Resistance to Etoposide in Human Leukemia HL-60 CELLS: Reduction in Drug-Induced DNA Cleavage Associated with Hypophosphorylation of Topoisomerase II Phosphopeptides

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SUMMARY

Tumor cell resistance to anthracyclines and epipodophyllotoxins can be due to reduced drug accumulation and/or alterations in the activity of topoisomerase II (TOPO II). HL-60 cells selected in 0.05 μM doxorubicin (DOX) are 10-fold and >20-fold resistant to DOX and etoposide (VP-16), respectively. The accumulation of [3H]VP-16 was 2-3-fold lower in the resistant cells (HL-60/DOX 0.05) than in similarly treated parent-sensitive cells (HL-60/S). However, compared with HL-60/S cells, the HL-60/DOX 0.05 cells required >20-fold higher concentrations of VP-16 to produce equivalent damage to DNA. The reduced formation of VP-16-stabilized DNA cleavable complex in the HL-60/DOX 0.05 cells was not due to differences in the amount of 170-kDa TOPO II α protein or enzyme catalytic activity between HL-60/S and HL-60/DOX 0.05 cells. Metabolic labeling with [32P]orthophosphoric acid and immunoprecipitation indicated that the level of phosphorylated 170-kDa TOPO IIα protein in the HL-60/S cells was 2.2 ± 0.4-fold higher than that in HL-60/DOX 0.05 cells. Hypophosphorylation (5-fold) of 170-kDa TOPO II protein in HL-60/S cells treated with the calcium chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester produced a >2-fold reduction in VP-16-induced TOPO II-mediated DNA cleavable complex formation. Two-dimensional mapping of phosphopeptides in complete tryptic digests demonstrated that the reduced phosphorylation of the 170-kDa TOPO IIα in HL-60/DOX 0.05 cells was due to the hypophosphorylation of at least three phosphopeptides characteristic of HL-60/S cells. Thus, the attenuated ability of TOPO II to form drug-stabilized DNA cleavable complex is related to the phosphorylated state of 170-kDa TOPO II, and in HL-60/DOX 0.05 cells, resistance may be related to hypophosphorylation of these phosphopeptides characteristic of HL-60/S cells.

Tumor cell resistance to chemotherapy is a serious problem confronting the effective treatment of human malignancies (1). The expression of resistance to antitumor agents that differ significantly in both structure and mechanism of action has been identified in experimental tumor models and clinical tumor specimens (2). This broad resistant phenotype is defined as MDR and is hypothesized to be exclusively mediated by the overexpression of a 150–180-kDa membrane glycoprotein, PGP (3, 4). Reduced drug accumulation in MDR cells is suggested to involve drug efflux across a concentration gradient by PGP (3, 4).

TOPO II, which is implicated in transcription, recombination, replication, chromosome segregation, and structure, is essential for cell function and represents a target for the clinically important MDR drugs DOX and VP-16 (5–7). Although resistance to the TOPO II inhibitors (e.g., anthracyclines or epipodophyllotoxins) and microtubule poisons (e.g., vinca alkaloids and taxol) is characteristic of MDR cells, resistance by mechanisms unrelated to drug accumulation defects has been identified with TOPO II inhibitors (8–11). Progressively DOX-resistant sublines with the MDR phenotype also exhibit alterations in TOPO II function as an important mode of resistance (7, 8, 12).

Resistance to inhibitors of TOPO II in experimental systems is usually associated with reduced drug-stimulated DNA cleavable complex formation (7). Although reduced levels of TOPO II may be associated with the expression of resistance (5–7), a precise understanding of other cellular events is necessary. The post-translational modification of TOPO II by phosphorylation regulates its activity, and site-specific phosphorylation during the cell cycle has been re-

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ABBREVIATIONS: MDR, multidrug resistance; DOX, doxorubicin; VP-16, etoposide; PGP, P-glycoprotein; MRP, multidrug resistance-related protein; TOPO II, topoisomerase II; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RIPT, radioimmuno precipitation assay.
ported (6, 13–15). The phosphorylated state of TOPO II is also altered in tumor cells resistant to amssacrine, DOX, VP-16, and teniposide (VM-26) (11, 12, 16–18). We recently reported in a study with HL-60 cells that DOX resistance is associated with the overexpression of mdr-1 versus MRP and is consistent with the evolution of 7q+ anomalies without remarkable changes in chromosome 16 band p13.1, to which MRP maps (19). In the current study, we characterized cellular determinants of resistance to the TOPO II inhibitor VP-16 in the DOX-resistant HL-60 model system. Our results demonstrate that (a) reduced DNA cleavable complex formation in the resistant subline is not due to changes in 170-kDa TOPO IIα enzyme level or catalytic activity; (b) in sensitive or resistant cells, hypophosphorylation of 170-kDa TOPO IIα is associated with decreased DNA cleavable complex formation; and (c) hypophosphorylation occurs in resistant cells of at least three 170-kDa TOPO IIα phosphopeptides characteristic of sensitive cells.

Materials and Methods

The parental HL-60/S (13) cells were obtained from Dr. Andrew Yen (College of Veterinary Medicine, Cornell University, Ithaca, NY.). Cultures of HL-60/S cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mm L-glutamine (M.A. Bioproducts, Walkersburg, MD) at 37° in a humidified 5% CO2/95% air atmosphere. The resistant sublines of HL-60 have been described previously (19) and were developed by culturing the cells in increasing concentrations of 0.025–0.05 μg/ml DOX. The resistant sublines were maintained in DOX-free medium for ≥2 weeks before preparation of stock frozen cultures and were subsequently not maintained in the presence of DOX for use in experiments. Under these conditions, the doubling time in vitro of the HL-60/S cells and the resistant sublines was 18–20 hr.

Cytotoxicity studies in vitro were carried out with a soft-agar colony assay after treatment with DOX or VP-16 for 1 hr (8, 9). The colony-forming efficiency of the HL-60/S cells and the resistant subline HL-60/DOX 0.05 was 29% and 10%, respectively.

Accumulation of VP-16 in HL-60/S and HL-60/DOX 0.05 cells was determined by treatment with [3H]VP-16 (specific activity, 768 mCi/ mmol; Moravek Biochemicals, Brea, CA) for 1 hr. After treatment, cells were centrifuged through silicone oil, digested in 0.2 N sodium hydroxide (20), and counted with a liquid scintillation counter using Ecolume (ICN Biochemicals, San Diego, CA) as the scintillation cocktail. Incorporation of [3H]VP-16 was expressed in mmol/10^6 cells.

The effect of VP-16 on induction of DNA damage was determined by measuring DNA single-strand break frequency by alkaline elution (21) and precipitation of DNA cleavable complex by the SDS-RCI technique (22, 23). Cells were labeled for 24 hr with 0.02–0.04 μCi/ml of [14C]ethymidine (specific activity, 53 mCi/mmole; American, Arlington Heights, IL). The HL-60/S and HL-60/DOX 0.05 cells were treated with 0.5–100 μM VP-16 for 1 hr, and damage to the DNA was determined. The measurement of DNA single-strand break frequency by alkaline elution was carried out as described previously (10).

The precipitation and analysis of drug-induced TOPO II-mediated DNA cleavable complexes were carried out by a modification of the SDS-RCI technique described by Rowe et al. (22) and Zwelling et al. (23).

Levels of 170-kDa TOPO IIα in HL-60/S and HL-60/DOX 0.05 cells was determined by SDS-PAGE and immunoblotting (10). Briefly, nuclei from HL-60/S and HL-60/DOX 0.05 cells were isolated in nucleic buffer supplemented with 0.5% Triton X-100 (10), and extracts were prepared in RIPA buffer (425 mm NaCl, 1% nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 10 μM β-mercaptoethanol, 50 mM TrisHCl, pH 8.0, supplemented with protease inhibitors/1 mm phenylmethylsulfonyl fluoride, 1 μM benzamidine, 10 μg/ml soybean trypsin inhibitor, 100 μg/ml leupeptin, 1 μg/ml pepstatin, 20 μg/ml aprotonin, and 10 μg/ml α-2-macroglobulin) (12). Protein content was determined with the Coomassie assay reagent (Pierce Biochemicals, Rockford, IL), and serial dilutions containing equivalent amounts of protein from HL-60/S and HL-60/DOX 0.05 cells were analyzed by SDS-PAGE (20, 24). After electrophoretography into nitrocellulose, the TOPO II protein was detected by following incubation with a rabbit polyclonal antibody that recognizes 170-kDa TOPO IIα (11, 25, 26) and by enhanced chemiluminescence (ECL kit; Amersham). The catalytic activity of TOPO II in nuclear extracts was determined by the P4 unknotted assay, and the reaction mixture without ATP served as the control (27).

The effects of clamping intracellular Ca2+ with BAPTA-AM (28) was carried out in HL-60/S cells. Cultures of HL-60/S cells were treated with 20 μM BAPTA-AM for 2 hr and metabolically labeled with [32P]orthophosphoric acid for phosphorylation experiments or treated with VP-16 for 1 hr. The DNA cleavable complex formation was determined by the SDS-RCI method (22, 23). The effect of BAPTA-AM on intracellular Ca2+ transients was determined according to Dubyak et al. (29).

Phosphorylation of TOPO II was determined in HL-60/S and HL-60/DOX 0.05 cells metabolically labeled with [32P]orthophosphoric acid (31). After isolation of nuclei and lysis in RIPA buffer (12), TOPO II in lysates containing equivalent amounts of protein was immunoprecipitated using a polyclonal antibody that recognizes the 170-kDa TOPO IIα protein (11, 12). Details of the technique for metabolic labeling and immunoprecipitation have been published previously (11, 12). Phosphorylated TOPO II protein levels were determined by densitometric scanning of autoradiograms or by the use of a PhosphorImager.

Phosphopeptide and phosphoamino acid analysis of the immunoprecipitated 170-kDa TOPO IIα was carried out according to Wells et al. (13) and Boyle et al. (30). Briefly, the band corresponding to the 170-kDa TOPO IIα protein was excised from the dried fixed gel and eluted overnight with 50 mM ammonium bicarbonate, 0.1% SDS, and 0.5% 2-mercaptoethanol. The protein was precipitated with 100% trichloroacetic acid and oxidized with performic acid. Samples hydrolyzed with 6 N hydrochloric acid for 1 hr at 110° were processed for phosphoamino acid analysis by two-dimensional electrophoresis at pH 1.9 and 3.5 (13, 30). Protein samples were digested overnight in N-tosyl-L-phenylalanine chloromethyl ketone (PMSF), and phosphopeptides were analyzed by electrophoresis with pH 1.9 buffer in the horizontal dimension and chromatography in the vertical dimension using n-butanol/pyridine/acetic acid/deionized water (6:4.5:14, v/v/v/v) as the buffer system (30).

Results and Discussion

The cytotoxic effects of DOX or VP-16 in the HL-60/S and HL-60/DOX 0.05 cells using a soft-agar colony assay are outlined in Fig. 1, A and B. Based on the IC50 values, the HL-60/DOX 0.05 cells were 10-fold and >20-fold resistant to DOX and VP-16, respectively.

Because the HL-60/DOX 0.05 cells overexpress PGP (19), the cellular accumulation of VP-16 was determined (Fig. 2). Cellular accumulation of [3H]VP-16 in HL-60/DOX 0.05 cells was 2–3-fold lower than that in similarly treated HL-60/S cells. However, as reported in another tumor model with the MDR phenotype (10), the magnitude of decrease in the cellular accumulation of VP-16 was not correlated with the level of resistance.

To address the possible relationship between DNA damage induced by VP-16 and the specific role of TOPO II, both DNA single-strand break frequency and drug-stabilized DNA cleavable complex formation were analyzed. The results (Fig. 3, A and B) demonstrate that damage to DNA induced by
Fig. 1. Cytoxic effects of DOX (A) and VP-16 (B) in parent-sensitive and DOX-resistant HL-60 cells. Data are the mean value from at least triplicate experiments. Bars, standard error.

Fig. 2. Cellular accumulation of [3H]VP-16 in HL-60/S and HL-60/DOX 0.05 cells. Data are the mean value of triplicate determinations from at least duplicate experiments. Bars, standard error.

DNA cleavable complex formation in the HL-60/DOX 0.05 cells versus the HL-60/S cells.

Alterations in drug-induced cleavable complex in resistant cells have frequently been associated with reduced TOPO II protein levels (5–7). To determine whether reduced a protein levels in HL-60/DOX 0.05 cells could be contributing to the observed changes in DNA cleavable complex formation, total protein levels (immunoblotting) and catalytic activity (P4 unknotted) of TOPO II in nuclear extracts were determined. Based on the results in Fig. 4A, it is apparent that 170-kDa TOPO IIα protein levels in nuclear extracts from HL-60/S and HL-60/DOX 0.05 cells are comparable. These results are also supported by results of the P4 unknotted assay (Fig. 4B), which demonstrated comparable enzyme catalytic activities for the HL-60/S and HL-60/DOX 0.05 cells.

Post-translational modification of TOPO II by phosphorylation is an important event in regulating activity (6, 31–33). Phosphorylation of the 170-kDa TOPO IIα isoform of TOPO II is a cell cycle phase-dependent event that regulates activity of the enzyme (6, 13–15, 31–33). Because phosphorylation of TOPO II could be modified in drug resistance (11, 12, 16–18), the phosphorylation state of 170-kDa TOPO IIα in the HL-60/S and HL-60/DOX 0.05 cells was examined after metabolic labeling with [32P]orthophosphoric acid. The data in Fig. 5 demonstrate that the 170-kDa TOPO II isoform of
Fig. 4. Detection by immunoblotting (A) of the 170-kDa TOPO II in HL-60/S (lanes 1–3) and HL-60/DOX 0.05 (lanes 4–6) cells; nuclear extracts were diluted to contain 50 µg of protein (lanes 1 and 4), 75 µg of protein (lanes 2 and 5), and 100 µg of protein (lanes 3 and 6). Catalytic activity of TOPO II was detected by P4 unknotted assay (B) in nuclear extracts from HL-60/S (lanes 1–4) and HL-60/DOX 0.05 (lanes 5–8) cells. Extracts were serially diluted to contain the following amounts of protein: lanes 1 and 5, 400 ng; lanes 2 and 6, 200 ng; lanes 3 and 7, 100 ng; and lanes 4 and 8, 50 ng. K, knotted; U, unknotted; L, linear P4 DNA.

TOPO II is a phosphoprotein in both HL-60/S and HL-60/DOX 0.05 cells. However, a notable difference is the markedly higher level of phosphorylated TOPO II protein in the HL-60/S cells than in the HL-60/DOX 0.05 cells. Quantification of differences in phosphorylation with a Phosphorimager or densitometric scanning of autoradiograms demonstrated that the level of phosphorylated α protein in the HL-60/S cells was $2.2 \pm 0.4$-fold higher (data from at least triplicate experiments) than in HL-60/DOX 0.05 cells.

To characterize specific differences in the phosphorylated state of TOPO II in the HL-60/S and HL-60/DOX 0.05 cells, phosphoamino acids and phosphopeptides in tryptic digests were analyzed by two-dimensional mapping (13, 30). In both HL-60/S and HL-60/DOX 0.05 cells, phosphoamino acid analysis revealed that TOPO II was almost exclusively phosphorylated on serine residues (data not shown). The phosphopeptide maps of HL-60/S cells (Fig. 6A) and of HL-60/DOX 0.05 cells (Fig. 6B) demonstrate obvious differences between the two cell lines, based on the hypophosphorylation of at least three phosphopeptides in the HL-60/DOX 0.05 compared with the HL-60/S cells. A mix of tryptic digests from HL-60/S and HL-60/DOX 0.05 cells was also found to support differences in phosphopeptide maps between the two cell lines (data not shown).

A possible link between the phosphorylated state of the 170-kDa TOPO II and drug-induced DNA cleavable complex formation is supported by data with the HL-60/S cells treated with BAPTA-AM to clamp intracellular calcium (28). As shown in Fig. 7, treatment of HL-60/S cells with BAPTA-AM results in >3-fold hypophosphorylation of the 170-kDa TOPO II. The results in Fig. 8 supporting an association between TOPO II phosphorylation and cleavable complex formation demonstrate that VP-16-induced TOPO II-mediated DNA cleavable complex formation is >2-fold lower in HL-60/S cells treated with BAPTA-AM. The clamping of intracellular calcium was verified by the absence of ATP-induced Ca$^{2+}$ transients in BAPTA-AM-treated cells measured in Fura-2-loaded cells (29, data not shown). Immunoblotting and VP-16 accumulation experiments demonstrated that reduced TOPO II phosphorylation and DNA cleavable complex formation in HL-60/S cells treated with BAPTA-AM were not due to changes in steady state levels of α or decreased drug accumulation (data not shown).

Tumor cell resistance to inhibitors of TOPO II associated

Fig. 5. Detection of phosphorylated 170-kDa TOPO II in HL-60/S (S, lane 1) and HL-60/DOX 0.05 (R, lane 2) cells. Nuclei from [32P]orthophosphoric acid-labeled cells were lysed in RIPA buffer, and lysate containing 250 µg of protein was immunoprecipitated with 170-kDa TOPO II-specific antiserum (22) and resolved by SDS-PAGE for autoradiography. Based on Phosphorimager or densitometric analysis, the level of phosphorylated TOPO II in HL-60/S cells was $2.2 \pm 0.4$-fold higher than that in HL-60/DOX 0.05 cells.

Fig. 6. Two-dimensional mapping of phosphopeptides from complete tryptic digests (25) of the 170-kDa TOPO II from HL-60/S (A) and HL-60/DOX 0.05 (B) cells. Samples contained 500 cpm. Arrows, phosphopeptides characteristic of HL-60/S (S) cells and hypophosphorylated in HL-60/DOX 0.05 (R) cells.
specifically, by the TOPO II-mediated DNA cleavable complex assayed by the SDS-KCl technique. A direct correlation of VP-16-induced TOPO II-mediated DNA cleavable complex and cytotoxicity in the soft-agar colony assay is also apparent for the HL-60/S or HL-60/Dox 0.05 cells. Taken together, these results suggest that the HL-60/Dox 0.05 cells with the MDR phenotype express resistance to VP-16 because of the attenuated ability of the 170-kDa TOPO IIα isofrom of TOPO II to form a stable DNA cleavable complex.

It is generally believed that levels of TOPO II predict drug sensitivity (6, 7). However, our data demonstrating equal amounts of TOPO II in the HL-60/S and HL-60/Dox 0.05 cells further support the observation that resistance is due to the compromised ability to form drug-stabilized cleavable complex. Also, the reduced formation of drug-stimulated cleavable complex by the HL-60/Dox 0.05 cells seems to be a selective modification of TOPO II because catalytic function of P4 DNA unknotting by TOPO II is unchanged. The possible role of phosphorylation as an event downstream of TOPO II levels in regulating drug-stimulated cleavable complex formation is supported by the data demonstrating a 2.2 ± 0.4-fold decrease in phosphorylation of TOPO II in the HL-60/Dox 0.05 cells compared with the HL-60/S cells. Also, band depletion studies (data not shown) performed after treatment with VP-16 suggested that the phosphorylated form of 170-kDa TOPO IIα is a major component of the cleavable complex. The hypophosphorylation of TOPO II in the resistant HL-60/Dox 0.05 cells is novel and seems to be related to the hypophosphorylation of at least three major phosphoproteins characteristic of the parental HL-60/S cells. Preliminary studies suggest that the hypophosphorylated phosphoproteins in HL-60/Dox 0.05 cells do not involve Ser1376 or Ser1524 (13) and are not the result of decreased levels of casein kinase II (data not shown). Other studies have also reported the association between reduced overall hypophosphorylation of TOPO II in VP-16 or teniposide-resistant cells (17, 18). Our results showing TOPO II hypophosphorylation in the DOX-resistant cells cannot be attributed to the overexpression of mdr-1 because the hypophosphorylation of TOPO II in the epipodophyllotoxin-resistant cells occurs in the absence of mdr-1 overexpression (17, 18). Although data from the current study and other reports with resistant cell systems suggest that hypophosphorylation of TOPO II is associated with resistance (11, 12, 17, 18), Takeoka et al. (16) observed hyperphosphorylation of TOPO II in VP-16-resistant KB human tumor cells. Because these resistant KB cells (16) had 10-fold lower TOPO II protein than the sensitive cells, this anomalous finding may represent a compensatory role of phosphorylation to fulfill catalytic activity of TOPO II in cell replication. Studies in vitro with Drosophila melanogaster TOPO II (34) have demonstrated that phosphorylation of TOPO II by casein kinase II and protein kinase C can decrease drug-stabilized DNA cleavable complex and increase DNA religation. A functional relationship between the phosphorylated state of TOPO II and DNA cleavable complex formation is supported by the present data in HL-60/S cells treated with BAPTA-AM. Because previous reports have suggested a role for Ca²⁺ in stabilizing DNA cleavable complex (35), the results of the current study demonstrate that clamping of intracellular Ca²⁺ can lead to reduced DNA cleavable complex formation and hypophosphorylation of the 170-kDa TOPO IIα. Overall, reduction in

with the MDR phenotype is suggested to be primarily due to reduced drug accumulation (3, 4). However, unlike the vinca alkaloids, resistance to TOPO II inhibitors (DOX or VP-16) in MDR cells is not correlated with the altered drug accumulation (9, 10). Furthermore, the reduced formation of drug-stimulated cleavable complex in isolated nuclei from MDR cells suggests the presence of a resistant enzyme (7, 8, 10). We reported previously that HL-60 cells selected for DOX resistance overexpress PGP (19), and the present results describe potential mechanisms of VP-16 resistance in this subtype. Although accumulation of VP-16 is compromised in the HL-60/Dox 0.05 cells, a comparison between the HL-60/S and HL-60/Dox 0.05 cells of the VP-16 accumulation (Fig. 2) and cytotoxicity (Fig. 1) data reveals that the HL-60/Dox 0.05 cells require markedly higher drug levels to achieve equivalent cell kill. The lack of correlation in HL-60/S and HL-60/Dox 0.05 cells between VP-16 accumulation and the magnitude of differences in VP-16-induced DNA cleavage or cytotoxicity is comparable to our previous data with the progressively DOX-resistant L1210 model system (10, 11). The observation that HL-60/Dox 0.05 cells accumulate higher drug levels than HL-60/S cells for an equivalent cytotoxic response is also apparent in the induction of DNA single-strand breaks measured by alkaline elution or, more
the 170-kDa TOPO II phosphorylation seems to be associated with decreased VP-16-induced DNA cleavable complex formation in HL-60/S or HL-60/DOX 0.05 cells; a novel finding in this study is the demonstration that reduced phosphorylation of TOPO II in the HL-60/DOX 0.05 cells seems to be primarily due to hypophosphorylation of at least three phosphopeptides.

In summary, the current results demonstrate that the 170-kDa TOPO II in DOX-resistant cells, which overexpress mdr-1 but not MRPI, is selectively altered with regard to the formation of VP-16-stabilized DNA cleavable complex. The reduction in cleavable complex formation and phosphorylation of TOPO II in the HL-60/DOX 0.05 cells is possibly due to the hypophosphorylation of phosphopeptides characteristic of HL-60/S cells. Thus, mapping of phosphoacceptor sites in either hypophosphorylated or hyperphosphorylated TOPO II from resistant cells labeled in vivo and analysis of site-specific mutant TOPO II should aid in establishing a functional role with reference to drug-stabilized DNA cleavable complex formation.

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References


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