Reduction of Drug Accumulation and DNA Topoisomerase II Activity in Acquired Teniposide-resistant Human Cancer KB Cell Lines

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ABSTRACT

We have isolated stable teniposide (VM26)-resistant cell lines from human cancer KB cells by stepwise exposure to increasing doses of the drug. At each step, we have purified VM26-resistant cell lines. KB/VM-a, KB/VM-b, KB/VM-1, KB/VM-2, KB/VM-3, and KB/VM-4 showed 3-, 6-, 12-, 16-, 74-, and 95-fold higher resistance to VM26 than did KB. We have further characterized KB/VM-2 and KB/VM-4 which showed about 15- and 100-fold higher resistance to VM26 or etoposide (VP16) than did KB. Both VM26-resistant cell lines showed 4- to 11-fold higher resistance to daunomycin and Adriamycin than did KB. Steady-state levels of the cellular accumulation of radioactive VP16 in KB/VM-2 and KB/VM-4 cells were about 40% of that of KB cells, whereas similar levels of radioactive daunomycin accumulation were observed in KB/VM-2 and KB/VM-4 cells as KB cells. Topoisomerase II activity of nuclear extracts of both KB/VM-2 and KB/VM-4 assayed by decatenation of kinetoplast DNA was consistently two-thirds or less the activity of KB cells. A similar reduction was seen in both immunoblot assays with specific anti-topoisomerase II antibody and Northern blot analysis with specific human DNA topoisomerase II complementary DNA. DNA topoisomerase I activity, however, was similar between the mutants and their parent. Furthermore, cell growth of KB/VM-2 and KB/VM-4 was more thermolabile than that of KB, while KB/VM-b already showed temperature-sensitive growth. KB/VM-1 did show reduced accumulation of VP16 as in KB/VM-2 or KB/VM-4, but it had a normal level of topoisomerase II content as in KB cells. These data suggest that the reduced expression of DNA topoisomerase II, possibly combined with decreased permeability to the drugs, can account for the acquired VM26 resistance of KB/VM-2 and KB/VM-4 cells and also that the temperature-sensitive phenotype might not be obligatorily coupled with the reduced expression of topoisomerase II or the decreased permeability.

INTRODUCTION

Podophyllotoxin derivatives, VP16 (etoposide) and VM26 (teniposide), have become prominent antitumor drugs. VP16 and VM26 stimulate the formation of DNA topoisomerase II-DNA cleavage complexes (1, 2). Several cell lines resistant to VP16, VM26, and other topoisomerase II-inhibitory drugs have been isolated and characterized (3, 4). Among the mechanisms that have been considered for VP16 or VM26 resistance are (a) reduced levels of topoisomerase II (5); (b) altered topoisomerase II with a reduced drug sensitivity (6, 7); (c) reduced cellular accumulation of VP16 or VM26 (8); and (d) reduction of topoisomerase II sensitivity because of altered nuclear factor(s) associated with the enzyme (4). It is of interest that VP16- or VM26-resistant cell lines often show pleiotropic multitud resistance to Vinca alkaloids, 4',9-acridinylaminomethanesulfon-m-aniside, and Adriamycin (6). In this study, we have established VM26-resistant cell lines by stepwise selection from the human cancer KB cell line. The mutants show changes in cell growth at high temperature, topoisomerase II, and membrane permeability.

MATERIALS AND METHODS

Cell Lines. VM26-resistant cell lines were obtained from human epidermoid carcinoma KB cells by sequential selection with increasing doses of VM26 without the use of mutagen. KB cells, its 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 of Bactopeptone (Difco Laboratories, Detroit, MI), 0.292 mg/ml of glutamine, 100 μg/ml of kanamycin, and 100 units/ml of penicillin (9–11).

Chemicals. cis-Platinum and VP16 were obtained from Nihon Kayaku Co., Tokyo, Japan; VM26 was from Bristol Myers Co., Kanagawa, Japan; and Adriamycin, daunomycin, actinomycin D, and vincristine were from Sigma Chemical Co., St. Louis, MO. Camptothecin was kindly obtained by Dr. Okada, Hiroshima University, Hiroshima, Japan. [3H]Daunomycin (2.1 Ci/mmol) and [3H]3'-P-[ATP (5000 Ci/mol) were purchased from New England Nuclear, Boston, MA. [3H]VP16 (385 mCi/mmol) was donated by Nihon Kayaku Co., Tokyo, Japan.

 Colony Formation and Growth Curves. 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 continuously incubated then for an additional 10 days with various drugs (9–11). During the entire colony-forming period, the drugs in contact with the cells were not changed. Colonies were counted after Giemsa staining. Drugs were prepared in dimethyl sulfoxide or ethanol, and controls were done adding the same amount of the solvent. To assess growth curves, 4 to 5 × 104 cells were plated, and incubation at the indicated temperatures was followed. The medium was changed every 2 days, and the number of surviving cells was estimated by trypan blue dye exclusion (12).

Preparation of Crude Nuclear Extracts. Crude nuclear extracts were prepared according to the method of Duguet et al. (13). Briefly, nuclei were obtained from 1 × 109 cells in early log-phase culture. Cells were washed 3 times with cold PBS (g/liter; NaCl, 8.0; Na2HPO4/2H2O, 2.9; KCl, 0.2; KH2PO4, 0.2), harvested with a rubber scraper, and pelleted by centrifugation. Cell pellets were then lysed in 10 mM Tris-HCl (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 TKCM buffer, and the suspension was layered over a 0.6-mL cushion of TKCM buffer containing 0.6 M sucrose. The mixture was centrifuged at 2000 x g for 10 min. The nuclear pellets were then washed once with 2 ml of TKM buffer, containing 0.25 M sucrose. The pellets were resuspended in 0.2 ml of TKM buffer and 20 μl of 0.2 M EDTA (pH 8.0), followed by addition of two volumes of 80 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 2 mM EDTA, 0.53 mM NaCl, and 20% glycerol. Extraction then proceeded on ice for 60 min. Precipitates were centrifuged at 17,000 x g for 15 min. The protein concentration in the extracts was immediately determined by a published method (14).

DNA Topoisomerase II Assays. The standard reaction for the DNA topoisomerase II assay contained 50 mM Tris-HCl buffer (pH 7.5), 85 mM KCl, 10 mM MgCl2, 5 mM dithiothreitol, 0.5 mM EDTA, bovine serum albumin (0.03 mg/ml), and 1 mM ATP. 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 Critidia fasciculata, which was obtained from Dr. P. Englund (The Johns Hopkins University School of Medicine).

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Received 11/2/89; accepted 6/4/90.

The abbreviations used are: MEM, minimal essential medium; PBS, phosphate-buffered saline; TKCM buffer, 50 mM Tris-HCl (pH 7.5)-25 mM KCl-2 mM CaCl2-5 mM MgCl2; TKM buffer, 50 mM Tris-HCl (pH 7.5)-25 mM KCl-2 mM CaCl2-5 mM MgCl2; TKM buffer, 50 mM Tris-HCl (pH 7.5)-25 mM KCl-2 mM CaCl2-5 mM MgCl2; kDNA, kinetoplast DNA; SDS, sodium dodecyl sulfate; cDNA, complementary DNA; SSC, standard saline citrate; PAGE, polyacrylamide gel electrophoresis.

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kDNA was purified from a Sarksol extract of the trypanosomes by cesium chloride-ethidium bromide density centrifugation as described (15). The reaction was terminated with 5 μl of 2% SDS-0.05% bromophenol blue-50% glycerol. Samples were then electrophoresed through a 1% agarose gel. After staining with ethidium bromide, gels were photographed under UV illumination.

**Cellular Accumulation Assays for [3H]VP16 and [3H]Daunomycin.** KB cells at 2.5 × 10⁶ cells/dish, or KB/VM-2 or KB/VM-4 cells at 4 × 10⁵ cells/dish were plated and incubated for 2 days at 37°C. The medium was then replaced with serum-free MEM, and the cells were incubated with 1 μCi/ml of [3H]VP16 or 0.25 μCi/ml of [3H]Daunomycin for the indicated times at 37°C. Cells were washed three times with ice-cold PBS, lysed with 200 μl of 0.05% SDS, and mixed thoroughly with 3 ml of Scinisol (Wako Chemical Co., Osaka, Japan). Radioactive samples were then counted (9, 10).

RNA Analysis with Topoisomerase II cDNA Probe. High-molecular-weight DNA was isolated from each cell line by the method of Maniatis et al. (16), and cytoplasmic RNA was isolated according to our previous reports (11, 12, 17). The human topoisomerase II cDNA probe (pBS-hTOP2) (18), obtained from J. C. Wang (Harvard University), and the genomic fragment from Ha160 for β-actin (19) were used as probes. In Northern blot hybridization, 20 μg of RNA samples were electrophoresed in a 1% agarose/2.2 M formaldehyde gels and transferred to nitrocellulose filters (11, 12, 17). 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 Determinations of DNA Topoisomerases I and II. Nuclear protein fractions extracted from 10⁶ cells were run in 7.5% SDS-PAGE. Protein fractions from the gel were electrophoretically transferred onto nitrocellulose filters in 25 mM Tris-HCl (pH 8.3)-92 mM glycine-20% methanol for 2 h at 20 V (12). Nitrocellulose membranes were further incubated with antibody against human DNA topoisomerase I (1:500) or 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 specification (Vectastain ABC-Go kit; Vector Laboratories, Burlingame, CA). Antibodies to the human DNA topoisomerases I and II were gifts of Dr. L. F. Liu (The Johns Hopkins University School of Medicine) (1).
Fig. 1. VP16 and daunomycin accumulation in KB, KB/VM-2, and KB/VM-4 cells. KB, KB/VM-2, and KB/VM-4 were seeded and then incubated with 5 μmol of [3H]VP16 (1 μCi/ml) or 5 μmol of daunomycin (DAU) (0.25 μCi/ml) for 5 min and 60 min, respectively. Cell-associated radioactivity was counted, and each value is the average of six independent trials. Columns, mean; bars, SD. * significantly (P < 0.001) different from the value for the KB cell line.

Fig. 2. Comparison of decatenation activities by nuclear extracts from KB, KB/VM-2, and KB/VM-4 cells. Decatenation assays were carried out as described in “Materials and Methods.” The reaction mixture was incubated in the absence of nuclear extract (control, C) or in the presence of various concentrations of nuclear extracts. The protein concentration diluted at 1/1 corresponded to 1.5 μg of nuclear extract protein in each cell line, and the protein concentrations in the extracts from all the three cell lines were equivalent. k, kDNA band; m, free minicircles.

of kDNA to minicircles. As seen in Fig. 2, decatenation of kDNA by serial dilution of crude nuclear extracts from KB, KB/VM-2, and KB/VM-4 cells showed two-thirds or less the activity of KB cells. DNA topoisomerase I assayed by relaxation of supercoiled pUC19 DNA shows similar activities in KB cells and the resistant mutants. 5

Mammalian topoisomerase II is homodimeric, M, 170,000 protein (24, 25). To quantitate DNA topoisomerase II, topoisomerase II levels in nuclear extracts of KB, KB/VM-2, and KB/VM-4 cells were compared by Western blot with antibody against the enzyme. Nuclear extracts of each cell line were fractionated, and M, 170,000 to 180,000 bands corresponding to topoisomerase II were seen (Fig. 3A). The relative intensities of the bands, when analyzed by densitometry of photographic negatives, showed that the amounts of topoisomerase II in both KB/VM-2 and KB/VM-4 cell lines were 18% and 21%, respectively, of the parental KB cells. By contrast, there appeared no apparent differences in the cellular levels of topoisomerase I among KB, KB/VM-2, and KB/VM-4 cells (Fig. 3B).

To examine whether the decreased levels of DNA topoisomerase II are due to decreased expression of the DNA topoisomerase II gene, Northern blotting analysis was done with the human DNA topoisomerase II cDNA (18). Consistent with a previous report (18), the topoisomerase II specific probe hybridized to a 6.2-kilobase RNA species (Fig. 4). Decreased expression of DNA topoisomerase II mRNA was observed in both KB/VM-2 and KB/VM-4 cells in comparison with KB cells. Densitometric analysis showed that topoisomerase II mRNA levels of KB/VM-2 and KB/VM-4 were 14% and 17%, respectively, of that of KB cells when normalized by β-actin mRNA levels.

Temperature-sensitive Cell Growth of KB/VM-2 and KB/VM-4 Cells. DNA topoisomerase II is involved in supercoiling DNA in eucaryotic cells (2, 26). The decreased topoisomerase II activity in VM26-resistant cell lines might affect their DNA replication and cell growth. We followed growth curves of KB, KB/VM-2, and KB/VM-4 cells at 37°C and also at 41.5°C (Fig. 5). At 37°C, growth rates of both KB/VM-2 and KB/VM-4 cells were slower than that of the parental KB cells. As seen in Fig. 5, growth of KB cells was slightly slowed at 41.5°C, but still continued exponentially. By contrast, growth of KB/VM-2 and KB/VM-4 cells stopped after one cell division at 41.5°C.

Both KB/VM-2 and KB/VM-4 cell lines had at least the three phenotypes: temperature-sensitive growth; reduced cellular accumulation of VP16; and reduced amounts of topoisomerase II. We examined whether the VM26-resistant cells had simultaneously acquired these three phenotypes during selection of the resistant cell lines (Table 2). Growth curves at 37°C and 41.5°C, cellular levels of topoisomerase II, and cellular accumulation of VP16 were determined with all the VM26-resistant cell lines, KB/VM-a, KB/VM-b, KB/VM-1, KB/VM-2, KB/VM-3, and KB/VM-4. KB/VM-a cells, which acquired only a 3-fold higher resistance to VM26 than did KB cells, already showed temperature-sensitive growth. Cellular accumulation of VP16 was reduced in KB/VM-1 cells, but a similar level of the accumulation as in KB cells was observed in KB/VM-b cells. Furthermore, reduced cellular levels of DNA topoisomerase II appeared in KB/VM-2 cells but not in KB/VM-1 cells (Table 2). Table 2 also demonstrates the doubling time

5 Unpublished data.
Fig. 3. Western blot analysis of DNA topoisomerases II (Topo II) and I (Topo I) from KB, KB/VM-2, and KB/VM-4 cells. Nuclear protein fractions of each cell line were run on 7.5% SDS-PAGE and transferred to the nitrocellulose filters. Nitrocellulose membrane filters were incubated with antibody against DNA topoisomerases II (A) and I (B) and then with biotinylated goat anti-rabbit immunoglobulin G. Nuclear proteins of 50 µg were used for this assay. The arrow indicates DNA topoisomerases II and I with molecular weights of about 170,000 and 97,000, respectively.

of 33 to 35 h in KB/VM-a, KB/VM-b, KB/VM-1, KB/VM-2, and KB/VM-3 cells and 42 h in KB/VM-4 cells compared with a 24-h doubling time for KB cells at 37°C.

DISCUSSION

The VP16/VM26-resistant cell lines, like other cell lines, are cross-resistant to multiple anticancer agents (4, 6, 8, 27, 28). The pleiotropic drug resistance in VP16/VM26-resistant cell lines appears, however, to be mediated through a mechanism unrelated to that for the classical multidrug resistance (6, 8). gp170, a specific membranous glycoprotein of typical multidrug-resistant cells, is expressed in multidrug-resistant VJ-300 cells (11), but not in KB/VM-2 and KB/VM-4 cells.5 The decreased accumulation of VP16 in VM26-resistant KB cells (Fig. 1) appears not to be due to enhanced efflux of VP16 (Ref. 6; Footnote 5). The cellular uptake of VM26 or VP16 is not

Fig. 4. Northern blot analysis for DNA topoisomerase II (Topo II) in KB, KB/VM-2 and KB/VM-4 cells. Ten µg of RNA from KB, KB/VM-2, and KB/VM-4 cells were used for hybridization with cDNAs of DNA topoisomerase II (A) and β-actin (B). Arrows indicate DNA topoisomerase II (6.2 kilobases) mRNA in A and β-actin mRNA in B.
yet understood, but a transport system in our KB/VM-2 or KB/VM-4 cells may be altered. Acquisition of drug resistance in KB/VM-2 and KB/VM-4 cells to daunomycin and Adriamycin that inhibit DNA topoisomerase II (23) might be caused by other mechanisms different from altered membrane transport. Cellular accumulation of daunomycin in KB/VM-2 or KB/VM-4 cells is similar to that in KB cells (Fig. 1). Possibly the alteration of DNA topoisomerase II or its related components may also be directly involved.

Altered topoisomerase II has been often related to the acquisition of drug resistance to VP16, VM26, and related other derivatives (29). Our VM26-resistant cell lines, KB/VM-2 and KB/VM-4, have decreased levels of topoisomerase II. However, the degree of decrease of DNA topoisomerase II and its mRNA appears to be greater than that of the decatenation activity in KB/VM-2 and KB/VM-4 cells (Figs. 2 to 4). Our present assays for the decatenation of kDNA are done with crude nuclear extracts and, thus, other activities similar to those of topoisomerase II might involve the decatenation. Assays with purified DNA topoisomerase II may clarify the above discrepancy. In most cases, loss of normal topoisomerase function appears to be correlated with VP16/VM26 resistance, for example, in the Chinese hamster ovary cell line, suggesting a close correlation of altered topoisomerase II with acquisition of VM26/VP16 resistance. In KB/VM-2, KB/VM-3, and KB/VM-4 cells, introduction of the VM26/VP16 resistance mutation appears to cause a quantitative loss in the DNA topoisomerase II (Table 2). By contrast, in KB/VM-a, KB/VM-b, and KB/VM-1 cells which show lower levels of VM26 resistance than do KB/VM-2 and KB/VM-4 cells, topoisomerase II levels are similar to that level in the parental KB cells (Table 2). KB/VM-a and KB/VM-b show temperature-sensitive growth (Fig. 5), and thus a quantitative loss of topoisomerase II appears not to be directly correlated with the temperature-sensitive growth. Furthermore, introduction of VM26 resistance mutation appears to modify drug accumulation in KB/VM-1 cells with wild-type levels of topoisomerase II (Table 2). Our present data suggest that different traits are acquired during development of increased VM26/VP16-resistant phenotypes in KB cells. However, it is not clear from our present study which, if any, of these traits are responsible for acquired resistance to VM26/VP16. Further study is necessary to characterize the molecular basis for the decreased expression of the DNA topoisomerase II gene and also for the reduced permeability of VP16 in our mutants.

ACKNOWLEDGMENTS

We thank D. Schlessinger (Washington University) for critical reading of this manuscript, and L. F. Liu and P. England (The Johns Hopkins University School of Medicine) and J. C. Wang (Harvard University) for invaluable probes for assaying DNA topoisomerases I and II. We also thank M. Ono and J. Kikuchi in our laboratory and W. T. Beck (St. Jude Children’s Research Hospital) for fruitful discussion.

REFERENCES


Table 2  Comparison of DNA topoisomerase II contents, VP16 accumulation, and temperature-sensitive growth of VM26-resistant cell lines

<table>
<thead>
<tr>
<th>Cloned cell lines</th>
<th>Relative resistance to VM26</th>
<th>Doubling time (h)*</th>
<th>VP16 accumulation (level)†</th>
<th>Topoisomerase II (level)†</th>
<th>Temperature-sensitive growth‡</th>
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* The doubling time (h) of each cell line was determined by following the growth curves at 37°C as seen in Fig. 5.
† The VP16 accumulation was determined after incubation for 5 and 60 min as seen in Fig. 1. The low level shows a 50 to 60% decrease of the normal level as in KB/VM-4 cells.
‡ Temperature-sensitive growth was determined by following the growth curves at 37°C and 41.5°C (see Fig. 5).
§ WT, temperature-resistant growth as in KB cells; TS, temperature sensitive.


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