD-1 human colon cancer cell line exhibits genomic instability derived from homozgyous mutation in the hMSH6 gene (26). Human MSH6 forms a protein complex with MSH2 and recognizes base-to-base mispairing of DNA molecules, such as G/T mismatch (27). Dysfunction of this mismatch repair gene leads to a genome-wide increase in the rate of mutation. In DLDSNR6 cells, the Gly360 (GCC) codon of the topoisomerase I gene was changed to serine (AGC). Furthermore, lack of the wild-type topoisomerase I enzyme is necessary for these cells to acquire high-level resistance to camptothecin derivatives, because camptothecin converts the wild-type topoisomerase I to a cellular poison. DLDSNR6 cells carried a missense mutation in one topoisomerase I allele accompanied by loss of the latter part of the remaining wild-type allele.

In most clinical protocols, camptothecin derivatives are given intermittently and they show a response rate of ~30%. Although some studies investigated topoisomerase I mutations in clinical specimens, most of them yielded negative results (28). In the present study, DLDSNR6 cells were established by long-term continuous exposure of cells to stepwise increasing concentrations of SN-38, and the drug concentrations used in the experiment were relatively low compared with the serum concentration that can be achieved in clinical use. This experimental condition might allow DLD-1 cells to generate two genetic processes. However, the drug concentration in vivo is affected by many factors and it may also be lower in target tissue. Therefore, the administration schedule of camptothecin derivatives must be carefully determined to avoid developing resistant mutants, especially when they are given continuously at low doses.

In conclusion, a novel missense mutation of topoisomerase I (G365S) causes resistance to camptothecin derivatives in DLDSNR6 cells, and investigation of this mutant may be useful not only for understanding enzyme function but also for developing new drugs that act on topoisomerase I.

References


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