Increased Phosphorylation of DNA Topoisomerase II in Etoposide-resistant Mutants of Human Cancer KB Cells

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ABSTRACT

We have isolated two etoposide (VP16)-resistant cell lines, KB/VP-1 and KB/VP-2, from human cancer KB cells after stepwise exposure to increasing doses of VP16. KB/VP-1 and KB/VP-2 showed 30- and 50-fold higher resistance to VP16 and also 20- and 30-fold higher resistance to teniposide than the parent cell line. Furthermore, both resistant cell lines showed more than 2-fold cross-resistance to Adriamycin and daunomycin than KB cells. The levels of accumulation and outward transport of radioactive VP16 were similar in KB/VP-1, KB/VP-2, and KB. The activity of nuclear extracts of DNA topoisomerase II for both KB/VP-1 and KB/VP-2 assayed by decaturation of kinetoplast DNA was consistently similar to that of KB. However, in both immunoblot assay with specific anti-topoisomerase II antibody and Northern blot analysis with specific human DNA topoisomerase II complementary DNA, cellular levels of topoisomerase II in both resistant cell lines were less than onetenth the level in KB. The cellular levels of DNA topoisomerase I, however, were similar between the mutants and their parent. A quantitative precipitation assay of covalent DNA-topoisomerase II complexes showed greatly reduced VP16-induced cleavages of 3'-32P-DNA by nuclear extracts of KB/VP-1 or KB/VP-2 cells in comparison with KB cells. The relative specific phosphorylation of DNA topoisomerase II was about 14- to 18-fold higher in the mutants than in the parental cells. Phosphoamino acid analysis of DNA topoisomerase II showed that serine was the phosphorylated amino acid in all three cell lines, KB, KB/VP-1, and KB/VP-2. These data suggest that reduced expression of DNA-topoisomerase II might account for the acquired VP16 resistance and reduced VP16-induced cleavages of DNA-topoisomerase II complexes in both VP16-resistant variants.

INTRODUCTION

DNA topoisomerases I and II in eukaryotic cells catalyze the topological conformation of DNA molecules by the concerted breakage of single or double strands (1). DNA topoisomerase II is involved in DNA replication, transcription, recombination, and mitosis. Podophyllotoxin derivatives, VP16 and VM26, are topoisomerase II-targeting antitumor agents (2-4). VP16/VM26 and other intercalating antitumor agents induce DNA topoisomerase II-mediated DNA cleavages (5-8). An enzyme subunit of topoisomerase II is covalently linked to the 5'-phosphoryl end of the nicked DNA forming a "cleavable complex" (9). VP16, VM26, and DNA-intercalating agents such as Adriamycin and m-amsacrine appear to stabilize the cleavable complex, leading to the inhibition of the strand passage, and consequently cause the accumulation of protein-associated DNA double-strand breakages (5, 7, 8).

Received 2/19/91; accepted 5/24/91.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1. This study was supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan; a Research Grant of the Princess Takamatsu Cancer Research Fund; and also by the U.S.-Japan cooperative cancer research program.

2. To whom requests for reprints should be addressed.

3. The abbreviations used are: VP16, etoposide; VM26, teniposide; MEM, minimal essential medium; PBS, phosphate-buffered saline (137 mM NaCl, 8 mM Na2HPO4, 2H2O, 2.7 mM KCl, 1.5 mM KH2PO4, 2 mM EDTA, 10 mM MgCl2, 10 mM CaCl2, 50 mM NaHCO3, 100 mM sodium citrate, pH 7.0).

One way to identify cellular lesions interacting with VP16/VM26 is to characterize somatic cell mutants with altered sensitivity to DNA topoisomerase II inhibitors (3). Among the possible mechanisms which may involve the altered sensitivity to VP16/VM26 are quantitative and qualitative changes of DNA topoisomerase II (10-14), altered drug accumulation (11), and altered double strand break rejoins in the repair of drug-induced lesions (15). We recently isolated VM26-resistant cell lines from human cancer KB cells that were characterized by decreased activities and contents of DNA topoisomerase II and also decreased cellular accumulation of VP16 (16). In the present study, we have independently isolated and characterized variants resistant to VP16 which have unique properties different from those previously isolated.

MATERIALS AND METHODS

Cell Lines. VP16-resistant cell lines were obtained from human epidermoid carcinoma KB cells by sequential selection with increasing doses of VP16 without the use of mutagen (16). KB cells, its VP16-resistant variants, and other cell lines were grown in monolayer in MEM (Nissui Seiyaku Co., Tokyo, Japan) containing 10% newborn calf serum (Microbiological Associates, Bethesda, MD), 1 mg/ml Bacto-Peptone (Difco Laboratories, Detroit, MI), 0.292 mg/ml glucose, 100 µg/ml kanamycin, and 100 units/ml penicillin (16-18).

Chemicals. cis-Platinum and VP16 were obtained from Nihon Kayaku Co., Tokyo, Japan; VM26 was from Bristol Myers Co., Kanagawa, Japan; Adriamycin, daunomycin, and vincristine were from Sigma Chemical Co., St. Louis, MO. Camptothecin was from Yakult Co., Tokyo, Japan. [3P]VP16 (388 mCi/mmol) was donated from New England Nuclear, Boston, MA. [3H]VP16 (388 mC/mmol) was donated from Nihon Kayaku Co., Tokyo, Japan.

 Colony Formation. To assay colony formation, we seeded 300 cells of KB and its variants in a 35-mm dish in the absence of drugs at 37°C for 18 h. Cells were then incubated for an additional 10 days with various drugs, freshly prepared in dimethyl sulfoxide or ethanol, and controls were done adding the same amount of the solvent (16-18).

Colonies were counted after Giemsa staining.

Preparation of Crude Nuclear Extracts. Crude nuclear extracts were prepared from 1 x 10⁶ cells in early log phase culture according to the method published by Duguet et al. (19). Briefly, cells were washed 3 times with cold PBS, harvested with a rubber scraper, and pelleted by centrifugation. Cell pellets were then lysed in 10 mM Tris-Cl (pH 7.5), 1.5 mM MgCl2, 10 mM NaCl, and 1% Nonidet P-40. Nuclei were centrifuged at 600 x g for 10 min and suspended in 2 ml of 50 mM Tris-Cl (pH 7.5)-25 mM KC1-2 mM CaCl2-5 mM MgCl2 buffer, and the suspension was layered over a 0.6-ml cushion of this buffer containing 0.6 M sucrose. The mixture was centrifuged at 2000 x g for 10 min. The nuclear pellets were washed once with 2 ml of 50 mM Tris-Cl (pH 7.5)-25 mM KC1-5 mM MgCl2-0.25 M sucrose and resuspended in 0.2 ml of TKM buffer, and 20 µl of 0.2 M EDTA (pH 8.0) were added followed by 2 volumes of 80% TA.TP (pH 7.5), 1 mM dithiothe-itol, 2 mM EDTA, 0.53 M NaCl, and 20% glycerol; the final concentration of NaCl was 0.35 M. Extraction then proceeded on ice for 60 min, and then the mixture was centrifuged at 17,000 x g for 15 min. The protein concentration in the extracts was determined by the method of Bradford (20). Small portions of the extract were stored in 40% glycerol with 1 mg/ml of bovine serum albumin at -80°C.
DNA Topoisomerase II Assays. The standard reaction for DNA topoisomerase II assay contained 50 mm Tris-HCl (pH 7.5), 85 mm KCl, 10 mm MgCl₂, 5 mm diithiothreitol, 0.5 mm EDTA, bovine serum albumin (0.03 mg/ml), and 1 mm ATP. The decatenation reaction of catenated DNA was carried out with serial dilutions of extract and 0.25 μg of kDNA in a final volume of 20 μl at 30°C for 30 min. The kDNA was prepared from Crithidia fasciculata, which was obtained from Dr. P. T. Englund (Johns Hopkins School of Medicine), and kDNA was purified from a Sarkosyl extract of the trypanosomes by cesium chloride-ethidium bromide density centrifugation as described by Englund (21) and Matsuo et al. (16). The reaction was terminated with 2 μl of 1.2 mg/ml proteinase K and 22 mm EDTA and followed incubation for 15 min at 37°C. Then, 5 μl of 0.05% bromophenol blue in 50% glycerol were added to the reaction mixture. Samples were then electrophoresed through a 1% agarose gel. After staining with ethidium bromide, gels were photographed under UV illumination.

Cellular Accumulation Assay for [³²P]VP16. KB or VP16-resistant variants at 2.5 × 10⁶ cells/well in a 24-well dish were plated and incubated for 2 days at 37°C. Medium was then replaced with serum-free MEM, and the cells were incubated with 1 μCi/ml [³²P]VP16 for various times up to 60 min at 37°C. Cells were washed three times with ice-cold PBS and lysed with 200 μl of 0.05% SDS and mixed thoroughly with 3 ml of Scintisol (Wako Chemical Co., Osaka, Japan), and samples were then counted (16). For the drug efflux assay, each culture well was washed after incubation for 60 min at 37°C with [³²P]VP16 3 times with PBS, followed by addition of fresh serum-free medium and incubation for various time intervals at 37°C. Cell-associated radioactivity was then determined.

RNA Analysis with Topoisomerase II cDNA Probe. High molecular weight DNA was isolated from each cell line by the method of Maniatis et al. (22), and cytoplasmic RNA was isolated according to methods previously reported (16, 23, 24). Human topoisomerase II complementary DNA probe (pBS-hTOP2) (25) was obtained from J. C. Wang (Harvard University), and genomic fragment from Ha160 for β-actin (16) were also used as probes. For Northern blot hybridization, 20-μg samples of RNA were electrophoresed in 1% agarose/2.2 M formaldehyde gel followed by transfer to nitrocellulose filters (23, 24). [³²P]-labeled complementary DNA fragments with a specific activity of 10⁶ cpm/μg DNA were prepared by random primer method (23). Hybridization was carried out in 50% deionized formamide, 5 × Denhardt’s solution, 5 × SSC, and 10% dextran sulfate at 42°C for 18 h. Filters were washed twice in 2 × SSC and 0.1% SDS at room temperature and then twice in 0.1 × SSC and 0.1% SDS.

Western Blot Determination of DNA Topoisomerase I and II. Nuclear protein fractions extracted from 10⁶ cells were run on 7.5% polyacrylamide gel electrophoresis. Protein fractions from the gels were electrophoretically transferred to nitrocellulose filters in 25 mm Tris-HCl (pH 8.3), 92 mm glycine, and 20% methanol for 2 h at 20 V (16). Nitrocellulose membranes were further incubated with antibody against human DNA topoisomerase I and II (1:2000) for 1 h at room temperature. The membranes were rinsed with PBS, treated with biotinylated secondary antibody, and developed according to the manufacturer’s specifications (Vectastain ABC-Go kit; Vector Laboratories, Burlingame, CA). Antibodies to the human DNA topoisomerase I and II were gifts of Dr. L. F. Liu (Johns Hopkins School of Medicine).

Cleavage Complex Formation Assay. The assay for precipitation of DNA-protein complexes was determined by the method of Liu et al. (9). Cleavage assay was done in a reaction mixture containing 10 mm Tris (pH 7.0), 1 mm MgCl₂, 0.5 mm EDTA, 10 μg/ml bovine serum albumin, nuclear extracts (50 μg/ml), about 5 × 10⁵ cpm [³²P]-labeled DNA, and various doses of VP16 in a final volume of 50 μl at 37°C for 5 min. For preparation of the [³²P]-labeled DNA, pUC19 was digested with SmaI under reaction conditions recommended by the manufacturer. The linear plasmid was then labeled at the 3’ ends with [³²P]dCTP by the Klenow fragment of DNA polymerase I and the labeled DNA was digested with EcoRI again. Unincorporated nucleotides were removed by three sequential precipitations with ethanol-ammonium acetate (22). The reaction was terminated by addition of 100 μl of a stop solution containing 2% SDS, 2 mm EDTA, and 0.5 mg/ml of salmon sperm DNA and heated to 65°C for 10 min. Precipitation of the topoisomerase-DNA complex was achieved by addition of 50 μl of 0.25 M KCl and the mixture was incubated on ice for 10 min. The precipitate was collected by centrifugation in an Eppendorf centrifuge 15 min in the cold room. The supernatant was aspirated and the pellet was washed once with 200 μl of a solution containing 10 mm Tris (pH 8), 100 mm KCl, 1 mm EDTA, and 100 μg/ml of carrier salmon sperm DNA at 65°C for 10 min. Following cooling on ice and recentrifugation, the pellet was resuspended in 200 μl of H₂O with heated to 65°C. The suspension was then quickly transferred to a vial containing 3 ml of scintillation fluid and counted.

DNA Topoisomerase II Phosphorylation. Phosphorylation and immunoprecipitation were done using a modification of a technique published by Seguchi et al. (26) and Hamanaka et al. (27). The cells were plated in 100-mm dishes. The cells were washed with PBS, 4 ml of phosphate-free, serum-free MEM were added, the mixture was incubated for 4 h and [³²P] (final 50 μCi/ml) was added, followed by incubation for 1 h prior to preparation of nuclear extracts (see above). The nuclear extracts were incubated with antibody against human DNA topoisomerase II for 1 h at 4°C in 500 μl of 50 mm Tris-HCl (pH 7.5)-150 mm NaCl-0.1% Nonidet P-40-1 mm EDTA-0.25% gelatin-0.02% sodium azide buffer. Protein A-Sepharose was added to the reaction mixture followed by incubation for 1 h at 4°C with gentle rocking. The mixture was then centrifuged 12,000 × g for 20 s, the supernatant was removed, and 1 ml of the Tris-HCl-NaCl-Nonidet P-40-EDTA-gelatin-sodium azide buffer was added. This procedure was repeated twice and the resulting mixture was finally washed with buffer (10 mm Tris-HCl, (pH 7.5)-0.1% Nonidet P-40) and centrifuged at 12,000 × g for 20 s at 4°C; the supernatant was removed. The supernatants were subjected to electrophoresis on 7.5% SDS-acrylamide gel. The gel was dried and autoradiographed.

Phosphoamino Acid Analysis. Phosphoamino acid analysis was done according to the published method of Saijo et al. (28). Briefly, phosphorylated proteins were separated by electrophoresis in 7.5% SDS-acrylamide gel. The bands corresponding to phosphorylated topoisomerase II were located by both Coomassie blue staining and autoradiography and excised. The gel slices were soaked in 45% ethanol for 30 min, 10% ethanol for 30 min, and 10% 2-propanol for 30 min and washed with water three times. The gel slices were crushed, and the polypeptides in the gel were hydrolyzed in N HCl for 2 h at 110°C. The samples were mixed with phosphoamino acid standards (1 mg/ml) and analyzed by thin-layer electrophoresis at 1000 V at pH 1.9 for 40 min and then pH 3.5 for 30 min. Standards were located by ninhydrin staining, and [³²P] was visualized by autoradiography.

RESULTS

Establishment of VP16-resistant Cell Lines of Human Cancer KB Cells. VP16-resistant cell lines were selected from KB cells after a stepwise selection by sequential treatment with increasing doses of VP16 with no added mutagen according to our published report (16). KB cells growing in 100-mm plastic dishes were first exposed to 100 ng/ml VP16, a dose to reduce the surviving fraction to 10% of the initial fraction, for 1 month, and then the cells were exposed to 200, 300, and 400 ng/ml of VP16, each for 1 month. During exposure to the drug, medium was replaced with fresh medium containing freshly prepared VP16 every 3 days. After exposure to 400 ng/ml for 1 month, colonies appearing in the presence of V16 were purified and cloned. A cell line, KB/VP-1, was isolated from the dish. Another cell line, KB/VP-2 was isolated and purified after exposure of KB/VP-1 to 500 ng/ml VP16 for 5 months. We have further characterized KB/VP-1 and KB/VP-2 cells.

Growth of the parental KB cells was inhibited by 100 and 500 ng/ml of VP16 but those of both resistant cell lines were unaffected by 500 ng/ml of VP16. Doubling times of KB/VP-1 and KB/VP-2 were 27 and 25 h, respectively, when that of the parental KB was 20 h.
The relative resistance of KB/VP-1 and KB/VP-2 to VP16 and other drugs was compared to KB cells by colony formation assays. Drug resistance was assessed by three independent trials of dose-response curves to each agent. Data are summarized in Table 1. KB/VP-1 and KB/VP-2 were 30- to 50-fold more resistant to VP16 than KB. KB/VP-1 and KB/VP-2, which exhibited 19- and 30-fold higher resistance to VM26 than KB, were 3- to 9-fold more cross-resistant to daunomycin and Adriaamycin and slightly more (1.7- to 2.0-fold) cross-resistant to vincristine (Table 1). KB/VP-1 and KB/VP-2 were not cross-resistant to camptothecin, a topoisomerase I-inhibitory antibiotic, and novobiocin, a gyrase-inhibitory antibiotic. With respect to cellular sensitivity to cis-platinum, both KB/VP-1 and KB/VP-2 were 2-fold more sensitive to cis-platinum than KB. The collateral sensitivity to cis-platinum of both cell lines was found to be reproducible, but the biological meaning remains to be determined. KB/VP-1 and KB/VP-2 show cross-resistance to other multiple agents similar to that of VM26-resistant mutants of KB which we have recently isolated (16), but the resistance spectrum appears to be different from those of typical multidrug-resistant cells selected in vincristine or colchicine (23, 24). There appeared to be no enhanced expression of a multidrug resistance gene mRNA in KB/VP-1 and KB/VP-2 cells.3

Drug Accumulation and Efflux of VP16. Decreased accumulation of VP16 might involve in VP16 resistance of KB/VP-1 or KB/VP-2 cells. Accumulation of [3H]VP16 in each cell line reached stationary levels within 5 min at 37°C. The cellular accumulation of [3H]VP16 in both KB/VP-1 and KB/VP-2 cells was about 80 to 90% of that in KB cells (Fig. 1). Outward transport of VP16 was very rapid and reached the stationary state within 5 min after incubation in drug-free medium (Fig. 1). No significant change was seen in the drug efflux of [3H]VP16 in both KB/VP-1 and KB/VP-2 cells in comparison with KB cells.

Cellular Levels of DNA Topoisomerase II. Antitumor activity of VP16 is mediated through interaction of the drugs with their target enzyme, and DNA topoisomerase II might be changed in KB/VP-1 and KB/VP-2 cells. DNA topoisomerase II assays with 0.35 mM NaCl nuclear extracts were carried out by measuring the decatenation of kDNA to minicircles. Decatenation of kDNA by serial dilution of the nuclear extracts from KB/VP-1 and KB/VP-2 cells showed activities similar to those of KB cells (Fig. 2).

Mammalian DNA topoisomerase I is a monomeric M, 100,000 protein and DNA topoisomerase II is a homodimeric M, 170,000 protein (29). To quantitate DNA topoisomerase I and II, topoisomerase I and II levels in nuclear extracts of KB, KB/VP-1, and KB/VP-2 were compared to Western blot with specific antibodies against both enzymes. As seen in Fig. 3, the cellular levels of topoisomerase I were similar in the three cell lines, but the cellular levels of topoisomerase II were apparently lower in both KB/VP-1 and KB/VP-2 than KB. The relative intensities of the bands showed that the amount of topoisomerase II in both resistant cell lines was much less than that in the parental cells. To examine whether the decreased levels of DNA topoisomerase II was due to decreased expression of the DNA topoisomerase II gene, Northern blot analysis was done with human DNA topoisomerase II cDNA (25). The topoisomerase II specific cDNA probe hybridized to a 6.2-kilobase RNA species, and decreased expression of the 6.2-kilobase topoisomerase II mRNA was observed in both KB/VP-1 and KB/VP-2 cells in comparison with KB cells (Fig. 4). Cellular levels of β-actin mRNA were similar among the three cell lines. Cellular

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**Table 1 Cross-resistance to other drugs of VP16-resistant cell lines of KB cells**

Relative resistance of KB/VP-1 and KB/VP-2 is presented when LD₉₀ of each cell line is divided by LD₉₀ of KB. LD₉₀ is the dose required to reduce the initial surviving fraction to 10% when dose-response curves of each cell line to various agents indicated are examined by colony formation assays. Averages for KB cells are derived from 8 separate experiments, and those for KB/VP-1 and KB/VP-2 cells are derived from 4 to 6 separate experiments.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>KB cells</th>
<th>Relative resistance</th>
<th>KB/VP-1</th>
<th>KB/VP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LD₉₀ᵃ</td>
<td>KB/VP-1</td>
<td>KB/VP-2</td>
<td>KB/VP-1</td>
</tr>
<tr>
<td>VP16</td>
<td>341.9 ± 97.2</td>
<td>28.0ᵃ</td>
<td>51.0ᵃ</td>
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<tr>
<td>VM26</td>
<td>38.5 ± 7.9</td>
<td>19.0ᵃ</td>
<td>30.0ᵃ</td>
<td></td>
</tr>
<tr>
<td>Daunomycin</td>
<td>11.1 ± 5.2</td>
<td>4.0ᵃ</td>
<td>3.5ᵃ</td>
<td></td>
</tr>
<tr>
<td>Vincristine</td>
<td>2.8 ± 0.4</td>
<td>2.0</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>Adriamycin</td>
<td>18.5 ± 5.5</td>
<td>5.0ᵃ</td>
<td>9.0ᵃ</td>
<td></td>
</tr>
<tr>
<td>Camptothecin</td>
<td>2241.4 ± 119.9</td>
<td>1.0</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>Novobiocin</td>
<td>196 ± 20.0</td>
<td>1.0</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>cis-Platinum</td>
<td>383 ± 44.1</td>
<td>0.6</td>
<td>0.5</td>
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ᵃ LD₉₀ for all drugs except novobiocin was derived from the report of Matsuo *et al.* (16). The LD₉₀ for novobiocin was estimated from dose-response curves in this study. Mean ± SD.

*b* Degree of resistance significantly different (*P* < 0.01) from that of KB cells.

*c* Concentration of LD₉₀ for novobiocin is in μmol.

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*H. Takano, unpublished data.*
ETOPOSIDE RESISTANCE MUTATION

A.

Topo I →

KB

KB/VP-1

KB/VP-2

B.

Topo II →

Fig. 3. Western blot analysis of DNA topoisomerase I and II from KB, KB/VP-1, and KB/VP-2 cells. Nuclear protein fractions of each cell line were run on 7.5% SDS-polyacrylamide gel and transferred to the nitrocellulose filters. Nitrocellulose membrane filters were incubated with antibody against DNA topoisomerases I (A) and II (B) and biotinylated goat anti-rabbit IgG. Fifty μg of nuclear proteins were applied to each lane. Arrow, DNA topoisomerase II with molecular weight of about 170,000 and DNA topoisomerase I with molecular weight of about 110,000.

levels of both DNA topoisomerase II and its mRNA were apparently much lower in the resistant cell lines than their parental counterpart.

Effect of VP16 on DNA Cleavages by DNA Topoisomerase II. To evaluate the effect of VP16 on the ability of DNA topoisomerase II to cleave DNA, we quantitated the protein-DNA complexes stabilized by VP16 in the absence of ATP, according to the method of Liu et al. (9). The presence of 50 μM doses of VP16 or higher caused about 3-fold increase in the amounts of cleavage mediated by the nuclear extracts from KB cells (Fig. 5). However, there appeared to be no significant increase in drug-mediated cleavage with nuclear extracts from both KB/VP-1 and KB/VP-2 cells. The differential effect of VP16 on cleavable complex formation between KB and KB/VP-1 or KB/VP-2 extracts was also apparent in the presence of ATP.

Phosphorylation of DNA Topoisomerase II. Both KB/VP-1 and KB/VP-2 cells had decreased levels of topoisomerase II, but they showed comparable enzymatic activities. We thus asked whether the topoisomerase II itself was activated or whether other topoisomerase II-related activity was compensatorily elevated in the resistant variants. DNA topoisomerase II is phosphorylated and phosphorylation increases the enzymatic activity (30–35). To examine whether phosphorylation may be responsible for the increased enzymatic activity in KB/VP-1 and KB/VP-2, we compared the cellular levels of the topoisomerase II phosphorylation in the parental and VP16-resistant cells. Immuno blot assay with specific antibody against DNA topoisomerase II again showed about one-twentieth of the enzyme levels in KB/VP-1 and KB/VP-2 cells as that of KB cells (Fig. 6A). By contrast, phosphorylation of the immunoreactive topoisomerase II of KB/VP-1 and KB/VP-2 cells was approximately similar to that of KB cells (Fig. 6B). The relative specific activity of incorporated phosphate in the topoisomerase II is presented in Table 2. Relative values were obtained after dividing the radioactivity of topoisomerase II by the total density of immunoreactive polypeptide from the immunoblots. The relative specific activity of the incorporated phosphate in the two resistant cell lines was 14- to 18-fold higher than that in KB cells. The increase in phosphorylation activity of DNA topoisomerase II in the resistant cell lines may contribute to similar catalytic activities in the nuclear extracts of KB and KB/VP-1 or KB/VP-2 cells. To examine which

Fig. 4. Northern blot analysis for DNA topoisomerase II in KB, KB/VP-1, and KB/VP-2 cells. Twenty μg of RNA from KB, KB/VP-1, and KB/VP-2 cells were used for hybridization with complementary DNAs of DNA topoisomerase II and β-actin. Arrows, DNA topoisomerase II (6.2 kilobases) mRNA and β-actin mRNA. Equal amounts of RNAs were applied to each lane.
Fig. 5. Effect of VP16 on DNA cleavage by DNA topoisomerase II in nuclear preparations from KB, KB/VP-1, and KB/VP-2 cells. 3'-End labeled 32P-pUC19 DNA was incubated with nuclear protein in the presence of various doses of VP16 (see "Materials and Methods"). Each point represents the average of 3 to 4 samples, with variability of less than 10%. Relative increase of 32P-DNA precipitable fraction from both KB/VP-1 and KB/VP-2 in the presence of VP16 significantly differed at each dose of VP16 (*, **, and ***; p < 0.05) from KB cells. Radioactivity of 32P-DNA-precipitable fraction was about 1.5-fold higher in both KB/VP-1 and KB/VP-2 than KB in the absence of drug. KB (O), KB/VP-1 (A), and KB/VP-2 (Δ).

amino acid was phosphorylated in DNA topoisomerase II, immunoreactive topoisomerase II of KB, KB/VP-1, and KB/VP-2 were hydrolyzed by acid treatment and analyzed by thin-layer electrophoresis. As seen in Fig. 7, the phosphorylated amino acid residue of topoisomerase II was serine in all the three cell lines.

DISCUSSION

Our VP16/VM26-resistant mutants apparently have new properties that are different from those previously published; KB/VP-1 and KB/VP-2 have approximately the parental levels of the topoisomerase II catalytic activity; whereas there appears to be a quantitative loss of the enzyme. Acquisition of VP16/VM26-resistant phenotype appears to be closely correlated with this quantitative loss. Decreased expression of about a 6.2-kilobase transcript appears in KB/VP-1 and KB/VP-2 cells in comparison with their parental cells. KB/VP-1 and KB/VP-2 grow with doubling times similar to that of KB. The present mutants are unlike the VP16/VM26-resistant mutants of KB which we previously selected; the previous mutants have decreased levels of activity and content of topoisomerase II and show slower growth rates than KB cells (16).

We wondered how the catalytic activity of topoisomerase II was increased in the resistant cell lines. Were there other enzymes carrying topoisomerase II activity, topoisomerase II-related cofactor(s), or any activation enzyme for the topoisomerase II that might compensate for the quantitative loss of the topoisomerase II? Published studies suggest that topoisomerase II is phosphorylated resulting in activation of the enzyme (30–35). Protein kinase C and casein kinase II can phosphorylate DNA topoisomerase II (30, 33, 34), and phosphorylation of topoisomerase II by protein kinase C (32) or by casein kinase II (31) enhances the enzyme activity. Saijo et al. (28) recently purified a protein kinase in the partially purified topoisomerase II fraction of mouse mammary cancer cells. This topoisomerase II-associated protein kinase is a serine kinase and phosphorylation by this kinase increases topoisomerase II activity by about 9-fold over that of unmodified enzyme (28). The cellular content of topoisomerase II in KB/VP-1 and KB/VP-2 is less than one-tenth that found in their parental KB. However, the relative specific rates of phosphorylation per enzyme protein are more activated in mutant topoisomerase II than wild type enzyme. Our present data suggest that phosphorylation of topoisomerase II may enhance catalytic activity in growing mammalian cell lines.

Table 2 Comparison of phosphorylation of DNA topoisomerase II in KB, KB/VP-1, and KB/VP-2 cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>A</th>
<th>B</th>
<th>A/B</th>
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<tbody>
<tr>
<td>KB</td>
<td>100.0</td>
<td>100.0</td>
<td>1.0</td>
</tr>
<tr>
<td>KB/VP-1</td>
<td>77.5</td>
<td>4.4</td>
<td>17.6</td>
</tr>
<tr>
<td>KB/VP-2</td>
<td>69.9</td>
<td>4.9</td>
<td>14.3</td>
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Fig. 6. Immunoprecipitation of 32P-labeled proteins with anti-topoisomerase II antibody. 32P-labeled nuclear protein samples were run on 7.5% SDS-polyacrylamide gel and transferred to the nitrocellulose filters. Nitrocellulose membrane filters were incubated with antibody against topoisomerase II and biotinated goat anti-rabbit IgG (Western immunoblot analysis (A). The same nitrocellulose membrane filters were autoradiographed on X-ray film (B). Arrows, DNA topoisomerase II.
Fig. 7. Phosphoamino acid analysis of phosphorylated DNA topoisomerase II. 32P-labeled topoisomerase II bands of KB (A), KB/VP-1 (B), and KB/VP-2 (C) cells were respectively excised from 7.5% SDS-polyacrylamide gel, and acid hydrolysates of the bands were analyzed by thin layer chromatography. The locations of phosphoamino acids were determined by authentic markers of phosphoamino acids: S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

cells. The amino acid which is phosphorylated in topoisomerase II of both KB and VP16/VM26-resistant cell lines is serine. Serine/threonine is the amino acid phosphorylated by protein kinase C (36), and the topoisomerase II-phosphorylating enzyme in KB or KB/VP-1 is indistinguishable from protein kinase C. However, no significant increase of topoisomerase II phosphorylation in KB and KB/VP-1 was observed when treated with phorbol esters, suggesting that phosphorylation enzyme in our cell lines might be different from protein kinase C. Definitive characterization of the topoisomerase II phosphorylation is required in order to gain a better insight of the mutant enzyme.

Many mutants resistant to VP16/VM26 or its related compounds have been isolated and characterized (see "Introduction") in order to understand whether qualitative or quantitative changes in topoisomerase II might involve VP16/VM26 resistance. Fernandes et al. (37) have indicated that the cytotoxic target for VP16/VM26 is the DNA topoisomerase II that is tightly bound to the nuclear matrix. A VP16/VM26-resistant CEM human leukemia cell line (CEM/VM-1) is low in catalytic activity and VM26-stimulated formation of the DNA-protein complex (13, 38). Fernandes et al. (14) further demonstrated that nuclear matrices isolated with 0.2 to 1.0 M NaCl have progressively lower specific activity in CEM/VM-1 cells than CEM cells. Acquisition of VP16/VM26-resistant phenotype is thus closely correlated with the quantitative change of topoisomerase II in the VP16/VM26-resistant cell lines derived from CEM (14) and also those derived from KB (11, 16). VP16/VM26-resistant cell lines derived from KB cells have been shown previously to have decreased content and activity of topoisomerase II as well as reduced cellular accumulation of VP16 (11, 16). It is possible that decreased topoisomerase II in the nuclear matrix might result in fewer strand breaks in nuclear matrix DNA and decreased cytotoxicity of VP16/VM26. In contrast, Sullivan et al. (39) demonstrated a qualitative change of topoisomerase II which was associated with VP16/VM26 resistance. This qualitatively altered topoisomerase II in nuclear matrix is demonstrated in a VP16/VM26-resistant cell line (Vmp8-5) derived from CHO. The Vmp8-5 enzyme is more heat labile than CHO enzyme while decatenation by both enzymes is inhibited equally by VP16 and both enzymes exhibit similar forms of DNA interaction in the high salt-resistant complexes (39). They suggested that VP16/VM26 can inhibit both religation and catalytic activity in wild-type CHO enzyme whereas it can inhibit the catalytic activity but not the religation in Vmp8-5 enzyme. These studies may propose a common notion for VP16/VM26 resistance that any qualitative as well as quantitative changes in topoisomerase II lead to a decreased interaction of VP16/VM26 with the matrix-bound enzyme and fewer breaks in newly replicated DNA.

KB/VP-1 and KB/VP-2 have decreased topoisomerase II contents but similar levels of the enzyme activity. The enhanced decatenation of topoisomerase II molecules in KB/VP-1 or KB/VP-2, possibly by phosphorylation, might be catalytically VP16 sensitive. This inhibition by VP16 of the catalytic activity is mediated through interference with the strand passing event, inhibition of ATP hydrolysis, or stabilization of a "post-cleavable complex" (2). VP16 induces much less DNA cleavage by nuclear extracts from both KB/VP-1 and KB/VP-2 cell lines than the parental KB cells (Fig. 5). The decreased formation of DNA-enzyme complex in the resistant lines may limit the number of DNA-topoisomerase II complexes for VP16-induced cleavage, resulting in diminution of the effect of VP16 on stabilization or cleavage of the DNA-enzyme complex. It appears likely that acquisition of VP16-resistant phenotype in KB/VP-1 or KB/VP-2 cells is rather closely correlated with decreasing levels of stable DNA-topoisomerase II complex formation in the presence of VP16 than the catalytic activity of topoisomerase II. A more precise analysis to determine whether VP16-resistant mutation may alter topoisomerase II itself or a modulator of the enzyme is required in order to verify this speculative notion.

ACKNOWLEDGMENTS

We thank Dr. G. Kobayashi (Washington University School of Medicine) for critical reading of the manuscript and Dr. F. Hanoaka (Riken Institute) for fruitful discussion. We thank Drs. J. C. Wang (Harvard University) and L. F. Liu (Johns Hopkins University School of Medicine) for invaluable probes and J. Kikuchi in our laboratory for her cooperation in isolating etoposide-resistant variants.

REFERENCES

7. Ross, W. E., Rowe, T., Glisson, B., Yalowich, J., and Liu, L. F. Role of


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