Biochemical Pharmacology, Vol. 52, 669–676, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00343-7

Effect of Acute and Chronic Intermittent Hypoxia on DNA Topoisomerase II_α Expression and Mitomycin C-Induced DNA Damage and Cytotoxicity in Human Colon Cancer Cells

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ABSTRACT. Recently, we reported that alterations in topoisomerase II (topo II) activity appear to contribute to mitomycin C (MMC) resistance in HT-29R13 human colon cancer cells under aerobic conditions. In this study, the expression of topo II_α and topo II_β in parent HT-29 and MMC resistant variant HT-29R13 cells was investigated under aerobic, acute hypoxic (after 4 hr in 95% N_2, 5% CO_2 <0.01% O_2), and chronic intermittent hypoxic (after 4 hr hypoxia/day × 7 days) conditions. Acute hypoxia induced topo II_α mRNA and protein, effects that were more pronounced in HT-29 cells. Chronic intermittent hypoxia caused a decrease in topo$_\alpha$ mRNA and protein, changes that were again more pronounced in HT-29 cells. The observed changes in topo II_α protein were associated with parallel changes in topo II activity under all conditions tested. Topo II_β mRNA was expressed at a very low level in both cell lines under aerobic and hypoxic conditions. Compared with cells under aerobic conditions, HT-29 cells were more sensitive to MMC under acute hypoxia but more resistant under chronic intermittent hypoxia. In contrast, the sensitivity of HT-29R13 cells was unchanged under acute hypoxia, but the cells were more resistant under chronic intermittent hypoxia. Under all conditions tested, the degree of cytotoxicity corresponded to the frequency of MMC-induced DNA cross-links and topo II_α protein levels and activity. Our results demonstrated that MMC cytotoxicity in hypoxic cells is highly dependent upon the type of hypoxia and the cell type. Hypoxia has significant effects on topo II_α expression in HT-29 and HT-29R13 cells which correlate with MMC cytotoxicity. BIOCHEM PHARMACOL 52;4:669–676, 1996.

KEY WORDS. mitomycin C; topoisomerase II; hypoxia; drug resistance

Solid tumors often contain two distinct classes of hypoxic cells, acutely hypoxic cells and chronically, transiently hypoxic cells [1, 2], which differ in their metabolic characteristics and therapeutic implications [3]. Some of these metabolic changes render cells resistant to radiation and many standard antineoplastic drugs [4, 5]. However, these hypoxia-induced changes may also serve as targets for cytotoxic drugs [6].

The antitumor antibiotic MMC† has preferential toxicity towards hypoxic cells under certain conditions in certain types of cells [6–9], a property that has stimulated much interest in its potential as an antineoplastic agent and as a radiation sensitizer. MMC is a bifunctional alkylating agent that, upon reductive activation [10], cross-links complementary strands of DNA *in vivo* and *in vitro* [11]. Damage to the integrity of DNA most likely represents the mechanism of MMC cytotoxicity and antitumor activity [12].

The findings presented here are an extension of our two previous reports [13, 14] that have identified the multifactorial nature of MMC resistance in the human colon cancer cell line HT-29R13, a variant of the HT-29 line that has exhibited stable MMC resistance over a 4-year period. HT-29R13 cells have a variety of similarities with parent HT-29 cells including expression of P-gp and high levels of baseline DT-diaphorase DTD activity. In addition to other factors, we demonstrated that MMC resistance in HT-29R13 under aerobic conditions is associated with reduced topo II activity. This was an interesting finding since MMC is not considered a topo II inhibitor [15]. HT-29R13 cells, in addition to MMC resistance, are highly cross-resistant to 5-FU [13], another agent that causes DNA damage unrelated to topo II. HT-29R13 cells are not cross-resistant to conventional topo II inhibitors such as doxorubicin or etoposide. The similar levels of P-gp, GSTπ, and DTD in both cell lines [14] may explain the lack of cross-resistance be-

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† *Abbreviations:* MMC, mitomycin C; topo II, topoisomerase II; cDNA, complementary DNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SSC, standard sodium citrate; P-gp, P-glycoprotein; DTD, DT-diaphorase; RT-PCR, reverse transcription-polymerase chain reaction; 5-FU, 5-flurouracil; and GAPDH, glyceraldehyde phosphate dehydrogenase.

Received 12 September 1995; accepted 25 March 1996.

tween them. A possible reason for MMC resistance in HT-29R13 cells is that MMC might not have been able to induce lethal amounts of DNA cross-links because of the decrease of topo II activity. However, such an association was not firmly established.

The ATP-dependent enzyme DNA topo II alters DNA conformation and plays an important role in DNA metabolism and structure [16]. Two isoenzyme forms of the topo II enzyme, α and β, are expressed in mammalian cells [17]. Topo II has been identified as the cellular target for many clinically active anticancer agents, but the relative contribution of topo II to MMC sensitivity is unknown. We have continued our work in this interesting model to examine the potential role of topo II (α or β) in MMC-induced DNA damage and cytotoxicity under aerobic and hypoxic conditions.

MATERIALS AND METHODS

Cell Culture

The HT-29 human colon cancer cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The MMC resistant HT-29R13 subline was isolated from the HT-29 line by repeated exposures to 2 μM MMC for 1 hr each week as previously described [13]. Cells were maintained in Ham's F10 medium with 5% fetal bovine serum at 37° in a 95% air/5% CO_2 environment. Cells in logarithmic growth phase were used in all experiments. MMC was provided by Mr. Sal Luciana of the Bristol-Myer-Squibb Co. (Princeton, NJ).

Generation of Hypoxia

Cells grown in 75 cm^2 flasks were incubated in a humidified environmental chamber (PLAS LABS, Lansing, MI) at 37°. Cells were treated in three different ways. For aerobic conditions, cells were gassed for 4 hr with air (+5% CO_2). For acute hypoxia, cells were gassed for 4 hr with N_2 (+5% CO_2, <0.01% O_2). For chronic intermittent hypoxia, cells were subjected repeatedly to hypoxia for 4 hr daily for 7 consecutive days, with fresh medium added each time after hypoxic treatment. Cells were initially plated at higher density in acute hypoxia experiments (4×10^4 cells/cm^2), compared with chronic intermittent hypoxia experiments (2×10^3 cells/cm^2), so that the final cell densities in both types of experiments were similar. Acute hypoxia had no effect on cell cycle kinetics as measured using flow cytometry in either cell line. Cells plated at the lower density and grown under aerobic conditions for up to 7 days had similar cell cycle kinetics. Both cell lines grown under chronic intermittent hypoxia underwent a similar increase in the G_1/G_0 fractions from 44 ± 7 to 72 ± 9%. Cultures were maintained under hypoxia in Ham's F10 medium containing 5% fetal bovine serum and 3 mM glucose, corresponding to a low normal physiologic level characteristic of ischemic tissue *in situ* [18]. After completion of aerobic or hypoxic treatment, cells were trypsinized for the preparation of nuclear protein or mRNA. There were no significant changes in medium pH during the described hypoxic periods. Cell viability after acute or chronic intermittent hypoxia was 92 ± 2 and 86 ± 3%, respectively.

Northern Blot

A 1.8 kb fragment of the human topo II_α cDNA (provided by Dr. L. Liu, Robert Wood Johnson Medical School, Piscataway, NJ), and a 1.8 kb fragment of the human topo II_β cDNA (from Dr. K. B. Tan, Smith Kline & French Laboratories, King of Prussia, PA) were labeled with [^{32}P]dCTP (3000 μCi/mmol, Amersham International, Arlington Heights, IL) with a specific activity of 10^9 cpm/μg DNA by random priming. The methods for total RNA extraction, electrophoresis, and transfer onto nitrocellulose membrane were essentially the same as previously described [19]. The RNA gel was stained with ethidium bromide prior to transfer to the membrane to monitor the integrity of 18S and 28S rRNA bands. The membrane was prehybridized in 1 M NaCl, 10% dextran sulfate, 50% formamide and 0.1% SDS at 42° overnight and then hybridized in 5× SSC, 1× Denhardt's solution, 0.1 mg/mL salmon sperm DNA, 10% dextran sulfate, 45% formamide, 0.05% SDS, and 10^6 cpm ^{32}P-labeled DNA topo II_α or topo II_β probe at 42° overnight. The membrane was treated with four 5-min washes in 2× SSC – 0.01% SDS at 24° and then three 15-min washes in 0.2× SSC – 0.1% SDS at 60°. Autoradiography was performed at −70°, and quantitation of the results was achieved by desitometric scanning and normalized to the signal for GAPDH.

Preparation of Nuclear Extracts

Nuclear fractions from HT-29 and HT-29R13 cells were isolated as described previously [14] with modifications. In brief, cells were trypsinized and washed twice with PBS and then permeabilized by incubation in hypotonic nuclear buffer (2 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM NaCl and 100 mM dithiothreitol, pH 6.5) for 10–15 min. The lysed cells were centrifuged at 1000 *g* for 5 min, and the nuclear pellet was collected and washed with hypotonic buffer. The nuclear protein was extracted from the nuclear pellet by incubation for 60 min in hypotonic buffer containing 0.35 M NaCl and 1 mM phenylmethylsulfonyl fluoride (PMSF). The nuclear DNA and debris were pelleted by centrifugation at 17,000 *g* for 30 min. The supernatant was saved as the nuclear protein extract. The extract was dialyzed for 24 hr at 4° against three changes in hypotonic buffer because hypoxia may alter cellular metabolism [20] and Ca^{2+}, Mg^{2+}, and Na^+ transport [21], all of which may interfere with topo II activity. Protein content of the nuclear extract was determined by the method of Bradford [22], and ATP levels were determined by luciferin-luciferase assay [23]. ATP concentrations in undialyzed and dialyzed nuclear extracts were 2.10 to 2.55 and 0.01 to 0.02 μM, respectively. Fresh preparations served as the source of protein for topo II

relaxation activity and for the topo II immunoblotting assay.

Reverse Transcription-cDNA Synthesis and PCR (RT-PCR)

For cDNA strand synthesis, 1 μg of total cellular RNA was reverse transcribed in a 10-μL reaction mixture containing 0.1 μg random hexamers, a 0.5 mM concentration of each deoxynucleoside triphosphate, and 100 U Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL, Grand Island, NY) in 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$ and 10 mM dithiothreitol. The reaction was incubated at 37° for 60 min. DNase-free RNase (2 μM; Worthington Biochemical, Freehold, NJ) was added for 5 min at 37°. Following phenol/chloroform extraction and precipitation with ethanol, the content of cDNA was measured by spectrophotometer and stored at −20°. PCR was performed in a Perkin–Elmer/Cetus DNA Thermal Cycler (Norwalk, CT). Five microliters of the above cDNA (0.01 to 100 ng) solution was incubated with 1 U Tag DNA polymerase (Perkin–Elmer/Cetus) in a 25-μL volume containing a 0.5 mM concentration of deoxynucleoside triphosphates, 0.2 μCi deoxycytidine 5′-[^{32}P]α-triphosphate (>3000 μCi/mmol, Amersham Corp.), a 37.5 mM concentration of each of the 5′ and the 3′ topo II_β primers (5′ 2079–2102, 3′ 2359–2382) [24], 40 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.8 mM $MgCl_2$, and 0.01% (w/v) bovine serum albumin. Fifty microliters of light mineral oil (Sigma Chemical Co., St. Louis, MO) was overlaid, and PCR was carried out for 35–55 cycles for 30 sec at 94°, 60 sec at 55° and 15 sec at 72°. Fifteen microliters of each sample was electrophoresed in an 8% acrylamide gel. Then gels were stained with ethidium bromide, visualized, and photographed on a UV transilluminator. Radioactivity of the gel fragments was measured by liquid scintillation counting. The sizes of bands were estimated by the migration of an *Msp*I digest of plasmid pBR322 DNA. Ribosomal RNA cDNAs (5′ 1501–1520 and 3′ 1827–1846) were used as internal controls.

Western Blot

One hundred micrograms of nuclear protein per lane from HT-29 and HT-29R13 cells was separated by 6% SDS–PAGE and electrophoretically transferred onto a nitrocellulose membrane (Bio-Rad, Richmond, CA). The membrane was incubated in blocking buffer (3% BSA, 5% nonfat dry milk in 0.05% Tween 20-PBS) for 2 hr at room temperature. Blots were then incubated in primary antibody against human DNA topo II_α (1:1000 in 0.05% Tween 20-PBS) (provided by Dr. L. Liu) for 1 hr at 24°. The bound antibody was visualized with alkaline phosphatase-linked goat anti-rabbit IgG, using 5-bromo-4 chloro-3 indolyl phosphate and nitroblue tetrazolium chloride substrates. The relative amounts of topo II_α protein levels were quantified using densitometric analysis.

DNA Topo II Activity

Quantitative measurements of DNA topo II relaxation activity in nuclear extracts of HT-29 and HT-29R13 cells were assayed by the ability of serial concentrations of nuclear protein to relax supercoiled pBR322 DNA (GIBCO, Gaithersburg, MD) [25]. One unit of topo II was defined as the amount of nuclear protein required to completely convert 1 μg of supercoiled pBR322 DNA into the relaxed form in 10 min at 37°.

Measurement of DNA Interstrand Cross-Linking

MMC-induced DNA intrastrand cross-linking in HT-29 and HT-29R13 cells was determined by the alkaline elution method [26]. Cells were seeded in 25-cm^2 flasks and labeled with [^{14}C]thymidine (0.02 Ci/mL; 56 mCi/mmol; New England Nuclear, Boston, MA) or [^{3}H]thymidine (0.5 Ci/mL) for 24 hr. The labeled cells were washed twice with cold PBS, and the radioactivity was chased by an additional 24-hr incubation in nonradioactive medium. Cells were labeled prior to aerobic or acute hypoxic drug treatment. For chronic intermittent hypoxic treatment, the labeling was done prior to the last hypoxia treatment. Cells were treated with MMC for 1 hr under aerobic or hypoxic conditions and then washed with ice-cold PBS containing 0.02% EDTA. Then control or drug-treated ^{14}C-labeled cells (0.5×10^6) were mixed with tritiated cells (0.5×10^6). Instead of irradiation, the cells were exposed to 100 μM H_2O_2 for 60 min on ice, corresponding to an irradiation dose of 300 rad [27]. The combined cell suspension was immediately prepared for alkaline elution as described by others [20]. Briefly, 1×10^6 ^{14}C- and ^{3}H-labeled cells were layered onto a polyvinyl chloride filter, washed with cold PBS, and lyzed with 5 mL lysis solution (2% SDS, 25 mM EDTA, 0.5 mg/mL proteinase K; pH 9.7) for 45 min. Filters were then washed twice with 2 mL of 25 mM EDTA (pH 10.3), and DNA was eluted from the filters with 30 mL tetrapropyl-ammonium hydroxide-EDTA (pH 12.1) containing 0.1% SDS at a rate of 0.035 mL/min in a dark environment at 25°. The filters and fractions were then processed as described by Kohn *et al.* [26]. Interstrand cross-linking (ISC) frequency was determined using the equation:

$$\text{ISC (rad eq)} = [((1 - R_0)/(1 - R_1))^{1/2} - 1] \times 300$$

where R_0 and R_1 are the relative retention of [^{14}C]DNA from control and MMC-treated cells, respectively, measured at the point where retention of [^{3}H]DNA equals 20%.

Hypoxic Cytotoxicity Assay

For acute hypoxia, cells were plated at a density of 2×10^5 cells in 20 cm^2 glass flasks (LUREX, Vineland, NJ) that were specially modified by adding a separate chamber for drug exposure as previously described by others [28]. The cells were allowed to grow for 24 hr. The medium was

removed and replaced with 2.5 mL of fresh medium, and 50 μL medium containing different concentrations of MMC was put in separate chamber of each flask. Then the flasks were put in the environmental chamber and gassed for 4 hr under aerobic or acute hypoxic conditions as described above. During the final hour, the cells were exposed to drug by mixing the MMC solution and the cell culture medium, so that no reoxygenation occurred between hypoxic and MMC treatment. After the incubation period, the chamber was opened and the flasks were immediately cooled on ice-cold aluminum trays. The drug-containing medium was removed, and the flasks were rinsed twice with ice-cold PBS followed by addition of 0.05% trypsin. After cell detachment, cells were resuspended in fresh medium, counted, and plated in 96-well flat-bottomed plates. Cells were allowed to grow to 4 days. The MTT assay was then carried out as previously described [29].

For chronic intermittent hypoxia, the cytotoxicity assay was essentially the same except that cells were initially seeded at a lower density (2×10^3 per flask) and subjected to hypoxia for 4 hr each day for 7 consecutive days as described above. Fresh medium was added each time after hypoxic treatment. On the final day (day 7), the cells were treated with MMC.

RESULTS

DNA Topo II mRNA Expression

Topo II mRNA expression under aerobic and hypoxic conditions was investigated using northern blotting. To determine which topo II isoform is expressed in these cells, northern blots were first hybridized with a topo II_α cDNA probe. The same membranes were later stripped of the probe and rehybridized with the topo II_β cDNA probe. Densitometric scans of the northern blots (Fig. 1) revealed that baseline topo II_α mRNA expression under aerobic conditions in resistant HT-29R13 cells was lower than that in HT-29 cells, indicating that the previously observed decrease of topo II activity in HT-29R13 cells [14] may occur at the transcriptional level. Acute hypoxia induced a 2.2-fold increase in topo II_α mRNA in HT-29 cells ($P < 0.05$) but induced only a 1.2-fold increase in HT-29R13 cells (P = NS). Under chronic intermittent hypoxia, HT-29 cells showed a 3.5-fold decrease and HT-29R13 cells a 2.8-fold decrease in topo II_α mRNA. The decreases in topo II_α mRNA in both cell lines were statistically significant ($P < 0.05$). Topo II_α mRNA levels in 7-day aerobic control cells showed no significant change as compared with cells cultured for 24–48 hr indicating that topo II_α expression may not be age dependent but instead may be cell-density dependent [30]. Topo II_β mRNA levels were almost undetectable in both cell lines under aerobic conditions (Fig. 2).

RT-PCR Amplification of Topo II_β mRNA

Since the levels of the topo II_β mRNA in HT-29 and HT-29R13 cells were almost undetectable by standard

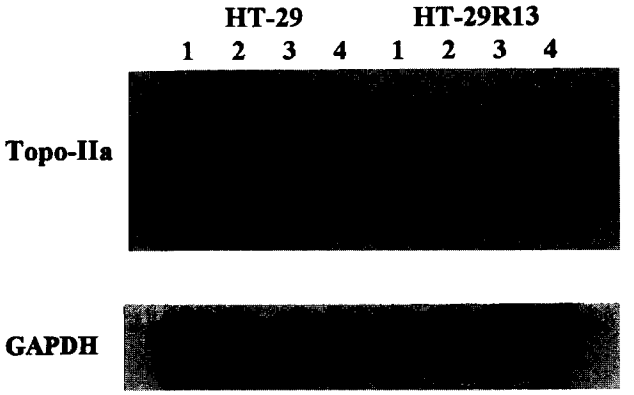

FIG. 1. Effect of hypoxia on topo II_α mRNA expression. Cells were incubated under aerobic or hypoxic conditions as described in Materials and Methods. Following incubation, 15 μg of total RNA was isolated, and mRNA was measured by northern blot hybridization with a ^{32}P-labeled topo II_α probe. Membranes were rehybridized with a GAPDH cDNA probe to control for the amount of RNA present in each lane. Shown is a northern hybridization from a representative experiment (N = 4). Lanes: 1, aerobic conditions for 24 hr; 2, acute hypoxia; 3, aerobic conditions for 7 days; and 4, chronic intermittent hypoxia.

northern blotting techniques, we sought to further analyze the levels of topo II_β mRNA in aerobic or hypoxic HT-29 and HT-29R13 cells with a PCR-based assay. As shown in the representative experiment displayed in Fig. 3, similar

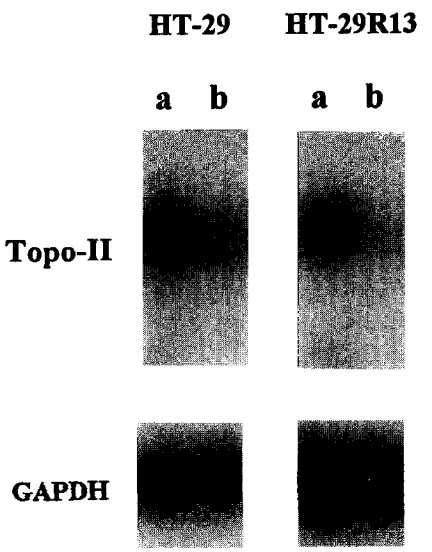

FIG. 2. Comparison of topo II_α and topo II_β mRNA expression. Cells were incubated under aerobic conditions as described in Materials and Methods. Following incubation, 15 μg of total mRNA was isolated, and mRNA was measured by northern blot hybridization with a ^{32}P-labeled topo II_α probe. The same membranes were stripped of the topo II_α probe, and then rehybridized with a ^{32}P-labeled topo II_β probe. The membranes were also rehybridized with a GAPDH cDNA probe to control for the amount of RNA present in each lane. Shown is a representative northern hybridization from one of the four experiments performed. Lanes: a, topo II_α; and b, topo II_β.

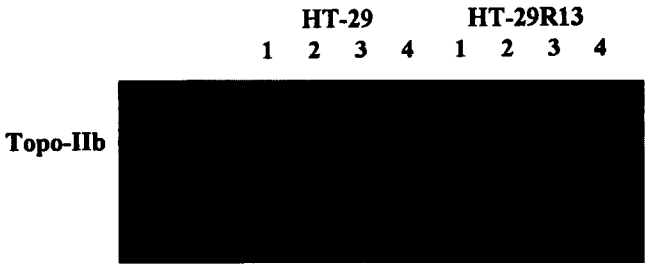

FIG. 3. Effect of hypoxia on topo II_β mRNA analyzed by RT-PCR. One microgram of total cellular mRNA was reverse transcribed, and PCR was performed as described in Materials and Methods. Samples were analyzed using 8% acrylamide gel electrophoresis and then stained with ethidium bromide. Ribosomal RNA cDNAs were used as internal controls. Shown is a representative gel from one of the three experiments performed. Lanes: 1, aerobic conditions for 24 hr; 2, acute hypoxia; 3, aerobic conditions for 7 days; 4, chronic intermittent hypoxia.

levels of topo II_β mRNA were found in all samples, indicating that acute or chronic intermittent hypoxia had no significant effect on topo II_β levels in the cells.

DNA Topo II_α Protein Levels

Nuclear protein extracted from HT-29 and HT-29R13 cells treated under aerobic or hypoxic conditions was subjected to western blotting with topo II_α antibody (Fig. 4). Under control (aerobic) conditions, topo II_α protein levels were greater in HT-29 than in HT-29R13 cells, suggesting that the previously demonstrated decrease of topo II activity in HT-29R13 cells [14] resulted from a decrease of topo II_α protein. Topo II_α protein levels showed no significant change when cells were grown under aerobic conditions for 7 days. Acute hypoxia significantly increased topo II_α protein levels in HT-29 cells by about 4-fold ($P < 0.001$), but only slightly increased topo II_α protein in HT-29R13 cells by about 1.5-fold (P = NS). In chronic intermittent hypoxia-treated HT-29 and HT-29R13 cells, topo II_α protein levels were decreased by 2.4- ($P < 0.01$) and 1.8-fold ($P < 0.05$), respectively, compared with aerobic cells.

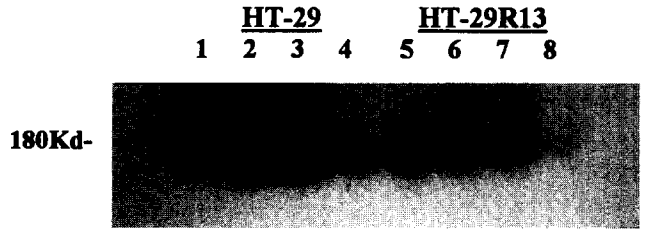

FIG. 4. Effect of hypoxia on topo II_α protein expression. Cells were incubated under aerobic or hypoxic conditions as described in Materials and Methods. Following incubation, nuclear protein was isolated, separated on a 6% SDS–PAGE gel, and then transferred to a nitrocellulose membrane. Topo II was detected by immunoblotting using an antibody against human topo II. Shown is an immunoblot from a representative experiment (N = 4). Lanes: 1 and 5, aerobic conditions for 24 hr; 2 and 6, acute hypoxia; 3 and 7, aerobic conditions for 7 days; and 4 and 8, chronic intermittent hypoxia.

DNA Topo II Activity

Topo II activity in 0.35 M NaCl extracts of nuclei isolated from control or hypoxia-treated cells was compared by using the topo II specific pBR322 DNA relaxation assay [25]. In ATP-depleted nuclear extracts, no topo I activity was noted using this assay. The results obtained with serial dilutions of nuclear protein extracts are shown in Figure 5. The activity of topo II in control (aerobic conditions) HT-29 cells was markedly (1.8-fold) higher than that in control HT-29R13 cells (Table 1). The activities were somewhat lower than those we reported before [14] because we used a lower serum concentration in the current experiments, which again demonstrates the proliferation dependence of topo II activity [30]. Acute hypoxia resulted in a 3.0-fold increase in topo II activity in HT-29 cells ($P < 0.05$), but lacked a significant effect in HT-29R13 cells. In chronic intermittent hypoxia-treated HT-29 and HT-29R13 cells, activities of topo II decreased 2.7-fold ($P < 0.05$) and 1.6-fold ($P < 0.05$), respectively, compared with their aerobic counterparts.

MMC-Induced DNA Interstrand Cross-Links

As shown in Fig. 6, MMC treatment under aerobic conditions induced DNA interstrand cross-links in both HT-29 and HT-29R13 cell lines in a concentration-dependent fashion, but the frequency of cross-links was about 2-fold higher in HT-29 cells than in HT-29R13 cells ($P < 0.05$). In HT-29 cells, MMC treatment under acute hypoxia resulted in an increased number of DNA cross-links, and treatment under chronic intermittent hypoxia resulted in a

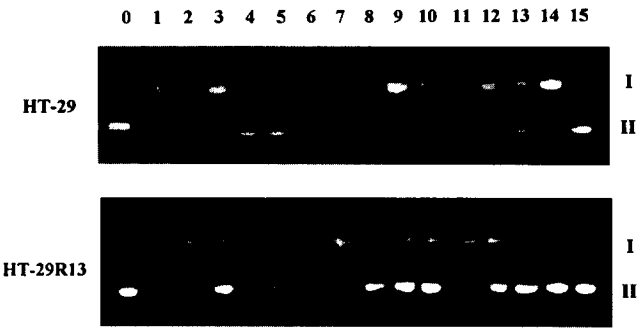

FIG. 5. Effect of hypoxia on topo II activity. Cells were incubated under aerobic or hypoxic conditions as described in Materials and Methods. After incubation, nuclear protein was extracted, and DNA topo II activity was assayed by the ability of serial dilutions of nuclear protein (0.25, 0.125, 0.0625, 0.0313, 0.0157 μg per well) to relax supercoiled pBR322 DNA. Shown is a representative gel from one of the four experiments performed. Ordinate I, "relaxed" form of DNA; II, supercoiled form of DNA; lanes: 0, control; 1–5, aerobic conditions; 6–10, acute hypoxia; and 11–15, chronic intermittent hypoxia.

TABLE 1. Effect of hypoxia on topo II activity

	Topo II activity (units)		
	Aerobic	Acute Hypoxia	Chronic intermittent Hypoxia
HT-29	68 ± 12	204 ± 51*	25 ± 15*†
HT-29R13	39 ± 10	46 ± 9	24 ± 7†

Cells were incubated under aerobic conditions, acute hypoxia (4 hr), and chronic intermittent hypoxia (4 hr/day × 7 days) as described in Materials and Methods. Nuclear protein was isolated, and topo II activity was determined using the pBR322 DNA relaxation assay. One unit of topo II was defined as the amount of nuclear protein required to completely convert 1 μg of supercoiled pBR322 DNA into the relaxed form in 10 min at 37°C. Values are means ± SEM, N = 5 independent experiments.

* $P < 0.05$ compared with aerobic cells.

† $P < 0.05$ compared with acute hypoxic cells.

decreased number of cross-links, compared to treatment under aerobic conditions (Fig. 6). The difference between the number of cross-links induced by MMC under aerobic, acute hypoxia, and chronic intermittent hypoxia was statistically significant ($P < 0.05$). In HT-29R13 cells, there was no significant difference in MMC-induced DNA cross-links under aerobic conditions or acute hypoxia (Fig. 6). However, MMC induced fewer DNA cross-links in HT-29R13 cells under chronic intermittent hypoxia than under aerobic conditions ($P < 0.05$). It is interesting to note that when the two cell lines were treated under the same conditions, the frequency of MMC-induced DNA cross-links was always significantly higher in HT-29 cells than in the resistant HT-29R13 cells.

MMC *Cytotoxicity*

Under aerobic conditions, there was an approximately 2.5-fold difference in IC_{50} values (Table 2) between HT-29 and HT-29R13 cells, consistent with our earlier study that used a higher serum concentration [14]. The serum concentration in this study was lowered to more closely resemble solid tumors *in vivo*. HT-29 cells were more sensitive to 1 hr MMC under acute hypoxia ($P < 0.01$). In contrast, acute hypoxia did not affect the sensitivity of HT-29R13 cells to 1 hr MMC. Under chronic intermittent hypoxia, both cell lines became significantly more resistant to MMC. HT-29 cells were about 3 times more resistant and HT-29R13 cells were about 1.5 times more resistant to MMC under chronic intermittent hypoxia than their aerobic controls (both $P < 0.01$). Overall, HT-29 cells were more sensitive to the effects of hypoxia than the MMC resistant subline HT-29R13.

DISCUSSION

MMC has been shown to be more cytotoxic towards certain cells under hypoxic conditions *in vitro* [6–9]. In these situations, enhanced activation of MMC occurs via hypoxic metabolism [10]. However, the antiproliferative effects of

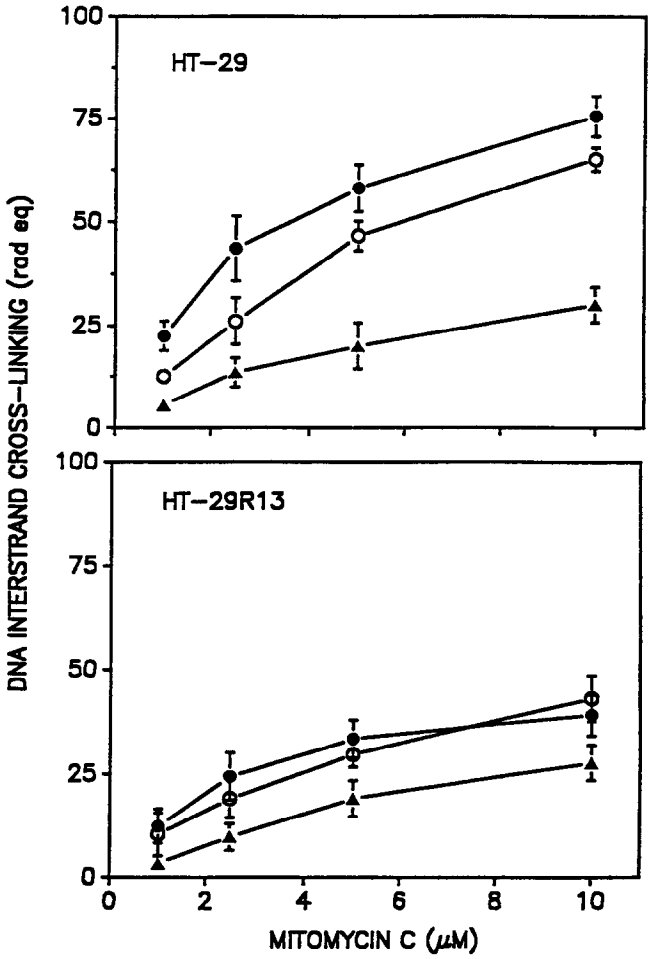

FIG. 6. Effects of hypoxia on MMC-induced DNA strand breaks. Cells were isolated under aerobic or hypoxic conditions and DNA fragments were isolated using the alkaline elution technique as described in Materials and Methods. Top panel, HT-29 cells. Bottom panel, HT-29R13 cells. Key: (○) aerobic conditions (●) acute hypoxia; and (▲) chronic intermittent hypoxia. Values are means ± SEM, N = 4 independent experiments.

MMC are not enhanced by increased activation in all *in vitro* systems [31]. This suggests that other factors may also affect MMC cytotoxicity, such as the ability of the drug to interact with DNA targets [12], or to interfere with DNA damage repair [32]. Since DNA topo II has been shown to

TABLE 2. Effect of hypoxia on MMC IC_{50} values

	IC_{50} (μM)		
	Aerobic	Acute hypoxia	Chronic intermittent hypoxia
HT-29	0.65 ± 0.11	0.28 ± 0.08*	1.91 ± 0.19*
HT-29R13	1.55 ± 0.16	1.42 ± 0.22	2.40 ± 0.16*

Cells were incubated under aerobic conditions, acute hypoxia (4 hr), and chronic intermittent hypoxia (4 hr/day × 7 days), and then treated during the last hour with MMC as described in Materials and Methods. Cytotoxicity was determined using the MTT assay. Values are means ± SEM, N = 4 independent experiments.

* $P < 0.01$ compared with aerobic cells.

play a vital role in DNA metabolism and repair [16], and decreased topo II activity was associated with decreased MMC cytotoxicity [14], it was important to further study the role of topo II in MMC-induced cytotoxicity and DNA cross-linking under hypoxic and aerobic conditions.

This study demonstrated by means of northern and western blotting that HT-29R13 cells possess lower levels of topo II_α mRNA and protein than HT-29 cells. Thus, our previous finding of lower topo II activity in HT-29R13 cells may be the result of a transcriptional level change of topo II_α expression. An unexpected observation was that topo II_β expression was not changed in HT-29R13 cells compared with HT-29 cells, either under aerobic or hypoxic conditions. This indicates that topo II_β is unlikely to contribute to MMC and 5-FU resistance in HT-29R13 cells and unlikely responsible for the hypoxia-induced alterations of MMC sensitivity in these cells.

Acute hypoxic stress significantly induced topo II_α mRNA, protein, and activity in HT-29 cells, but had little effect in HT-29R13 cells. Chronic intermittent hypoxia resulted in decreased topo II_α mRNA levels in both HT-29 and HT-29R13 cells, accompanied by decreased protein content and activity, indicating transcriptional regulation of topo II_α expression. It is interesting that the effects of hypoxic conditions on topo II_α expression and activity were more pronounced in HT-29 cells.

Decreased topo II expression/activity is felt to be responsible for cellular resistance to a variety of anticancer agents [33]. Such drugs appear to exert their cytotoxicity by forming topo II–drug–DNA cleavable complexes, resulting in DNA damage [34]. Qualitative assays of topo II catalytic and cleavage activity revealed that up to 100 μM MMC had no significant effect on topo II activity (data not shown), suggesting that topo II itself is not a direct target of MMC. The differences in topo II_α expression and activity in HT-29 and HT-29R13 cells treated under aerobic, acute, and chronic intermittent hypoxic conditions correlate with the ability of MMC to induce DNA interstrand cross-linking (Fig. 6). These results support our hypothesis that topo II_α affects MMC-induced DNA damage by increasing the accessibility of DNA targets to MMC.

The results also demonstrate that baseline or hypoxia-induced differences in topo II_α activity (Fig. 5, Table 1) correlate with differences in MMC-induced cytotoxicity. Under acute hypoxia, HT-29 cells were more sensitive to MMC, similar to results observed with acute hypoxia in other cell lines [31]. In contrast, HT-29R13 cells showed no significant change in MMC sensitivity under acute hypoxia. Chronic intermittent hypoxia decreased the sensitivity of both cell lines to MMC. MMC sensitivity was decreased about 3-fold in HT-29 and 1.5-fold in HT-29R13 cells (Table 2). The changes in MMC cytotoxicity induced by chronic intermittent hypoxia were not due to cell density effects *per se* since the sensitivity of control (aerobic) cells up to 7 days in culture remained unchanged (data not shown).

We have concentrated our attention on the association between the levels of expression of topo II_α and MMC-induced DNA cross-linking and cytotoxicity. The results presented here suggest a correlation between cellular sensitivity to MMC and topo II_α expression and activity under aerobic or hypoxic conditions. It is certainly possible that hypoxia may also induce alterations in mRNA stability and metabolism and in other mechanisms associated with MMC resistance. We have not seen any hypoxia-induced alterations in DTD activity under the conditions used in this study (unpublished observations), although others have noted induction of DTD mRNA with a single longer (8 hr) hypoxic treatment [35]. The chronic intermittent hypoxia-induced changes in topo II_α expression and activity may be explained, at least partially, by the observed increase in G_1/G_0 fraction (see Materials and Methods). However, the topo II_α alterations that occurred under acute hypoxia were not accompanied by any changes in cell cycle kinetics. This suggests that factors in addition to cell cycle regulate topo II under hypoxic conditions. The effects of hypoxia on topo II metabolism, P-gp expression, and MMC activation or detoxification enzymes are being studied currently in our laboratory.

We speculate that topo II affects the accessibility of targets in DNA which are damaged by drugs such as MMC and 5-FU. This appears different from the role that topo II plays in DNA damage repair or the formation of the cleavable DNA–topo II–drug complex. Furthermore, the results of this study demonstrate that hypoxia-induced alterations of chemotherapy sensitivity are highly dependent upon the type of hypoxia and the particular cell line.

This work was supported, in part, by Grant CDA 93-283 from the American Cancer Society and the Sentara Endowment Fund. The authors very much appreciate the help of Dr. Janos Luka with the PCR techniques and thank Diane K. Fox for preparing the manuscript.

References

1. Thomlinson RH and Gray LH, The histological structure of some human lung cancers and the possible implications for radiotherapy. *Br J Cancer* **9:** 539–549, 1955.
2. Chaplin DJ, Olive PL and Durand RE, Intermittent blood flow in a murine tumor: Radiobiological effects. *Cancer Res* **47:** 597–601, 1987.
3. Vaupel P, Kallinowski F and Okunieff P, Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: A review. *Cancer Res* **49:** 6449–6465, 1989.
4. Bedford JS and Mitchell JB, The effect of hypoxia on the growth and radiation response of mammalian cells in culture. *Br J Radiol* **47:** 687–696, 1974.
5. Born R, Hug O and Trott KR, The effect of prolonged hypoxia on growth and viability of Chinese hamster cells. *Int J Radiat Oncol Biol Phys* **1:** 687–697, 1976.
6. Kennedy KA, Hypoxic cells as specific drug targets for chemotherapy. *Anticancer Drug Des* **2:** 181–194, 1987.
7. Iyer VN and Szybalski W, Mitomycins and porfiromycin: Chemical mechanism of activation and cross-linking of DNA. *Science* **145:** 55–58, 1964.

8. Sartorelli AC, Therapeutic attack of hypoxic cells of solid tumors: Presidential address. *Cancer Res* **48:** 775–778, 1988.
9. Workman P, Keynote address. Bioreductive mechanisms. *Int J Radiat Oncol Biol Phys* **22:** 631–637, 1992.
10. Keyes SR, Fracasso PM, Heimbrook DC, Rockwell S, Sligar SG and Sartorelli AC, Role of NADPH: Cytochrome *c* reductase and DT-diaphorase in the biotransformation of mitomycin C. *Cancer Res* **44:** 5638–5643, 1984.
11. Dorr RT, Bowden GT, Alberts DS and Liddil JD, Interaction of mitomycin C with mammalian DNA detected by alkaline elution. *Cancer Res* **45:** 3510–3516, 1985.
12. Kumar S, Lipman R and Tomasz M, Recognition of specific DNA sequences by mitomycin C for alkylation. *Biochemistry* **31:** 1399–1407, 1992.
13. Perry R, Graves BR and Kang Y, Development and initial characterization of a mitomycin C-resistant colon cancer cell line variant. *Cancer Chemother Pharmacol* **32:** 326–328, 1993.
14. Perry RR, Kang Y and Greaves B, Biochemical characterization of a mitomycin C resistant colon cancer cell line variant. *Biochem Pharmacol* **46:**1999–2005, 1993.
15. Liu LF, DNA topoisomerase poisons as antitumor drugs. *Annu Rev Biochem* **58:** 351–375, 1989.
16. Maxwell A and Gellert M, Mechanistic aspects of DNA topoisomerases. *Adv Protein Chem* **38:** 69–107, 1986.
17. Drake FH, Zimmerman JP, McCabe FL, Bartus HF, Per SR, Sullivan DM, Ross WE, Mattern MR, Johnson RK, Crooke ST and Mirabelli CK, Purification of topoisomerase II from amscacrine-resistant P-388 leukemia cells: Evidence for two forms of the enzyme. *J Biol Chem* **34:** 16739–16747, 1987.
18. Webster KA, Discher DJ and Bishopric NH, Induction and nuclear accumulation of *fos* and *jun* proto-oncogenes in hypoxic cardiac myocytes. *J Biol Chem* **268:** 16852–16858, 1993.
19. Kang Y and Perry RR, Effect of α-interferon on p-glycoprotein expression and function and on verapamil modulation of doxorubicin resistance. *Cancer Res* **54:** 2952–2958, 1994.
20. Kvietikova I, Wenger RH, Marti HH and Gassmann M, The transcription factors of ATF-1 and CREB-1 bind constitutively to the hypoxia-inducible factor-1 (HIF-1) DNA recognition site. *Nucleic Acids Res* **25:** 4542–4550, 1995.
21. Kozlowski RZ, Ion channels, oxygen sensation and signal transduction in pulmonary arterial smooth muscle. *Cardiovasc Res* **30:** 318–325, 1995.
22. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
23. Beutler E, *Red Cell Metabolism: A Manual of Biochemical Methods*, pp. 92–96. Grune & Stratton, New York, 1971.
24. Chung TD, Drake FH and Tan KB, Characterization and immunological identification of cDNA clones encoding two human DNA topoisomerase II iosenzymes. *Proc Natl Acad Sci USA* **86:** 9431–9435, 1989.
25. Wang JC, DNA topoisomerases. *Annu Rev Biochem* **54:** 665–697, 1985.
26. Kohn KW, Erickson LC, Ewig RAG and Friedman CA, Fractionation of DNA from mammalian cells by alkaline elution. *Biochemistry* **14:** 4629–4637, 1976.
27. Manome Y, Datta R, Taneja N, Shafman T, Bump E, Hass R, Weichselbaum R and Kufe D, Coinduction of *c-jun* gene expression and internucleosomal DNA fragmentation by ionizing radiation. *Biochemistry* **32:** 10607–10613, 1993.
28. Prekeges JL, Rasey JS, Grunbaum Z and Krohn KH, Reduction of fluoromisonidazole, a new imaging agent for hypoxia. *Biochem Pharmacol* **42:** 2387–2395, 1991.
29. Kang Y and Perry R, Modulatory effects of tamoxifen and recombinant human α-interferon on doxorubicin resistance. *Cancer Res* **53:** 3040–3045, 1993.
30. Woessner RD, Mattern MR, Mirabelli CK, Johnson RK and Drake FH, Proliferation and cell cycle dependent differences in expression of the 170 kilodalton and 180 kilodalton forms of topoisomerase II in NIH-3T3 cells. *Cell Growth Differ* **2:** 209–214, 1991.
31. Plumb JA and Workman P, Unusually marked hypoxic sensitization to indoloquinone E09 and mitomycin C in a human colon-tumor cell line that lacks DT-diaphorase activity. *Int J Cancer* **56:** 134–139, 1994.
32. Hughes CS, Irvin CG and Rockwell S, Effect of deficiencies in DNA repair on the toxicity of mitomycin C and porfiromycin to CHO cells under aerobic and hypoxic conditions. *Cancer Commun* **3:** 29–35, 1991.
33. Fry AM, Chresta CM, Davies SM, Walker MC, Harris AL, Hartley JA, Masters JRW and Hickson ID, Relationship between topoisomerase II level and chemosensitivity in human tumor cell lines. *Cancer Res* **51:** 6592–6595, 1991.
34. de Jong S, Zijlstra JG, de Vries EGE and Mulder NH, Reduced DNA topoisomerase II activity and drug-induced DNA cleavage activity in an Adriamycin-resistant human small cell lung carcinoma cell line. *Cancer Res* **50:** 304–309, 1990.
35. Dwyer PJ, Kao KS, Ford P, Godwin AK and Clayton M, Effects of hypoxia on detoxicating enzyme and expression in HT29 colon adenocarcinoma cells. *Cancer Res* **54:** 3082–3087, 1994.