Topoisomerase II-mediated DNA Breaks and Cytotoxicity in Relation to Cell Proliferation and the Cell Cycle in NIH 3T3 Fibroblasts and L1210 Leukemia Cells

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ABSTRACT

The DNA intercalator, 4′-(9-acridinylamino)methanesulfon-m-anisidine (m-AMSA) and the nonintercalator, etoposide (VP-16) produce topoisomerase II-mediated protein-linked DNA strand breaks. This function of topoisomerase II was investigated in relation to cell proliferation and cell cycle. Mouse fibroblasts NIH 3T3 and mouse leukemia L1210 cells stop proliferation when they reach a certain density. Nuclei were isolated from proliferative or quiescent cells and then treated with drug for 30 min. DNA modifications were assayed by alkaline elution. We found that the frequencies of m-AMSA- or VP-16-induced DNA-protein links were higher in nuclei from exponentially growing than in those from quiescent cells in both the 3T3 and the L1210 lines. Drug-induced protein-associated DNA breaks were also studied as a function of the cell cycle in 3T3 cells that had been arrested by contact inhibition in medium containing 1% calf serum and then stimulated to proliferate by replating at a lower cell density in medium containing 10% serum. In these synchronized cells, a large peak of PHthymidine incorporation occurred 18–30 h after replating. The yield of DNA-protein cross-links produced by 30-min drug treatments of nuclei isolated at various times after growth initiation increased concomitantly with the peak of the DNA synthesis. The topoisomerase II activity of nuclear extracts, as measured by kine-toplast DNA decatenation followed a similar pattern. Using colony-forming assays, we also observed that m-AMSA and VP-16 were most cytotoxic in proliferative cells and during DNA synthesis. These results suggest that alkaline elution measurement of m-AMSA- or VP-16-induced protein-linked DNA breaks reflects the association of topoisomerase II with DNA. This association is increased during DNA replication, making the cells more vulnerable to m-AMSA and VP-16 at this time.

INTRODUCTION

The diverse activities of eukaryotic type II topoisomerases have been well reviewed (1–3). These enzymes play an important role in the organization and behavior of chromosomal DNA. It was demonstrated that type II topoisomerase is a major component of mitotic chromosome scaffold and the nuclear matrix (4, 5). Using different topoisomerase II phenotypes (mutants) of yeast, Holm et al. (6) demonstrated the indispensability of yeast topoisomerase II for cell growth. Recent studies have shown that one of the essential roles of eukaryotic topoisomerase II is its ability to unknot and decatenate chromosomal DNA, thus enabling the segregation of intertwined, newly replicated DNA molecules during mitosis (7). However, it is unlikely that topoisomerases act only in the mitotic phase of the cell cycle in mammalian cells. It is strongly suggested that these enzymes are also present during the G1 and the S phases of the cell cycle. Topoisomerases also control, by their relaxing effects on supercoils, the superhelical density of DNA in chromatin, perhaps in conjunction with mammalian type I topoisomerase (1–3). The initiation step of replication of bacteriophage T4 may also depend upon the phage-encoded type II topoisomerase (8). Duguet et al. (9) reported that topoisomerase II activity increased in regenerating rat liver. This result suggested a possible correlation between the activity of mammalian topoisomerase II and cell proliferation. Surprisingly, the studies of Tricoli et al. (10) showed no differences in topoisomerase II activity (measured by the ATP-dependent catenation reaction with PM2 DNA) in proliferating versus non-proliferating mouse embryonic fibroblast (C3H10T/2) cells, nor in the G1, S, and M phases of synchronized C3H10T/2 cells. Sullivan et al. (11) found that topoisomerase II DNA cleavage activity was severalfold higher in exponential phase versus plateau phase CHO cells but not strikingly different in HeLa or L1210 cells.

Further evidence has been accumulating that mammalian type II topoisomerases, which transiently break and rejoin double-stranded DNA, are targets of many DNA intercalative antineoplastic drugs, such as Adriamycin, daunomycin, the ellipticines, and m-AMSA, and of the nonintercalative epipodophyllotoxins VP-16 and VM-26 (teniposide) (12–16). These drugs trap topoisomerase II on the DNA within DNA–enzyme complexes. The complexes consist of topoisomerase II homodimers bound to DNA double- or single-strand breaks in such a way that the breaks are bridged by the enzyme, and one or both subunits are covalently linked to the 5'–ends of the DNA. DSB, SSB, and DNA-protein cross-links can be easily detected in mammalian cells by alkaline elution (17). These lesions have been characterized as drug-induced protein-linked DNA strand breaks (16, 18–20).

A correlation has been observed between the induction of such lesions by these drugs and their cytotoxic effects (21–26). We used the alkaline elution method to examine the formation of protein-linked DNA breaks by topoisomerase II inhibitors as a function of the proliferation state of the cells, and more specifically as a function of the cell cycle in synchronized cells. We also investigated whether the cytotoxicity of these drugs correlated with the production of DNA lesions in different phases of the cell cycle.

MATERIALS AND METHODS

Drug and Chemicals. m-AMSA (NSC 249992) was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, NCI. VP-16 (VP-16-213, NSC 141540) was a generous gift from Bristol-Myers Co. (Syracuse, NY). m-AMSA and VP-16 were dissolved in dimethyl sulfoxide at a concentration of 10 mM. m-AMSA stock solutions were kept frozen at −20°C for no longer than 15 days, while VP-16 stock solutions were prepared immediately before use. (methyl-
Plasma contamination in these cell lines.

Cells were labeled by adding [2-14C]dThd (0.02 Ci/ml) to the culture medium containing only 1% calf serum. These quiescent cells were used either to test drug effects in the nonproliferating state or to perform cell synchrony experiments.

In the latter case, quiescent cells were trypsinized and induced to grow by replating at low density (5-6 x 10^6 cells/flask) into medium containing 10% calf serum.

Cell Radiolabeling and Drug Treatment. Asynchronously proliferating 3T3 cells were labeled for 40 h with 0.02 µCi/ml of [2-14C]dThd. 3T3 cells were then washed and chased for 1 h immediately prior to drug treatment or the preparation of nuclei (see below). Exponentially growing L1210 cells were labeled throughout the experiment, and nuclei were prepared at various times with no chase period. Quiescent 3T3 cells were treated with drugs. Under these conditions, the cells were never allowed to reach confluence, and their doubling time was 18-19 h. Periodic testing (Flow Laboratories, McLean, VA) demonstrated the absence of Mycoplasma contamination in these cell lines.

To obtain a synchronized 3T3 population, 5-6 x 10^6 cells were seeded per 25 cm^2 flask in complete MEM. After two doublings, when cells had reached 70-80% of confluence, the medium was removed, the cells were briefly rinsed with MEM and then grown for 48-72 h in medium containing only 1% calf serum. These quiescent cells were used to test drug effects in the nonproliferating state or to perform cell synchrony experiments.

Cells were replated and stimulated to grow in synchrony. At various times after replating, nuclei were isolated by tryptic digestion period, wherein the cells were replated and stimulated to grow in synchrony. At various times after replating, nuclei were isolated by tryptic digestion and subsequently treated with drugs.

Drug treatments under all experimental conditions (cells or nuclei) were for 30 min at 37°C. Treatments of 3T3 cells were terminated by rinsing the flasks twice with 10 ml of cold HBSS containing 0.02% EDTA. Treatments of isolated nuclei of both cell types were stopped by 15-fold dilutions into ice-cold nucleic buffer.

Isolation of Cell Nuclei. The procedure for nuclei isolation has been described previously (22). 3T3 cells were scraped into 4°C nucleus buffer (150 mM NaCl, 1 mM KH2PO4, 5 mM MgCl2, 1 mM ethyleneglycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 0.1 mM di-thiothreitol, 10% (v/v) glycerol, and 0.1 mM phenylmethylsulfonyl fluoride, pH 6.4) and centrifuged (460 x g for 10 min). L1210 cells were centrifuged and resuspended in nucleus buffer. Both cell lines were then centrifuged at 670 x g for 20 min. The supernatants were used in the prelabeled template DNA.

Decatenation Activity of 3T3 Nuclear Extracts. Salt extracts of isolated nuclei were prepared as described previously (31). All procedures were performed at 4°C. Briefly, nuclei as described above were centrifuged (460 x g for 10 min), washed, centrifuged again, and resuspended in 1 ml nucleus buffer containing 0.35 M NaCl (final concentration). The sale extraction was performed by gentle rotation for 30 min. The nuclei were then centrifuged at 670 x g for 20 min. The supernatants were collected and centrifuged again at 12,000 x g for 10 min. The topoisomerase II activity of these extracts was immediately determined (31, 32). Serial dilutions of these extracts were reacted with 0.2 µg [3H]Topoisomerase-labeled kinetoplast DNA (a generous gift from Dr. Paul Englund, Department of Physiological Chemistry, Johns Hopkins School of Medicine, Baltimore, MD) for 30 min at 37°C in 40 µl of decatenation buffer (10 mM Tris, 80 mM KCl, 5 mM MgCl2, 0.1 mM Na2EDTA, and 15 µg/ml bovine serum albumin, pH 7.4). The reaction products were run in 1% agarose gels and stained with ethidium bromide. The kinetoplast DNA and minicircle bands were cut and counted by liquid scintillation spectrometry. Decatenation was determined as the ratio of the dpm in the minicircle band to the total dpm.

RESULTS

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the frequencies of DNA SSB and DSB produced by m-AMSA in quiescent and in asynchronously proliferating 3T3 cells. As shown in Fig. 1, exposure of proliferating cells to various concentrations of m-AMSA produced higher frequencies of DNA SSB and DSB than those which occurred when quiescent cells were similarly exposed. The saturation level for SSB appeared at an m-AMSA concentration of about 1 µM and for DSB at a slightly lower concentration.

m-AMSA- and VP-16-induced DPC and DSB in Nuclei Isolated from Proliferating and Quiescent 3T3 Cells. In these experiments, isolated cell nuclei were studied in order to exclude any potential plasma membrane transport difference. When nuclei isolated from proliferating 3T3 cells were treated with m-AMSA, dose-dependent increases in both DPC and DSB were observed (Fig. 2, A and B). The DPC/DSB frequency ratios indicated in Fig. 2 were consistent with results previously obtained in nuclei derived from exponentially growing L1210 cells (30, 33). Exposure of nuclei from quiescent 3T3 cells, however, produced no detectable DNA damage (Fig. 2, A and B). Treatment of nuclei from proliferating and quiescent cells with VP-16 resulted in a similar pattern: the production of DPC and DSB was markedly dependent on cell proliferation (Fig. 2, C and D). In nuclei from quiescent cells, a low level of DPC was detected following 40 µM VP-16, but not under any other conditions tested. The responses of nuclei isolated from proliferating cells showed no evidence of a plateau over the concentration ranges (1–10 µM m-AMSA; 20–40 µM VP-16) tested, suggesting that the trapping of topoisomerase II-DNA complexes was not saturated at these drug levels.

Cell Proliferation Dependence of m-AMSA- and VP-16-induced DPC in Nuclei Isolated from Mouse Leukemia L1210 Cells. In contrast to 3T3 cells, which arrest at relatively low cell densities, mouse leukemia L1210 cells continue to proliferate to the point of nutritional deprivation. The question was investigated as to whether the type of proliferative arrest seen in L1210 cells would also be associated with reduced topoisomerase II activity. Nuclei were isolated and assayed at four different times from the same original L1210 suspension culture: from early, middle, and late exponentially growing cells and from growth-arrested cells. Eighteen h before the first measurement cells were inoculated at low density (~0.3 x 10^5 cells/ml), and grown in the same medium for 5 days to plateau phase (18 x 10^5 cells/ml). Fig. 3 shows a typical growth curve for L1210 cells, having a doubling time of ~15–16 h. Every 24 h over a 4-day period, an aliquot was removed from the growing L1210 culture. Nuclei were prepared and then treated with either 1 µM m-AMSA or 20 µM VP-16. After drug treatment, alkaline elution was carried out on the nuclei to determine the DPC frequency.
As shown in Fig. 3, the frequencies of DPC induced by both drugs was highest in nuclei isolated from the cell culture at a density of $7 \times 10^5$ cells/ml. This cell density represents an intermediate point between the density of mid- and late-log phase cells. When the cell growth plateaued (cell concentrations $\geq 15 \times 10^5$ cells/ml), the frequency of DPC induced by m-AMSA or VP-16 clearly decreased. In nuclei from control cells, there was no significant elevation in DPC frequency except for a slight increase as the cells entered stationary phase.

It is interesting to note that the maximum frequency of DPC produced by 1 $\mu$M m-AMSA or by 20 $\mu$M VP-16 in L1210 cell nuclei (Fig. 3) is higher than the frequency of DPC produced by similar drug concentrations in nuclei from proliferating 3T3 cells (Fig. 2).

Protein-linked DNA Breaks in Synchronized 3T3 Cells. Stimulation of protein-linked DNA breakage by m-AMSA or VP-16 was higher in proliferating than in quiescent 3T3 cells. It was also higher in exponential than in plateau L1210 cells. This difference could be related either to an increase in the amount and/or the activity of topoisomerase II during a specific phase of the cell cycle or to a more global increase related to cell proliferation. To test this hypothesis, quiescent 3T3 cells were stimulated to proliferate synchronously by replating at low density in fresh medium containing 10% calf serum. A large peak of DNA synthesis occurred approximately 16–28 h after release of the cells from quiescence (Fig. 4). Around 95–100% of the cells divided between 34 and 36 h. After the first mitosis, the cells grew in partial synchrony and a second and third mitosis occurred at intervals of 18–20 h (not shown), corresponding to the normal doubling time of the asynchronous, proliferating 3T3 cells.

The cells were radiolabeled by adding [14C]thymidine to the medium beginning in the cell proliferation period prior to quiescence and continuing to the end of the experiment. At different times after replating, nuclei were isolated and treated with m-AMSA or with VP-16 for 30 min at 37°C (Fig. 5). The use of isolated nuclei ensured that any difference in DNA damage was related to topoisomerase II and not to drug uptake. Protein-linked DNA breaks were monitored by measuring DPC using alkaline elution. In the absence of drug, a small peak of DPC occurred 5–6 h after the cells were released from quiescence. After a drop to a minimum value at about 9 h, the control DPC frequency rose to a low plateau which showed no further change during S phase.

In drug-treated nuclei, the early peak of DPC occurring at 5–6 h was similar to that seen in the non-drug-treated controls. After dropping to a minimum at 9 h, DPC frequency rose sharply to a peak at 22 h. This peak usually preceded the peak of DNA synthesis by approximately 2 h. Another experiment (not shown) also demonstrated little or no drug-induced enhancement of the 5- to 6-h DPC peak relative to control, but a marked stimulation of DSB frequency (2- to 4-fold) in this period was observed in the same nuclei.

In order to distinguish between effects on replicating and nonreplicating regions of DNA, experiments were performed in which cells were radiolabeled only during the period of proliferation leading to quiescence. No label was present after release from quiescence. Under these conditions, in contrast to the results using the preceding labeling protocol, the magnitude of drug-induced DPC was relatively low and did not show a clear peak during S phase (Fig. 6). The controls for both labeling protocols were similar. The results are consistent with the possibility that the drug-induced DPC complexes occur preferentially in replicating DNA regions, probably equally with template and daughter strands, and that the complexes in a region become reduced in number soon after that region is replicated.

Topoisomerase II activity was also determined by measuring the decatenation activity of nuclear extracts prepared at several times following release from quiescence (Table 1). Decatenation activity was measurable at all time points, but was greatest at the time (21 h) of the DPC peak in S phase. This is consistent with the hypothesis that the drug-induced DPC are a measure of topoisomerase II activity.
In the past decade, studies have revealed mammalian topoisomerase II to be an important enzyme with multiple biological functions (1, 2). There is some evidence that topoisomerase II is a target for several antineoplastic compounds. As a consequence of its DNA-breaking-rejoining activity, this enzyme forms covalent DNA-protein complexes (protein-linked DNA strand breaks) which are stabilized by antineoplastic compounds such as m-AMSA and VP-16, and can be detected as DNA SSB, DSB, and DPC using alkaline elution.

To determine if the topoisomerase II activity varied as a function of cell proliferation, we examined two cell lines, nonmalignant NIH 3T3 fibroblasts and mouse leukemia L1210 suspension cultures. The effects of two well-characterized topoisomerase II inhibitors, VP-16 and m-AMSA, were measured at different stages of proliferation in both cell types. To circumvent any confusion which might arise from proliferation-dependent drug transport differences, we determined DPC frequencies in isolated nuclei.

Our results showed clear differences between proliferating and quiescent cells in regard to the DPC responses produced by treatment of isolated nuclei with m-AMSA or VP-16. This observation was true for both NIH 3T3 and L1210 cells, and is consistent with the observation of Duguet et al. (9), that topoisomerase II activity is higher in regenerating than in normal rat liver.

When we investigated drug-induced DPC frequencies as a function of cell cycle, we found consistently reproducible DPC formation occurring during the period 5 to 6 h after release from quiescence. This low but significant level of DPC (300 rad equivalents) occurred in nuclei without any drug treatment, and in the absence of DNA synthesis (Fig. 5). It is unclear whether these DPC result from DNA topoisomerase II activity.

During S phase, DPC frequencies were examined on total DNA and more specifically on parental strands. In both cases, the m-AMSA-induced increase of DPC frequencies occurred simultaneously with, or slightly before, the wave of DNA synthesis. This phenomenon may reflect an increasing amount of enzyme interacting with the DNA, which would be consistent with the results of the decatenation assay. However, the frequencies of DPC and the shapes of the DPC curves with time were different in the total versus newly replicated DNA (Fig. 6). These differences seen at discrete points within the S phase suggest a preferential association of topoisomerase II with newly replicated DNA.

A recent study by Sullivan et al. (11) has shown the proliferation dependence of VP-16- and m-AMSA-induced protein-linked DNA breaks in CHO cells. These authors reported that topoisomerase II activity was higher in proliferative than in quiescent CHO cells, whereas only a small difference was observed in HeLa or L1210 cells. Our findings using 3T3 cells are consistent with the CHO cell results. However it is unclear why these authors did not find proliferation-related differences in L1210 cells.

A relationship between drug-induced protein-linked DNA breaks and cytotoxicity has been suggested by a number of
experimental approaches (21-26). The present data also support this hypothesis since m-AMSA- and VP-16-induced cytotoxicity were correlated with the drug-induced DNA lesions in synchronized 3T3 cells.

REFERENCES

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