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Down-Regulation of DNA Topoisomerase IIα in Human Colorectal Carcinoma Cells Resistant to a Protoberberine Alkaloid, Berberrubine

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ABSTRACT

Berberrubine, a protoberberine alkaloid that exhibits antitumor activity in animal models, has been identified as a specific poison of DNA topoisomerase II in vitro. To better understand the mechanisms of cellular response to berberrubine, human colorectal carcinoma cells (AMC5) were selected for resistance to berberrubine. The resulting cell line (AMC5/B1) was 5.3-fold resistant to berberrubine in the absence of MDR1 overexpression. The AMC5/B1 line was cross-resistant to topoisomerase II-targeted drugs but showed no cross-resistance to other antitumor drugs. The patterns of cross-resistance to various drugs led us to examine the cellular contents of topoisomerase II. Topoisomerase II activity was ~2.8-fold lower in AMC5/B1 cells compared with parental cells. The AMC5/B1 line contained ~5-fold decrease in topoisomerase IIα protein level and ~2.5-fold decrease in topoisomerase IIα mRNA level. A comparison of the degradation kinetics of topoisomerase IIα mRNA demonstrated that there was no difference in mRNA stability between the two cell lines. Furthermore, the activity of topoisomerase IIα promoter in AMC5/B1 cells was about 25% of that in AMC5 parental cells when transient transfection experiments were performed with the promoter-luciferase reporter gene. These results indicate that down-regulation of topoisomerase IIα in AMC5/B1 cells occurs at the transcriptional level. Nucleotide sequencing of the topoisomerase IIα promoter regions revealed no mutations in AMC5/B1 cells. In summary, resistance to berberrubine in AMC5 cells is associated with decreased level of catalytically active topoisomerase IIα, suggesting that topoisomerase IIα is the cellular target of berberrubine in vivo.

Eukaryotic DNA topoisomerase II is essential for cell survival and has been implicated in many important cellular processes such as replication, transcription, recombination, and chromosomal segregation (DiNardo et al., 1984; Holm et al., 1985; Liu and Wang, 1987; Bae et al., 1988). Topoisomerase II modulates the topological states of DNA via transient double-strand breaks in DNA coupled with subsequent strand passage step (Osheroff, 1989; Chen and Liu, 1994; Watt and Hickson, 1994; Wang, 1996). There are two closely related isoforms, topoisomerase IIα and IIβ (Chung et al., 1989; Jenkins et al., 1992; Tan et al., 1992). These isoforms exist as homodimers, and their amino acid sequences show homology at regions believed to be functionally significant. However, topoisomerase IIα and IIβ isoforms differ in important biochemical and pharmacological properties including sensitivity to topoisomerase II-targeting drugs, thermal stability, cellular localization, and cell cycle regulation (Drake et al., 1989a).

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Topoisomerase II is the intracellular target for a variety of active agents currently used in the treatment of human cancers (Corbett and Osheroff, 1993; Pommier et al., 1994; Froelich-Ammon and Osheroff, 1995). By stabilizing the covalent enzyme-associated DNA complexes, these drugs shift the DNA cleavage/religation equilibrium of the enzyme reaction toward the cleavage state. These drugs are able to convert biological intermediate in topoisomerase II activity into a lethal one ultimately leading to cell death and thus act as cellular poisons. Unlike the topoisomerase II poisons, catalytic inhibitors have been reported to inhibit topoisomerase II activity without significantly stabilizing cleavable complexes. These drugs inhibit DNA topoisomerase II activity at a step before the formation of the cleavable complex and thus act as antagonists of DNA topoisomerase II poisons (Drake et al., 1989b; Jensen et al., 1990; Tanabe et al., 1991; Permana et al., 1994). Agents identified as poisons and/or catalytic inhibitors have proven to be useful in understanding the mechanisms of the topoisomerase II-catalyzed reactions in addition to their clinical use in cancer chemotherapy.

Complications such as resistance in solid tumors and subsequent genetic changes severely limit the efficacy of

ABBREVIATIONS: k-DNA, kinetoplast DNA; MDR, multidrug resistance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; P-gp, P-glycoprotein.

topoisomerase II inhibitors (Sobulo et al., 1997). For these reasons, the development of new drugs that supplement such problems is needed to improve clinical cancer chemotherapy. In tumor cells selected for resistance to topoisomerase II-targeted drugs, the most common mechanism of drug resistance involves enhanced drug efflux associated with either MDR1 (Gros et al., 1986) or MDR-associated protein expression (Grant et al., 1994; Zaman et al., 1994) and alterations in the activity/levels of topoisomerase II (Mo et al., 1997; Matsumoto et al., 1997; Le Mee et al., 2000; Morgan et al., 2000; Son et al., 1998). Although the relationship between topoisomerase II level and drug sensitivity in tumor cells expressing altered topoisomerase II has been described, many questions concerning the role of topoisomerase II in the development of multidrug resistance still remain.

Protoberberine alkaloids are a new class of organic cations that exhibit topoisomerase poison activity (Gatto et al., 1996; Makhey et al., 1996; Li et al., 2000). Coralyne and its derivatives were shown to be inducers of topoisomerase I-DNA cleavable complexes, whereas the structurally similar benzophenanthridine alkaloid nitidine showed a dual poison activity for topoisomerases I and II (Pilch et al., 1997; Sanders et al., 1998; Makhey et al., 2000). In previous work from our laboratory, we have demonstrated that berberrubine has a potent activity as DNA topoisomerase II poison by stabilizing topoisomerase II-mediated cleavable complex in vitro (Kim et al., 1998). Berberrubine is an isoquinoline alkaloid isolated from *Berberis vulgaris* L. and is readily derived from berberine. Despite much resemblance in chemical structure, other protoberberine alkaloids such as berberine and palmatine did not act on topoisomerase II. Furthermore, it has been shown that berberrubine and its derivatives exhibit antitumor activity in mouse models, and a hydroxyl group at the 9-position of berberrubine is essential for the manifestation of antitumor activity (Ikekawa and Ikeda, 1982).

To better understand the mechanisms of cellular response to protoberberine alkaloid, we selected human colorectal carcinoma cells for resistance to berberrubine and biochemically characterized the resistant clone. The resistant cell line exhibits a relatively modest resistance to berberrubine in the absence of MDR1 overexpression, and drug resistance in this cell line is limited to topoisomerase II-targeted drugs. The resistant cell line has decreased level of topoisomerase IIα mRNA and its protein, suggesting that the resistance is associated with down-regulation of DNA topoisomerase IIα gene expression.

Materials and Methods

Selection of Berberrubine-Resistant Cell Lines. The human colorectal carcinoma cell line AMC5 (Kim et al., 1996) was provided by Dr. J. C. Kim (College of Medicine, University of Ulsan, Seoul, Korea). Both AMC5 cells and resistant AMC5/B1 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. AMC5/B1 cells were selected by intermittent exposure of surviving cells to increasing concentrations of berberrubine. AMC5 cells in 100-mm plastic dishes were first exposed to berberrubine at 10 μM to reduce the surviving fraction to 20 to 30% and were allowed to grow in the absence of selecting drug until they reached the initial cell density or greater. The cells were then cultured in medium containing progressively increasing concentrations of berberrubine at 25 and 50 μM, respectively. One of the resulting colonies was cloned and further exposed to berberrubine at 100 μM. After 6 months of intermittent exposure, the berberrubine-resistant cell line was purified, cloned, and named AMC5/B1. Before each experiment, the cells were grown for two or three passages in the absence of drug.

Cytotoxicity Assays. Cytotoxicity was determined by the MTT assay (Alley et al., 1988). Exponentially growing cells were plated into 96-well microtiter plates at 3×10^3 cells/well in 200 μl of culture medium. After drug exposure for 96 h, 50 μl of MTT dye (2 mg/ml) was added to each well, and the cells were incubated for an additional 4 h. The metabolic activity of cells was measured by quantifying the conversion of the yellow MTT to its purple formazan, and absorbance was read at 540 nm using a microplate reader. The concentration of drug that produced a 50% inhibition of growth (IC_{50}) was calculated from linear regression analysis of the linear portion of the growth curves.

Topoisomerase II Catalytic Activity Assays. Topoisomerase II catalytic activity was assayed by the ATP-dependent decatenation of k-DNA (Kim et al., 1998). The decatenation reactions were performed in a total volume of 20 μl of assay buffer (50 mM Tris-HCl, pH 7.6, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, and 30 μg/ml bovine serum albumin) containing 0.2 μg of k-DNA and the indicated amounts of nuclear extracts. After incubation for 15 min at 37°C, the reactions were stopped by the addition of 5 μl of 5% sarcosyl, 0.025% bromphenol blue, and 25% glycerol, and the products were analyzed on a 1% agarose gel containing 0.5 μg/ml of ethidium bromide. The amount of decatenated DNA was quantified by densitometric analysis using the Eagle Eye II imaging system (Stratagene, La Jolla, CA). Topoisomerase I activity was assayed by relaxation of supercoiled pBlueScript. The relaxation reactions were performed in a total volume of 20 μl of assay buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 100 mM NaCl) containing 0.2 μg of negatively supercoiled DNA and the indicated amounts of nuclear extracts. The reactions were incubated for 15 min at 37°C and stopped by the addition of 0.1 volume of 10% SDS. DNA samples were then analyzed on a 1.2% native agarose gel.

Western Blot Analysis of Topoisomerase IIα and IIβ Proteins. Nuclear extracts were separated on 7% SDS polyacrylamide gel, and electroblotted onto Hybond-ECL membranes (Amersham Biosciences, Piscataway, NJ). The membranes were probed with rabbit antiserum raised against a synthetic peptide corresponding to a unique region of each human topoisomerase II isoform (TopoGEN, Inc., Columbus, Ohio). Topoisomerase I was detected with human serum from a scleroderma patient (TopoGEN). The immunoblot signals were visualized by enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences).

Northern Blot Analysis of Topoisomerase IIα and IIβ mRNA. Total RNA was isolated from exponentially growing cells with TRIzol reagents (Invitrogen, Carlsbad, CA). RNA samples (20 μg/ml) were separated on 1% formaldehyde-agarose gel and vacuum-transferred to Hybond N^+ membranes (Amersham Biosciences). Topoisomerase IIα and IIβ mRNA were detected with an *Eco*RI fragment (3.03 kb) from the topoisomerase IIα cDNA and an *Eco*RI-*Pst*I fragment (1.8 kb) from the topoisomerase IIβ cDNA, respectively. All probes were labeled with [α-^{32}P]dCTP using a random-primer DNA labeling system (Amersham Biosciences). The membrane was exposed to X-ray film, and the relative intensities of the bands were determined with a BioImaging Analyzer (Fujix, Tokyo, Japan).

Stability of Topoisomerase IIα mRNA. To determine the stability of topoisomerase IIα mRNA, the drug-sensitive and drug-resistant cells were treated with 100 ng/ml actinomycin D, and total RNA was prepared at 0, 3, 6, 12, and 24 h after addition of actinomycin D (Kubo et al., 1995). Total RNA was analyzed by hybridization with ^{32}P-labeled topoisomerase IIα cDNA probe. The mRNA levels were determined with a BioImaging Analyzer.

Transfection and Luciferase Expression Assays. Cells plated onto six-well plates were grown to 70% confluence before transfection. Test constructs (2 μg) were cotransfected with 2 μg of β-galac-

tosidase expression plasmid, pCH110 (Amersham Biosciences), into AMC5 and AMC5/B1 cells using LipofectAMINE (Invitrogen) according to the manufacturer's protocol. After 48-h incubation, the cells were harvested, and lysates were prepared. Amounts of lysates employed for the luciferase activity assays were normalized to the β-galactosidase activities.

Results

Selection of Berberrubine-Resistant Cells. Berberrubine-resistant cell line, AMC5/B1, was selected from human colorectal carcinoma AMC5 cells by adding stepwise increasing drug concentrations to the culture medium as described under *Materials and Methods*. Cell cytotoxicity induced by berberrubine was determined for AMC5 and AMC5/B1 cells (Fig. 1). The IC_{50} value of berberrubine for AMC5/B1 was 187.5 ±17.7 μM compared with an IC_{50} value of 35.1 ± 14.3 μM for AMC5. This resistance level remained stable in cells grown in the absence of selecting drug for several months. In addition to berberrubine, the sensitivity of AMC5/B1 cells to its analog, berberine, was examined. Although berberrubine and berberine are very similar in chemical structure, no increase in resistance was observed for berberine (Fig. 1). These data indicate that cellular changes occurred during the selection process are specific for resistance to berberrubine.

Cross-Resistance to Other Antitumor Agents. The sensitivities of the resistant cells to a variety of antitumor agents are shown in Table 1. AMC5/B1 cells were 5.3-fold resistant to berberrubine compared with the parental AMC5 cells and were cross-resistant to topoisomerase II-targeted drugs such as etoposide, doxorubicin, and mitoxanthrone, but they showed low levels of resistance to the other topoisomerase II-targeted drugs such as 4′-(9-acridinyl-amino)methanesulfon-*m*-anisidide (amsacrine) and ellipticine. In contrast, AMC5/B1 cells exhibited no or little cross-resistance to antitumor agents whose mechanism of action does not directly involve topoisomerase II such as camptothecin, cisplatin, and vinblastine. Lack of cross-resistance to these compounds suggests that the MDR phenotype may be not expressed in the resistant cells. To determine whether the drug-resistant phenotype was caused by the overexpression of the *mdr*-1 gene-encoded P-glycoprotein (P-gp), immunoblot analysis was performed using P-gp specific-antibody. There was no evidence of induction of P-gp in AMC5/B1 cells compared with parental AMC5 cells (Fig. 3A).

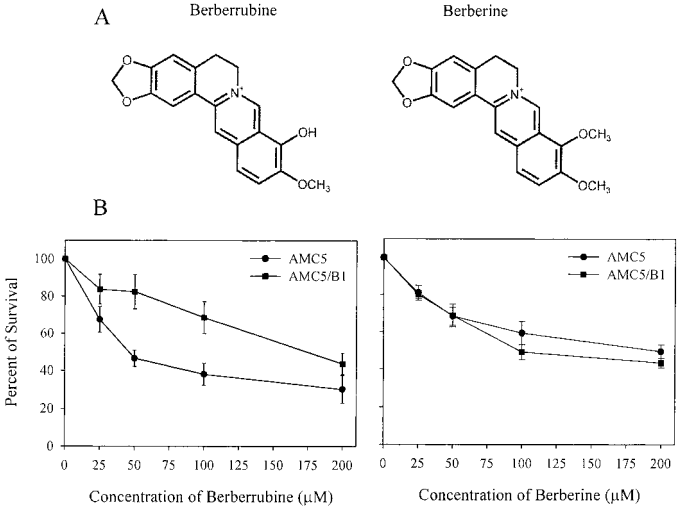

Fig. 1. Cytotoxicity of berberrubine and berberine on AMC5 and AMC5/B1 cells. A, chemical structures of berberrubine and berberine are shown. B, AMC5 and AMC5/B1 cells were treated with the indicated concentrations of berberrubine and berberine. Cell proliferation was measured by MTT assay after 96 h of continuous drug exposure. Values are the mean ± S.D. of at least three independent experiments.

Topoisomerase II Catalytic Activity. The cross-resistance pattern of AMC5/B1 cells to the topoisomerase II-targeted drugs suggests that quantitative and/or qualitative alterations of the topoisomerase II catalytic activity might be involved in the resistance phenotype of the cells. The ability of topoisomerase II to decatenate k-DNA in the berberrubine-resistant cells was determined in nuclear extracts derived from AMC5 and AMC5/B1 cells. Topoisomerase II decatenation activity (per identical amounts of nuclear extract proteins) was 2.8-fold lower in AMC5/B1 cells compared with parental AMC5 cells as determined by comparison of the banding intensities of the minicircles in several dilutions (Fig. 2A). To determine whether the reduced topoisomerase II activity in the resistant cells reflected a generalized phenomenon of altered gene expression after cellular drug exposure, we compared the catalytic activity of the related nuclear enzyme topoisomerase I by the relaxation of the supercoiled plasmid. Topoisomerase I activities were equivalent in the parental and resistant cells (Fig. 2B).

Decreased Levels of Topoisomerase IIα Protein and mRNA in Berberrubine-Resistant Cells. We next examined the amounts of topoisomerase IIα and IIβ proteins present in the nuclear extracts from AMC5 and AMC5/B1 cells using antibodies that are specific for each isoform on Western blots. The topoisomerase IIα protein level was decreased approximately 5-fold in AMC5/B1 cells compared with parental AMC5 cells (Fig. 3A). In contrast, the amounts of topoisomerase IIβ and topoisomerase I protein were not modified in the berberrubine-resistant cells compared with the AMC5 control. To determine whether the observed reduction of the topoisomerase IIα protein was due to reduced level of topoisomerase IIα mRNA, total RNA was extracted from AMC5 and AMC5/B1 cells and analyzed by Northern blotting. The berberrubine-resistant cells contained about 2.5-fold decreased topoisomerase IIα mRNA compared with the

TABLE 1
Sensitivity of berberrubine-resistant cells to various antitumor drugs
IC_{50} values were measured using the MTT assay. Values are means ± S.D. of three or more determinations. Resistance factor (R) was calculated as the ratio of IC_{50} values between AMC5/B1 and AMC5 cells.

Drug	AMC5 (IC_{50})	AMC5/B1	
		IC_{50}	R
	μM	μM	
Berberrubine	35.1 ± 14.3	187.5 ± 17.7	5.3
Etoposide	54.2 ± 6.0	293.6 ± 42.9	5.4
Doxorubicin	2.2 ± 1.8	10.5 ± 0.3	4.8
Mitoxantrone	0.8 ± 0.09	2.1 ± 0.04	2.6
m-AMSA	8.6 ± 2.6	16.5 ± 0.9	1.9
Ellipticine	0.9 ± 0.05	1.7 ± 0.63	1.9
Camptothecin	1.0 ± 0.04	1.1 ± 0.19	1.1
Cisplatin	41.9 ± 4.7	54.3 ± 6.9	1.3
Vinblastine	0.5 ± 0.02	0.5 ± 0.02	1.0

m-AMSA, 4′-(9-acridinyl-amino)methanesulfon-*m*-anisidide (amsacrine).

AMC5 control (Fig. 3B). In consistent with protein data, topoisomerase IIβ mRNA level was similar between AMC5 and AMC5/B1 cells.

Because topoisomerase IIα expression is cell-cycle regulated, the apparent down-regulation of topoisomerase IIα in the resistant cell line could be simply a consequence of alterations in cell-cycle distribution. For example, an increase in G_1 phase cell population would decrease topoisomerase IIα mRNA and protein levels, with little effect on the topoisomerase IIβ or topoisomerase I levels. To address this issue, we determined cell-cycle distributions in AMC5 and AMC5/B1 cell lines by FACS analysis after staining with propidium iodide. There was no significant difference in the number of cells in G_1 phase between the two cell lines (Table 2).

Half-Life of the Topoisomerase IIα in Berberrubine-Resistant Cells. Decreased level of topoisomerase IIα mRNA in the resistant cell line might be due to altered stability of the mRNA. To examine this hypothesis, Northern blot analysis was performed in the presence of the RNA synthesis inhibitor, actinomycin D. The degradation kinetics of topoisomerase IIα mRNA revealed that the half-lives of topoisomerase IIα for AMC5 and AMC5/B1 cells, were about 16 h and 17.5 h, respectively (data not shown). These results indicate that the substantial reduction of topoisomerase IIα mRNA in the resistant cell line seemed not to have been caused by an altered mRNA stability.

Topoisomerase IIα Promoter Activity in Berberrubine-Resistant Cells. To examine the possibility that mutations in the promoter region of topoisomerase IIα lead to its reduced expression, we cloned by polymerase chain reaction the promoter region of topoisomerase IIα gene between −554 and +87 in AMC5/B1 line. Nucleotide sequencing of the resistant cell line revealed no mutation in the promoter region. To test whether the decreased level of topoisomerase IIα is caused by transcriptional down-regulation, we analyzed the promoter activity by introducing topoisomerase IIα promoter-luciferase reporter constructs into AMC5 and AMC5/B1 cells, respectively. The luciferase activity driven by a 554-bp upstream region of topoisomerase IIα promoter was reduced by about 75% in AMC5/B1 cells compared with parental AMC5 cells (Fig. 4). When DNA fragments with nested 5′-deletions of the promoter sequence were placed upstream of the luciferase reporter gene, similar reduction in luciferase activity was obtained in AMC5/B1 cells. These results confirmed that down-

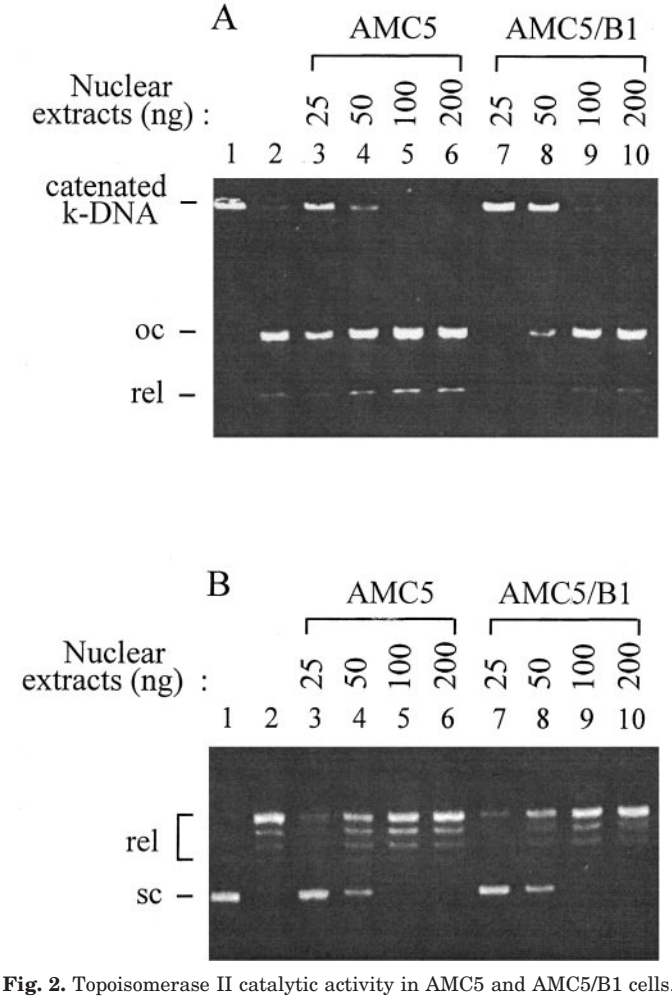

Fig. 2. Topoisomerase II catalytic activity in AMC5 and AMC5/B1 cells. A, topoisomerase II activities in nuclear extracts from AMC5 (lanes 3–6) and AMC5/B1 cells (lanes 7–10) were measured by the decatenation assay of k-DNA. Reaction mixtures were incubated in the presence of various dilutions of nuclear extracts as indicated. Lane 1 contained catenated k-DNA, and lane 2 contained decatenated k-DNA. The positions of decatenated open circular (oc) and relaxed (rel) k-DNA are indicated. B, topoisomerase I activity assays were performed by relaxation of supercoiled pBlueScript. Reaction mixtures were incubated in the presence of various dilutions of nuclear extracts from AMC5 (lanes 3–6) and AMC5/B1 cells (lanes 7–10). Lane 1 contained supercoiled DNA (sc), and lane 2 contained relaxed DNA (rel).

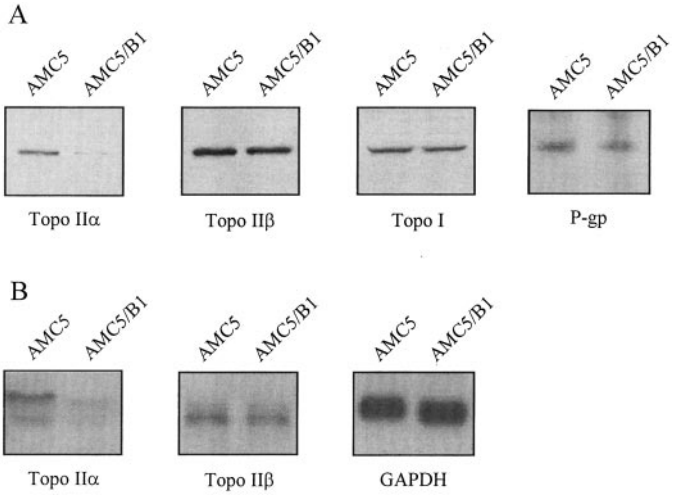

Fig. 3. Expression levels of topoisomerase II protein and mRNA in AMC5 and AMC5/B1 cells. A, immunoblot analysis showing expression of topoisomerase IIα, IIβ, and topoisomerase I. For the analysis of MDR-1, the membrane proteins were fractionated and detected using a monoclonal antibody against P-gp. B, Northern blot analysis of topoisomerase IIα and IIβ mRNA levels in AMC5 and AMC5/B1 cells. A human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe was used for normalization of gel loading.

TABLE 2

Cell cycle distribution in AMC5 and AMC5/B1

Cell cycle distribution was determined by flow cytometry after staining with propidium iodide. Values are the mean ± S.D. of three experiments.

Cell Line	Cell Cycle Distribution		
	G_1	S	G_2/M
	%		
AMC5	65.1 ± 7.6	9.2 ± 0.9	25.7 ± 6.6
AMC5/B1	64.2 ± 4.6	10.4 ± 0.5	25.4 ± 4.1

regulation of topoisomerase IIα in AMC5/B1 cells occurred at the transcriptional level.

Berberrubine Sensitivity in Doxorubicin-Resistant Cells. Previously, a human stomach-adenocarcinoma cell line (MKN-45) was selected for resistance to doxorubicin by stepwise exposure to increasing amounts of this agent (Son et al., 1998). Like AMC5/B1, the doxorubicin-resistant cell line (MKN/ADR) contained a reduced topoisomerase II catalytic activity compared with parental MKN-45. Topoisomerase IIα protein levels were lower in MKN/ADR cells than in parental MKN-45 cells. In contrast, topoisomerase IIβ levels were similar for both cell lines. We evaluated alterations in the berberrubine sensitivity of MKN-45 and MKN/ADR cell lines. A higher level of resistance in MKN/ADR cells was observed for berberrubine (6.2-fold) and other topoisomerase II-targeted drugs (Table 3). Such difference between the two lines was not observed for berberine. These results indicate that a quantitative reduction in topoisomerase IIα is associated with berberrubine resistance.

Discussion

DNA topoisomerase II is one of the most important molecular targets currently used in clinical cancer treatment (Corbett and Osheroff, 1993; Pommier et al., 1994; Froelich-Ammon and Osheroff, 1995). Despite the availability of wide range antitumor drugs, a recurrent problem of resistance and secondary complication hampers the efficacy in cancer chemotherapy (Sobulo et al., 1997). To overcome such hindrance, the need for development of new drugs or treatment strategies increases with time. Previously, berberrubine has been shown to be a new class of antitumor agent which exhibits the topoisomerase II poison activity in vitro (Kim et al., 1998). To further study the interaction between berberrubine and topoisomerase II in vivo, the berberrubine-resistant cell line, AMC5/B1, was obtained from a human colorectal carcinoma cell line by stepwise selection of the parental AMC5 cells. Because P-glycoprotein overexpression was not detected in AMC5/B1 cells, resistance to berberrubine cannot be ascribed to MDR. This idea is further supported by the observation that the cross-resistance pattern of the resistant cell line was not similar to that expected for MDR. AMC5/B1 was cross-resistant to topoisomerase II-targeted drugs but showed no cross-resistance to other antitumor drugs. These results suggest that topoisomerase II could be an intracellular target of berberrubine.

Topoisomerase II catalytic activity in AMC5/B1 cells was reduced 2.8-fold compared with AMC5 cells as measured by the decatenation of k-DNA, whereas topoisomerase I catalytic activity was almost identical in extracts from AMC5 and AMC5/B1 cells. Resistance to berberrubine is specifically associated with reduced expression of the topoisomerase IIα isoform. The discovery that topoisomerase IIα protein level is lower in AMC5/B1 cells compared with the parental cells is consistent with the finding that total enzyme activity is reduced in the resistant cells. In contrast, topoisomerase IIβ and topoisomerase I protein levels were the same in AMC5 and AMC5/B1 cells. It would be possible that reduced translational efficiency of the message and/or an increase in ubiquitination might lead to more rapid degradation of the topoisomerase IIα protein. Changes in translational efficiency or in topoisomerase IIα stability could be caused by point mutation(s). Although we have not yet sequenced the mutant gene in its entirety, post-translational modification is unlikely to be a determining factor in the berberrubine-resistant phenotype considering reduced level of topoisomerase IIα mRNA in AMC5/B1 cells.

The finding that AMC5/B1 cells contained reduced level of topoisomerase IIα mRNA suggests that either transcriptional or posttranscriptional regulation could account for the decrease in the topoisomerase IIα protein and activity. We found no significant difference in topoisomerase IIα mRNA half-lives between AMC5 and AMC5/B1 cells. In contrast, we observed reduced topoisomerase IIα promoter activity in AMC5/B1 cells when transient transfection experiments were performed with the topoisomerase IIα promoter-luciferase reporter constructs. Changes in *cis*-elements of the promoter region such as mutation could lead to a decrease in gene expression. However, nucleotide sequencing of AMC5/B1 revealed no mutation in the topoisomerase IIα promoter region. These results suggest that reduced transcription activity was not caused by alteration of *cis*-elements in its promoter; rather, changes in the levels of *trans*-acting factor(s) could regulate gene expression. Although we have not comprehensively tested all possible transcription factors that might regulate topoisomerase IIα expression, our initial data suggest that transcriptional down-regulation significantly contributes to the reduced expression of topoisomerase IIα protein and is associated with berberrubine resistance in AMC5 cells.

Because the rearrangement and hypermethylation of the topoisomerase IIα gene may be associated with reduced gene expression in cells selected for resistance to topoisom-

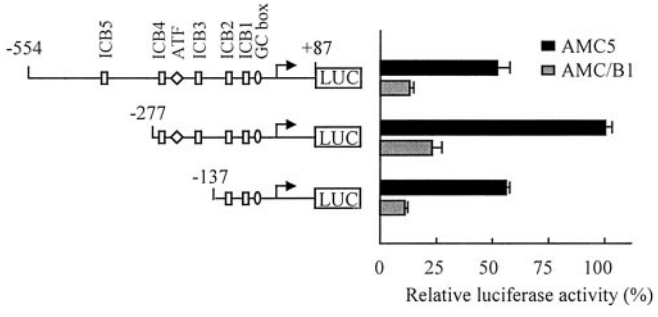

Fig. 4. The promoter activity of topoisomerase IIα gene in AMC5 and AMC5/B1 cells. DNA fragments containing different lengths of the topoisomerase IIα promoter were placed upstream of the luciferase reporter gene and transfected into AMC5 and AMC5/B1 cells. Relative positions of the 5′-ends of deleted promoter in each construct are marked. A β-galactosidase expression plasmid was cotransfected as an internal control. Amounts of cell lysates employed for the luciferase activity assay were normalized to the β-galactosidase activity, and the relative luciferase assay of each construct was expressed as a percentage of that of −277/+87 construct. Data are averages of four independent experiments.

TABLE 3

Sensitivity of doxorubicin-resistant cells to various antitumor drugs

IC_{50} values were measured using the MTT assay. Values are means ± S.D. of three or more determinations. Resistance factor (R) was calculated as the ratio of IC_{50} values between MKN/ADR and MKN-45 cells

Drug	MKN-45 (IC_{50})	MKN/ADR	
		IC_{50}	R
	μM	*μM*	
Berberrubine	32.3 ± 1.7	201.0 ± 26.9	6.2
Etoposide	24.0 ± 1.4	161.0 ± 46.7	6.7
Doxorubicin	1.5 ± 0.4	9.7 ± 0.7	6.5
Mitoxantrone	1.9 ± 0.1	4.8 ± 1.3	2.5
Berberine	25.0 ± 8.5	25.8 ± 9.5	1.0

erase II-targeted drugs (Chandler et al., 1986; Tan et al., 1989; McPherson et al., 1993), we evaluated chromosome rearrangement and CpG methylation for topoisomerase IIα gene in AMC5 and AMC5/B1 cells. The Southern blot data indicate that reduced level of topoisomerase IIα in AMC5/B1 cells seems to be unrelated to chromosomal rearrangement or hypermethylation of the topoisomerase IIα gene (data not shown).

Given the fact that topoisomerase II level/activity is one of the major determinants of cellular sensitivity to agents targeted this enzyme, we concluded that topoisomerase IIα is a significant cellular target for berberrubine in vivo. Despite much resemblance in chemical structure between berberrubine and berberine, AMC5/B1 cells were not cross-resistant to berberine. These results are well consistent with the previous reports that berberrubine is a much more potent poison of topoisomerase II than berberine (Kim et al., 1998). Furthermore, it has been shown that berberrubine had a strong antitumor activity in mouse models, but berberine had no antitumor activity (Ikekawa and Ikeda, 1982). Berberrubine has a hydroxyl group at the 9-position, whereas berberine contains a methoxy group at this position. Such difference in chemical structure is essential for antitumor activity of berberrubine. In summary, resistance to berberrubine is associated with reduced topoisomerase II catalytic activity. Transcriptional down-regulation in AMC5/B1 cells contributes to the reduced expression of topoisomerase IIα protein.

Acknowledgments

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