Journal of Cellular Biochemistry 75:46–55 (1999)

Topoisomerase II Is Nonfunctional in Polyamine-Depleted Cells

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Abstract The polyamines—putrescine, spermidine, and spermine—are essential for normal cell proliferation. Polyamine depletion affects DNA structure and synthesis. Topoisomerase II (topo II) is also necessary for normal cell proliferation, and it has been shown in vitro that polyamines may affect topo II activity. In order to investigate the effect of polyamine depletion on topo II activity, we treated Chinese hamster ovary cells with either α-difluoromethylornithine (DFMO) or 4-amidinoindan-1-one-2′-amidinohydrazone (CGP 48664), which are polyamine biosynthesis inhibitors. Treatment with the topo II inhibitor etoposide results in DNA strand breaks only if there is active topo II in the cells. By quantitating DNA strand breaks after etoposide treatment using single cell gel electrophoresis, we were able to estimate intracellular topo II activity. We also quantitated topo II activity in crude nuclear extracts from control and polyamine biosynthesis inhibitor-treated cells. Using single cell gel electrophoresis, we noted a clear decrease in the function of topo II in polyamine biosynthesis inhibitor-treated cells, as compared with untreated control cells. However, the topo II activity in crude nuclear extracts did not differ significantly in control versus polyamine biosynthesis inhibitor-treated cells. Taken together, these results indicate that although the function of topo II in polyamine-depleted cells was impaired, topo II remained functional in an in vitro assay. Using the single cell gel electrophoresis assay, we also found that spermine depletion itself caused DNA strand breaks. J. Cell. Biochem. 75:46–55, 1999.

Key words: CGP 48664; DFMO; DNA strand breaks; etoposide; single cell gel electrophoresis

The polyamines—putrescine, spermidine, and spermine—are essential for cell proliferation [Luk and Casero, 1987]. They are polycations, and targets of their action are negatively charged macromolecules such as proteins, RNA, DNA, and phospholipids [Watanabe et al., 1991]. Polyamines have been shown to stabilize double-helical structures such as DNA, as well as stems and loops in rRNA and mRNA. Spermine stabilizes the tRNA conformation through binding to specific sites [Heby and Persson, 1990]. Polyamines have also been implicated in DNA replication [Geiger and Morris, 1980; Morris, 1991; Fredlund et al., 1994; Fredlund and Oredsson, 1997].

Topoisomerase II (topo II) is also essential for normal cell proliferation [Wang, 1985]. It is required to maintain the integrity of the DNA helix in replication, transcription, and chromosome condensation in mitosis [Gellert, 1981; Wang, 1985]. Topo II is part of the DNA synthesome [Coll et al., 1996] and it is thought to be localized at or near DNA-matrix attachment sites [Berrios et al., 1985; Earnshaw et al., 1985]. The absence of active topo II at DNA-nuclear matrix attachment sites may result in high supercoiling stress in DNA during replication [Basu and Marton, 1995]. Topo II changes the topology of DNA by catalyzing the passage of one double-stranded segment of DNA through a double strand break it has cut in a second DNA double strand segment [Roca et al., 1996]. After the passage, topo II religates the double strand break. This multistep reaction is ATP and Mg^{2+} dependent. Etoposide causes a stabilization of the cleavable complexes of topo II and DNA [Glisson and Ross, 1987; Liu, 1989], which leads to impaired DNA synthesis and cell cycle arrest [Del Bino et al., 1991]. Etoposide is a semisynthetic derivative of the naturally occurring anti-proliferative agent epipodophyllotoxin [Henwood and Brogden, 1990], and it is a nonintercalative specific inhibitor of topo II.

Grant sponsor: Swedish Natural Science Research Council; Grant sponsor: Swedish Cancer Foundation; Grant sponsor: Hierta-Rezius foundation; Grant sponsor: IngaBritt and Arne Lundbergs Research Foundation.

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Received 8 October 1998; Accepted 1 March 1999

After removal of proteins attached to DNA, the damage caused by etoposide can be measured as DNA single and double strand breaks [Long et al., 1985].

Polyamines have been shown to alter topo II activity in the test tube [Kreuzer and Cozzarelli, 1980; Krasnow and Cozzarelli, 1982; Pommier et al., 1989]. Polyamine biosynthesis inhibitors have indirectly been shown to affect topo II in cells in culture [Dorr et al., 1986; Bakic et al., 1987; Desiderio et al., 1997]. However, in none of these studies has the function of topo II in DNA cleavage in the cell been investigated. The present paper addresses the issue of topo II activity in cells treated with two different polyamine biosynthesis inhibitors. α–Difluoromethylornithine (DFMO) is a highly specific, enzyme-activated, irreversible inhibitor of ornithine decarboxylase, which is the first enzyme in the polyamine biosynthetic pathway [Metcalf et al., 1978]. 4-Amidinoindan-1-one 2′-amidinohydrazone (CGP 48664) inhibits S-adenosylmethionine decarboxylase, which is another key enzyme in the polyamine biosynthetic pathway [Stanek et al., 1993]. We also estimated the topo II activity in crude nuclear extracts from control and inhibitor-treated cells, which gives the total cellular pool of potential enzyme activity. Our results show that although there is no difference between topo II activity in crude nuclear extracts of control or inhibitor-treated cells, topo II appears unable to cleave DNA in polyamine biosynthesis inhibitor-treated cells, as detected using the single cell gel electrophoresis assay.

MATERIALS AND METHODS

Materials

Etoposide was purchased from Sigma Chemical Co. (St. Louis, MO). Low-melting-point agarose, Nusieve® GTG, and agarose gel support medium, Gel Bond®, were purchased from FMC BioProducts (Rockland, ME). Growth medium components were purchased from Biochrom KG (Berlin, Germany). Tissue culture plastics were purchased from Nunc, Roskilde, Denmark. The Topo II assay kit was purchased from TopoGEN (Columbus, OH). DFMO was a generous gift from the Merrell Dow Research Institute (Strasbourg, France). CGP 48664 was a generous gift from Dr. Helmut Mett (Ciba Geigy, Basel, Switzerland).

Cells and Experimental Design

Chinese hamster ovary (CHO) cells were cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS) and supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml). The cells were subcultured twice weekly and incubated at 37°C in a water-saturated atmosphere of 5% CO_2 in air. For the experiments, cells were seeded into fresh medium in tissue culture flasks (75 cm^2) in the absence or presence of 5 mM DFMO or 20 µM CGP 48664. The concentrations of DFMO and CGP 48664 were chosen to produce similar growth inhibition [Fredlund et al., 1994; Fredlund and Oredsson, 1997]. For the growth curve experiments, cells were seeded at a density of 0.25×10^6 per flask in 15 ml of medium for all treatment groups. For all other experiments, the seeding density used when treating cells with DFMO or CGP 48664 was 1×10^6 per flask in 15 ml of medium. Control cells were seeded at a density of 1×10^6, 0.5×10^6, or 0.25×10^6, to be harvested at days 1, 2, or 3–4, respectively, after seeding. The proliferation rate of the cells was the same at the different seeding densities. The higher seeding densities were used to provide adequate numbers of cells for the analysis. When used, etoposide (100 µM) was added to cultures 16 h before harvest. The etoposide concentration used did not result in apoptosis or necrosis. Etoposide was dissolved in DMSO and stored at −20°C as a 100-mM stock solution.

Cell Cycle Phase Distribution

The cells were harvested by trypsinization, pelleted at 700*g* for 5 min, and the medium was decanted. The cells were resuspended in a buffer consisting of 40 mM Na-citrate pH 7.6, 250 mM sucrose, 0.7 M dimethylsulfoxide (DMSO) at a density of 2×10^6 cells per ml and stored at −20°C. Before flow cytometric (FCM) analysis of the cell cycle phase distribution, cellular DNA was stained with propidium iodide (phosphate-buffered saline [PBS] containing 50 mg/ml propidium iodide, 100 mg/ml RNAse, and 0.6% Nonidet P-40 [NP-40]). The staining solution was added during vigorous agitation, and the samples were incubated in the dark for 30 min at 4°C. The nuclear suspension obtained after the staining procedure was suctioned three times through a cannula (0.7-mm diameter) and filtered through a 50-µm nylon mesh in

order to remove nuclear aggregates. Analysis of the DNA content was performed in an Ortho Cytoron Absolute flow cytometer (Ortho Diagnostic Systems, Raritan, NJ) equipped with a 15-mW air-cooled argon-ion laser. The laser line at 488 nm was used for excitation of the fluorochrome. Ten thousand nuclei per sample were analyzed at approximately 200 nuclei per second. The fluorescence signals were digitized by a 4096 multichannel analyzer and stored as list data files according to the FCM standard 1.0 by acquisition software, running on a PC, onto a data file server (VAX/VMS, Digital Equipment) for further computerized analysis. Contributions from nuclear aggregates were minimized by electronic threshold settings in the red area versus red peak bivariate distributions [Baldetorp et al., 1989]. For the computerized analysis, Multi2D® and MultiCycle® software programs (Phoenix Flow Systems, San Diego, CA) were used.

Topoisomerase II Activity in Nuclear Extracts

Crude nuclear extracts were prepared according to De Isabella et al. [1990]. All steps were performed at 0–4°C. After harvesting by trypsinization, the cells were washed once with PBS (pH 7.3: 8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4, 0.2 g/L KH_2PO_4). After pelleting, the cells were resuspended in 500 µl of nuclear buffer consisting of 100 mM NaCl, 1 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM β-mercaptoethanol, 10% (v/v) glycerol, and 0.5 mM phenylmethylsulfonylfluoride (PMSF). Then, 4.5 ml of nuclear buffer containing 0.35% Triton X-100 was added, and the suspension was incubated for 30 min under gentle rotation. The suspension was centrifuged and the pellet washed once with nuclear buffer. The pellet was then resuspended in lysis buffer consisting of 100 mM NaCl, 1 mM KH_2PO_4, 1 mM EGTA, 10 mM β-mercaptoethanol, 10% (v/v) glycerol, 0.5 mM PMSF, and 10 mM $NaHSO_3$. NaCl (4 M) was slowly added to a final concentration of 0.35 M. The nuclei were incubated for 1 h under gentle rotation. The sample was centrifuged and the supernatant was immediately used for the analysis of topo II activity using the topo II assay kit from TopoGEN. In brief, the activity was measured by monitoring the ability of the crude nuclear extract to decatenate catenated DNA. The extracts were diluted 10, 50, 100, 500, and 1,000 times before being added to the reaction mixture containing catenated DNA. The samples were then incubated for 30 min at 37°C. The reaction was terminated by putting the samples on ice and adding a stop and loading buffer. The degree of DNA decatenation was analyzed by agarose gel electrophoresis. The gel contained ethidium bromide. The DNA was visualized by UV transillumination. Photographs were taken of the gels and the negatives were analyzed by densitometric scanning using a Sun-computer and the Bioimage scanning and analyzing program (Bioimage, Millipore, Ann Arbor, MI).

Single Cell Gel Electrophoresis Assay

The single cell gel electrophoresis (SCGE) assay was used to investigate the presence of DNA strand breaks in control and inhibitor-treated cells grown in the absence or presence of etoposide. The assay was performed under alkaline conditions according to the procedures of Singh et al. [1991] and Olive et al. [1992] with the adjustments noted below. A standard protocol for comet preparation and analysis was adopted after examining several different parameters.

Standard protocol. Direct light was avoided during all steps. The cell layer was rinsed with 3 ml of trypsin-EDTA solution (4°C, 0.05% trypsin, 0.02 mM EDTA, pH 7.2–7.3). Then, 1 ml of trypsin-EDTA solution (4°C) was added, and the cells were incubated at 37°C for 3 min. The reaction was promptly stopped by the addition of complete medium, and the cell suspension was transferred to a test tube on ice. The cells were counted, pelleted at 700*g* for 5 min, and diluted in PBS to 2×10^4 cells/µl. Low melting agarose (1%) was mixed with PBS, melted, and kept at 37°C. Each 30-µl cell suspension was diluted in 1 ml of the low melting agarose-PBS mixture. A total of 70 µl of the agarose-cell mixture was pipetted onto the hydrophilic side of a piece of Gel Bond® membrane (size: 24 × 60 mm) and another piece of Gel Bond® was placed on top with the hydrophobic side facing the gel. The gel was allowed to solidify at 4°C for 7 min. Before removing the upper Gel Bond®, the gel was kept at room temperature (RT) for 2 min. A total of 70 µl of agarose was added as a protective layer on top of the now solid agarose-cell mixture, and another piece of Gel Bond® was placed on top. When the second layer of agarose was solid, the upper membrane was removed and the cells were lysed for 1 h at 4°C by placing the mem-

branes with the gels in lysis solution (2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, 10% DMSO, 10 mM Tris-HCl, pH 10). The gels were then put in electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) and agitated for 30 min at RT, allowing the DNA to unwind. After electrophoresis in fresh electrophoresis buffer for 20 min at 30 V/32 cm and 560 mA, the gels were incubated for three times 5 min each in neutralization buffer (0.4 M Tris-HCl, pH 7.5). The DNA was stained for 15 min with ethidium bromide (10 µg/ml); the gels were then destained by washing four times with distilled water. Excess fluid was removed and coverslips placed on top of the gels. The samples were then viewed in a fluorescence microscope as described below.

Evaluation of DNA damage. Observations were made at 40× magnification, using an epifluorescence microscope (Olympus BX90). Photos were taken with a Kodak DCS 420 digital camera and the image processing program Adobe Photoshop™ version 3.0 was used. The analysis was performed on a Power Macintosh 8100/100AV, using the public domain NIH Image version 1.56 (written by Wayne Rasband at the U.S. National Institutes of Health and available from Internet by anonymous FTP from zippy.nimh.nih.gov).

The objects of interest appeared as comets, with heads and tails, in the fluorescence microscope. The comet head represents the remnants of the nucleus, the nucleoid, and the comet tail represents migrated DNA, which in most cases was less fluorescent than the nucleoid (Fig. 1). Care was taken to avoid measuring comets near the edges of the gels and the objects were illuminated for only a short period of time. Each comet was randomly captured, avoiding comets clustered together and/or without a clearly identifiable head and/or tail. At least five objects/slide were photographed. The image analysis program displayed 256 levels of intensity. Thresholding was used when calculating the areas of interest.

The following procedures were used for calculation (Fig. 1):

1. To measure the cells, an image of a scale photographed with the same magnification as the comets was used as a standard.
2. The nucleoid area (NA) and nucleoid average intensity were measured.
3. The total area (NA+TA) and total average intensity were measured.

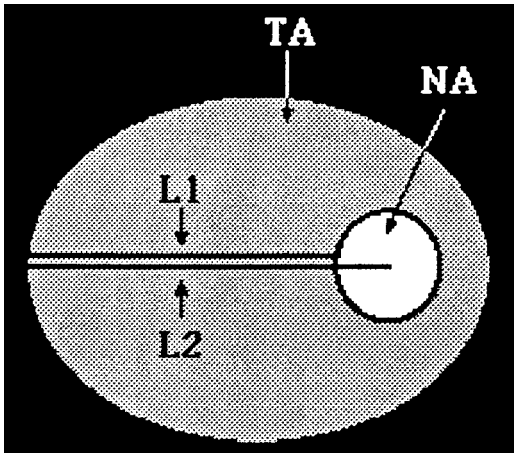

Fig. 1. Schematic representation of measurements made when analyzing comets obtained after single cell gel electrophoresis. NA, nucleoid area. TA, tail area. L1, the length from the edge of the nucleoid to the end of the tail. L2, the length from the center of the nucleoid to the end of the tail.

4. The tail area (TA) and tail average intensity were measured.
5. Two different lengths were measured. L1 was measured from the trailing edge of the nucleoid to the end of the tail and L2 was measured from the center of the nucleoid to the end of the tail.
6. Background intensity (BI) was determined as one intensity level below the lowest intensity level in the tail.

Steps 1–6 were repeated for each image and the measurements were stored automatically in a file.

Calculations were made using abbreviations and formulas according to Hellman et al. [1995]:

BI: background intensity
TA: tail area (µm^2)
TI: tail average intensity − BI
NA: nucleoid area (µm^2)
NI: nucleoid average intensity − BI
TDNA: percentage of DNA in tail
TMOM: tail moment

$$TDNA = TA \cdot TI / [(TA \cdot TI) + (NA \cdot NI)]$$

$$L\ (\text{tail distance}, \mu m) = L2 - L1/2$$

$$TMOM = TDNA \cdot L$$

TMOM is a measure of DNA damage. The more DNA that has left the nucleoid area due to DNA damage, the higher TMOM.

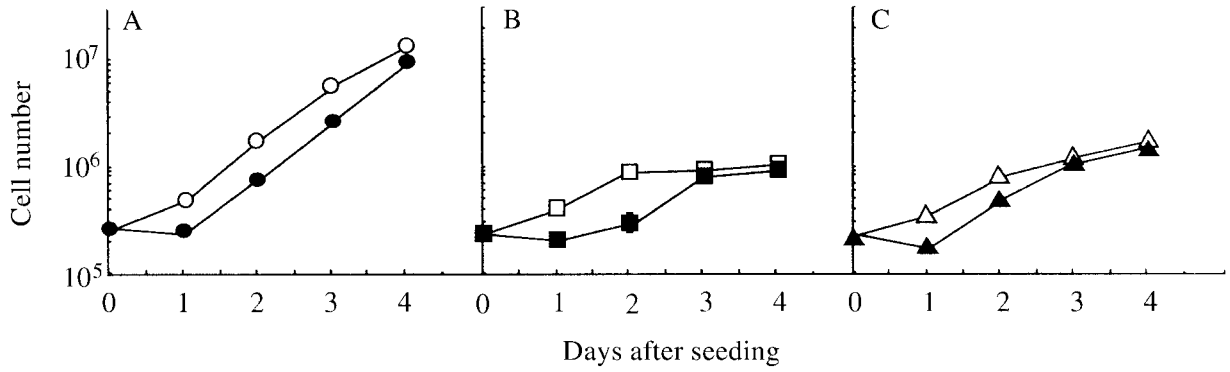

Fig. 2. Effect of etoposide on the growth of CHO cells seeded in the absence or presence of polyamine biosynthesis inhibitors. Cells were seeded at time 0 in the absence (○, ●) or presence of 5 mM DFMO (□, ■) or 20 μM CGP 48664 (△, ▲). At 16 h before harvest, 100 μM etoposide was added (●, ■, ▲). The standard error of the mean (n = 6 from three different experiments) was calculated. Error bars are hidden within the symbols.

RESULTS

Cell Growth

As shown previously [Fredlund et al., 1994; Fredlund and Oredsson, 1997], DFMO or CGP 48664 added at the time of seeding inhibits the proliferation of CHO cells, which is apparent two days after seeding (Fig. 2). When etoposide was added to control cells 16 h before harvesting, the cells slowed or ceased proliferating, resulting in reduced cell numbers at days 1–4 after seeding as compared with cells not treated with etoposide (Fig. 2A). In DFMO- and CGP 48664-treated cultures, the cell numbers were lower in the etoposide-treated cell cultures days 1 and 2 after seeding, but not on days 3 and 4 (Figs. 2B,C).

Topo II Activity in Nuclear Extracts

The topo II assay kit used is specific for topo II (type I activities will not be detected by this assay). As shown in Figure 3, catenated kinetoplast DNA (kDNA) remains in the well after electrophoresis because of its size (C). When catenated kDNA is cleaved by topo II, two decatenation products with different electrophoretic mobility are obtained (D). Decatenated nicked open circular kDNA has a lower rate of mobility than that of decatenated relaxed kDNA. A potential problem with this assay is contamination by nucleases whose presence can result in linearization of the catenated kDNA. To ascertain that there is no or limited linearization, linearized kDNA is included as a control (L). The linearized kDNA has a slightly higher electrophoretic mobility than the decatenated nicked open circular kDNA. In addition, the results of nuclease contamination are ATP independent, whereas topo II requires ATP. The topo II assay kit gives the total pool of potential topo II activity in the cells.

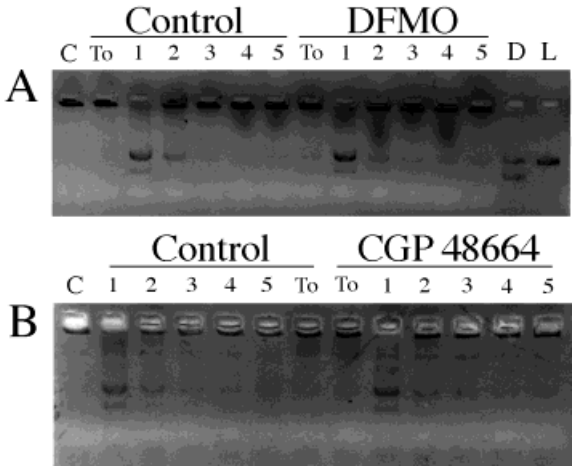

Fig. 3. Effect of DFMO or CGP 48664 treatment on the topo II-mediated cleavage of catenated kDNA. The ability of crude nuclear extracts from control, DFMO- and CGP 48664-treated CHO cells to decatenate kDNA was measured by incubating the extract with catenated kDNA. The reaction products were then separated by agarose gel electrophoresis, and visualized by UV transillumination after staining the gel with ethidium bromide. Cells were seeded at time 0 in the absence or presence of 5 mM DFMO or 20 μM CGP 48664 and harvested 1–4 days after seeding. Results from day 3 are shown. Each experiment was repeated three times; similar results were obtained for all treatment groups and days. In **lanes To,** the reaction was stopped immediately after addition of undiluted crude nuclear extract to the decatenation assay. **Lanes 1–5**, crude nuclear extracts diluted 1:10, 1:50, 1:100, 1:500, and 1:1,000, respectively; **lane C**, pure catenated kDNA; **lane D**, decatenated kDNA showing nicked open circular kDNA with lower electrophoretic mobility and relaxed kDNA with higher electrophoretic mobility; **lane L**, linearized kDNA.

When the decatenation reaction was stopped immediately after the addition of nuclear extracts from control or inhibitor-treated cells, all

the catenated kDNA was found in the well (Fig. 3, lane To). When the nuclear extracts from any of the treatment groups were diluted 1:10 and incubated with kDNA for 30 min, all kDNA was decatenated and found as nicked open circular and relaxed (Fig. 3, lane 1). With increasing dilution of the nuclear extracts, less kDNA was decatenated. There was no difference between the treatment groups at days 1–4 after seeding (Fig. 3 shows day 3 after seeding). Although the difference in migration mobility was small, the linear kDNA always migrated farther than the decatenated nicked open circular DNA indicating that nuclease activity was absent or minimal in the nuclear extracts. Assays run without ATP did not result in any linearization of catenated kDNA, further diminishing the possibility of nuclease contamination (results not shown).

Topo II Activity in the Cell

When cells were treated with etoposide alone, there was a substantial increase in the number of DNA strand breaks as compared with untreated control cells based on the calculation of the TMOM of comets in the SCGE assay (Fig. 4A). Control cells treated with etoposide had a TMOM value approximately twice as large as that observed in cells not treated with etoposide. The increase in TMOM in control cells after etoposide treatment was essentially the same at days 1–4 after seeding. In DFMO-treated cells, TMOM was the same as that in control cells at day 1 after seeding but increased at days 2–3 after seeding (Fig. 4A,B). Day 4 after seeding there was no significant difference in TMOM between control and DFMO-treated cells. When DFMO-treated cells were treated with etoposide, TMOM was increased at day 1 after seeding, but not at days 2–4 after seeding, as compared with cells treated with DFMO alone. The TMOMs of DFMO- and etoposide-treated cells were significantly lower than those of control cells treated with etoposide (Fig. 4A,B). In CGP 48664-treated cells, TMOMs were significantly increased as compared with those of untreated control cells at days 1–4 after seeding (Fig. 4A,C). Etoposide treatment resulted in a slight but insignificant further increase in TMOM in CGP 48664-treated cells (Fig. 4C). The addition of putrescine to DFMO-treated cells and of spermidine and spermine together with aminoguanidine to CGP 48664-treated cells essentially reversed the effects of the polyamine biosynthesis inhibitors. The resulting TMOMs were similar to those obtained with etoposide alone (results not shown).

Cell Cycle Phase Distribution

In control cells, the cell cycle phase distributions were severely affected by etoposide treatment at days 1–4 after seeding (Fig. 5). Cells accumulated in the S and G2 phases after etoposide treatment. DFMO- or CGP 48664-treated cells were as affected as control cells by etoposide treatment at days 1–2 after seeding; that is, they accumulated in the S and G2 phases. However, at days 3–4 after seeding the effects of etoposide treatment on the cell cycle phase

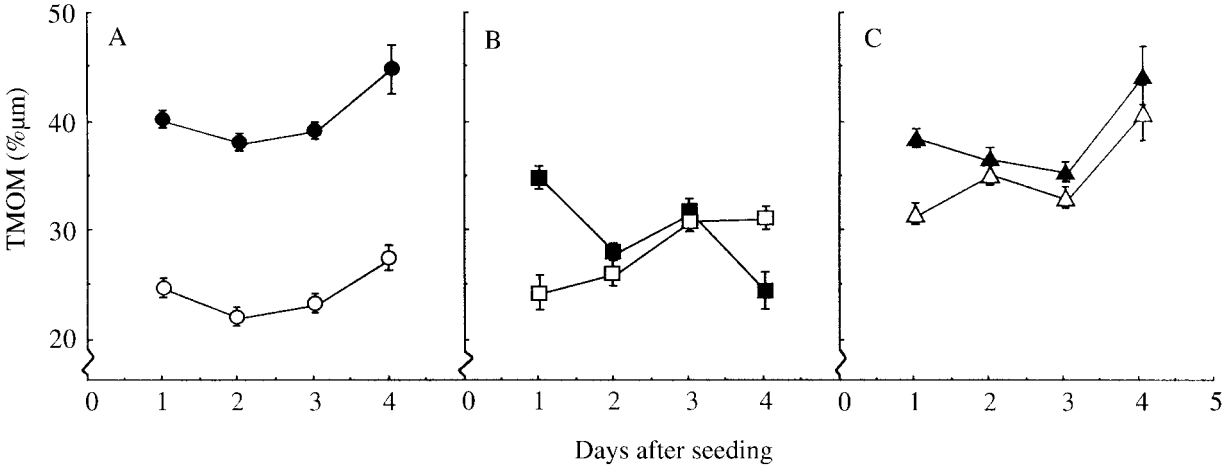

Fig. 4. Effect of etoposide on DNA damage as evaluated by the SCGE assay in CHO cells seeded in the absence or presence of DFMO or CGP 48664. Cells were seeded in the absence (○, ●) or presence of 5 mM DFMO (□, ■) or 20 μM CGP 48664 (△, ▲). At 16 h before harvest, 100 μM etoposide was added (●, ■, ▲). TMOM is a measure of DNA damage. Error bars represent standard error of the mean ($n > 30$ from three different experiments).

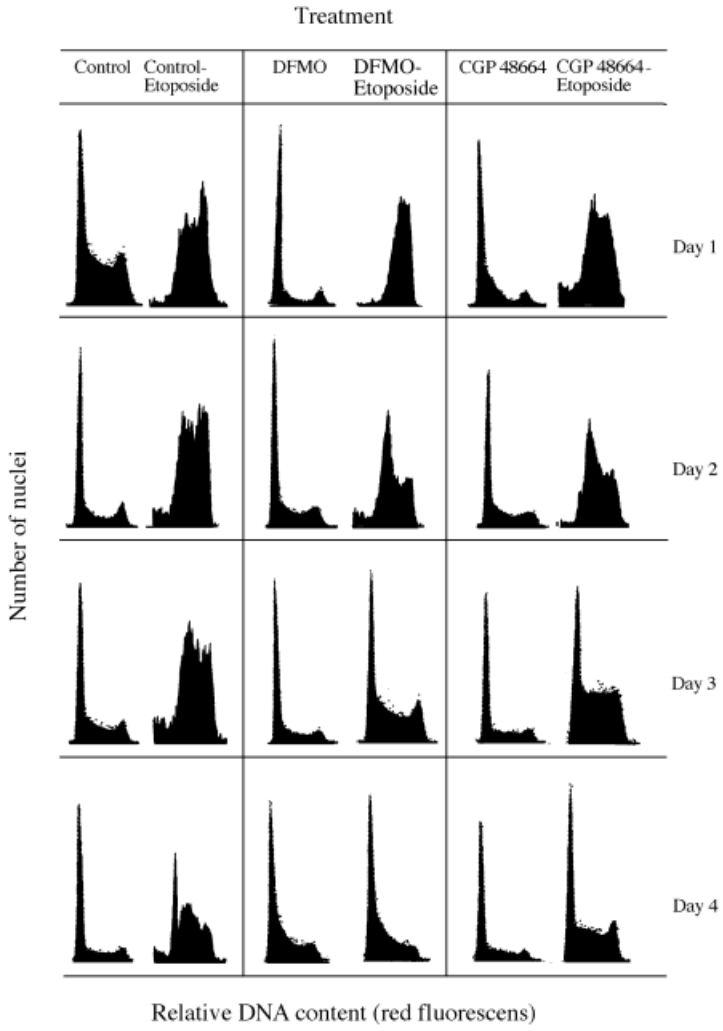

Fig. 5. Effect of etoposide on the cell cycle phase distribution of CHO cells seeded in the absence or presence of DFMO or CGP 48664. Cells were seeded and treated as described in the text of Figure 2. The results for days 1–4 after seeding are shown. They are representative of two different experiments with n = 4.

distribution of DFMO- or CGP 48664-treated cells were not as significant as those at days 1–2 after seeding. The addition of putrescine to the DFMO-treated cells, and of spermidine and spermine together with aminoguanidine to the CGP 48664-treated cells essentially reversed the effects of the polyamine biosynthesis inhibitors (results not shown).

DISCUSSION

Our results show that although the function of topo II in polyamine-depleted cells was impaired, topo II remained functional in an in vitro assay. There was no difference in the topo II activity measured in crude nuclear extracts from control or inhibitor-treated cells, even after four days of treatment with polyamine biosynthesis inhibitors. The topo II activity measured in crude nuclear extracts gives a measure of the total cellular pool of potential enzyme activity. To estimate the functional intracellular topo II activity, we used an alkaline SCGE assay that detects both single and double strand breaks in DNA [Singh et al., 1991; Olive et al., 1992]. Control and DFMO- or CGP 48664-treated cells were treated with the topo II inhibitor etoposide, which induces DNA single and double strand breaks when bound to active topo II [Long et al., 1985]. The more functional topo II (i.e., topo II bound to DNA) that is present in cells, the greater the number of DNA strand breaks that will be detected by the SCGE assay after etoposide treatment. As expected, the number of DNA strand breaks increased in cells

when they were treated with etoposide. In DFMO- or CGP 48664-treated cells, etoposide treatment increased the number of DNA strand breaks at day 1 after seeding. At days 2–4 after seeding in the presence of DFMO or CGP 48664, etoposide treatment did not result in an increase in the number of DNA strand breaks. Taken together, these results show that the function of topo II in the cell was severely affected by polyamine depletion while the enzyme itself was not directly affected as reflected by the lack of difference in kDNA cleaving activity in crude nuclear extracts from the different treatment groups. These results are supported by a number of studies, although none of those has directly estimated the topo II function in the cell [Dorr et al., 1986; Bakic et al., 1987; Desiderio et al., 1997]. The cytotoxicity of etoposide was found to be significantly lower in polyamine biosynthesis inhibitor-treated cells compared with control cells, as determined by colony forming efficiency assay [Desiderio et al., 1997]. Using Western blot, Desiderio and colleagues found that the enzyme protein level was not affected by the polyamine biosynthesis inhibitor treatment. Bakic et al. [1987] showed that DFMO-induced putrescine and spermidine depletion decreased etoposide-induced DNA single strand breaks, as measured by alkaline elution, in several cell lines. This decrease was not caused by reduced uptake of etoposide, which was actually increased in DFMO-treated cells, nor was it cell cycle phase dependent. The addition of putrescine reversed the effect of DFMO on etoposide-induced DNA single strand cleavage. Smirnov et al. [1994] noted a similar decrease in etoposide cytotoxicity after polyamine analog treatment of human brain tumor cells. In contrast to these results, showing an antagonistic effect of DFMO treatment on etoposide, Dorr et al. [1986] found an increased number of etoposide-induced DNA single strand breaks in DFMO-treated L1210 cells. Their results are in stark contrast to those of Bakic et al. [1987] using the same cell line.

We used two different polyamine biosynthesis inhibitors to obtain different polyamine deficiency profiles to determine whether the results could be conferred to a specific polyamine. As shown earlier, treatment of CHO cells with DFMO results in total depletion of putrescine and spermidine after 1 day of treatment, while the spermine level remains essentially unchanged [Fredlund et al., 1994]. Treatment of CHO cells with CGP 48664 results in increased putrescine levels at day 1 of treatment. Decreased levels of spermidine and spermine are obvious at day 1 of treatment, and at day 2 of treatment, spermidine and spermine are essentially depleted [Fredlund and Oredsson, 1997].

DFMO treatment alone did not seem to cause any DNA damage, as the TMOMs of control and DFMO-treated cells were similar. CGP 48664 treatment, however, resulted in increased DNA damage as compared with the damage observed in control and DFMO-treated cells. This increase in DNA damage may be the result of the lowered spermine and spermidine pool and/or the increased putrescine pool in CGP 48664-treated cells. Putrescine has a much lower binding affinity for DNA than spermidine or spermine [Morgan et al., 1986]. Therefore, our point of view is that the increased DNA damage detected by the SCGE assay in CGP 48664-treated cells, compared with control or DFMO-treated, was caused by the lowered spermine and spermidine pool, rather than the increased putrescine pool. The lowered putrescine and spermidine pools in DFMO-treated cells did not give rise to DNA damage. Since the absence of spermidine did not cause any DNA damage in DFMO-treated cells, we believe that the decrease in the spermine pool was responsible for the increased DNA damage in CGP 48664-treated cells. Polyamines bind to DNA in both specific and unspecific ways and spermidine and spermine have a stabilizing and protective effect on DNA [Morgan et al., 1987; Williams et al., 1994; Snyder and Sunkara, 1990]. Spermine deficiency may render DNA more vulnerable to certain cellular processes that cause DNA strand breaks.

The polyamines have been implicated in playing an important and dynamic role in chromatin structure and function [Morgan et al., 1987; Hougaard et al., 1987; Feuerstein et al., 1990]. Polyamine depletion has been shown to negatively affect the structure of chromatin [Basu et al., 1992; Snyder, 1989]. Topo II activity has been shown to be dependent on the DNA structure [Krasnow and Cozzarelli, 1982], which implies that changes in DNA structure caused by polyamine depletion could affect the function of the enzyme. We have shown that etoposide treatment did not cause an increase in DNA strand breaks in polyamine-depleted cells, in contrast to our results in cells containing normal polyamine levels. As mentioned above, eto-

poside only binds to topo II that is bound to DNA, thereby causing a stabilization of DNA strand breaks induced by the enzyme. Our results imply that although present in the cell, topo II was less functional in polyamine-depleted cells, resulting in an insensitivity to etoposide. Desiderio et al. [1997] have also speculated that a lowered cytotoxicity of etoposide in polyamine-depleted cells may be caused by changes in chromatin structure. Our results suggest that both spermine and spermidine have an important role in maintaining the chromatin structure in an optimal configuration for the function of topo II.

Etoposide treatment markedly affected the cell cycle phase distribution of polyamine biosynthesis inhibitor-treated cells at day 2 after seeding (Fig. 5), when there was no significant effect on the DNA strand breaks (Fig. 4). A lesser effect of etoposide on the cell cycle phase distribution of inhibitor-treated cells was observed at day 3 (DFMO and CGP 48664) and at day 4 (CGP 48664) after seeding the cells. At those time points, the TMOM values for inhibitor- and etoposide-treated cells are above those of cells treated only with inhibitor (Fig. 4). Although this is not a statistically significant increase, it may still imply that etoposide gave rise to a slight increase in DNA strand breakage, which in turn inhibited cell cycle progression. An alternative explanation may be that etoposide has another mechanism of action not related to topo II, although we have not found any support for this contention in the literature.

Topo II is vital for DNA replication [Wang, 1985]. Without functioning topoisomerases, the cells can initiate DNA replication and replicate a few thousand base pairs [Kim and Wang, 1989], but further elongation will be delayed. We and others have shown that the elongation step of DNA replication is impaired in polyamine-depleted cells [Geiger and Morris, 1980; Fredlund et al., 1994; Fredlund and Oredsson, 1997]. The present investigation gives rise to the hypothesis that DNA elongation is impaired in polyamine-depleted cells at least partly because topo II cannot function optimally.

In conclusion, we have investigated the total topo II activity in crude nuclear extracts as well as the functional intracellular topo II activity in control and polyamine biosynthesis inhibitor-treated cells. We found that the total topo II activity in crude nuclear extracts was unaltered by polyamine depletion, while the topo II activity in the cells was severely impaired. Since topo II activity has been shown to be dependent on DNA structure, these results imply that polyamine depletion may cause changes in DNA structure which renders the enzyme less efficient. Our results imply that spermidine and spermine have important functions in maintaining chromatin structure in an optimal state for the function of topo II. An impairment in the function of topo II may be responsible for the reduced DNA elongation rates found in polyamine-depleted cells. We have also shown that there is an increase in DNA strand breakage in spermine-depleted cells.

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