Altered Formation of DNA in Human Cells Treated with Inhibitors of DNA Topoisomerase II (Etosipside and Teniposide)

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ABSTRACT

We have investigated the importance of DNA topoisomerase II for the formation of mammalian DNA replication intermediates. Treatment with the DNA topoisomerase II inhibitor etosipside (teniposide) prevents the formation of large intermediates, such as 10-kilobase DNA, but allows the formation of small intermediates, i.e., Okazaki fragments. In untreated cells, there is a distinct stage in which the 10-kilobase DNA intermediates are joined before the appearance of mature chromatin. We find that pretreatment with etosipside (teniposide) prevents the appearance of this stage. When the protocol is reversed and the cells contain labeled 10-kilobase DNA before exposure to the drugs, one can detect the stage.

INTRODUCTION

Mammalian DNA synthesis occurs by a semicontinuous bidirectional mechanism. Small replication intermediates are joined into larger intermediates, and finally adjacent replicon-accordance with 18 U.S.C. Section 1734 solely to indicate this fact.1 This work was supported by grants from the Swedish Cancer Society and the Karolinska Institute.

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of this, it is of interest to examine the influence of the enzyme on a defined stage in DNA synthesis.

To analyze the effect on DNA synthesis of inhibition of DNA topoisomerase II, we treated cells with etoposide (or teniposide), which is believed to be a specific inhibitor of DNA topoisomerase II (13, 14). We find that the drug treatment prevents the appearance of the large 10-kilobase DNA intermediate and abolishes the appearance of the post-elongation stage.

MATERIALS AND METHODS

Cells, Culture Medium, and Labeling with [3H]Thymidine. A human melanoma cell line (CRL 1424), obtained from Flow Laboratories, Irvine, United Kingdom, was grown as monolayers at 37°C in 5% CO2 in air. The culture medium was Eagle's minimal essential medium with Earle's salts, containing 2 mM l-glutamine, 10% fetal calf serum, and antibiotics.

For experiments the cells were seeded in small culture dishes (35 x 10 mm) containing 3 ml of medium. Twenty-four h later, the cells were incubated with 50 μCi of [3H]thymidine (22 Ci/mmol; Amersham, Inc., Amersham, United Kingdom) for the desired length of time. To obtain prelabeled DNA, the cells were incubated for 24 h in [3H]thymidine-containing medium and then for another 24 h in medium without thymidine.

Etoposide and teniposide were obtained from a local pharmacy.

Cell Lysis. The incubation medium was drained from the culture dish, and the cells were rinsed twice with cold phosphate-buffered saline.

Cell lysis was performed in the dark at 0°C by the addition of 2.25 ml of 0.03 M NaOH (pH 12.1). After 30 min the solution was neutralized with 0.9 ml of 0.067 M HCl/0.02 M NaH2PO4. For a more detailed description, see Refs. 1 and 15. The sample was then either digested with nuclease S1 (see below) or immediately made 0.5% with regard to SDS.3

Digestion with Nuclease S1. Immediately after the neutralization of the dilute alkali used to lyse the cells, 300 μl of 300 mM sodium acetate (pH 4.6)-0.5 mM zinc acetate-750 mM NaCl were added. Two-hundred IU/ml of nuclease S1 (Sigma Chemical Co., St. Louis, MO) were then added, and the mixture was incubated for 30 min at 37°C. The digestion was stopped by making the solution 1% SDS-0.02 M EDTA.

Agarose Gel Electrophoresis. Agarose gels (0.75%) were made as described, see Refs. 1 and 15. The sample was then either digested with nuclease S1 (see below) or immediately made 0.5% with regard to SDS.3

Treatment of Nuclei in Salt Solutions. Isolated nuclei were suspended in buffer (0.25 M sucrose-50 mM Tris-HCl (pH 7.5)-25 mM KCl-1 mM CaCl2-1 mM MgCl2). KCl was added to a final concentration of 0.3 M or 0.6 M, and the nuclei were incubated at 37°C in order to reduce the nucleosome repeat length (16, 17). In nuclei incubated in 0.3 M KCl the repeat length is 160 to 170 base pairs and, in nuclei incubated in 0.6 M KCl, it is 140 to 145 base pairs. The nucleosome repeat length in nuclei incubated in sucrose-buffer alone is 190 to 195 base pairs.

KCl/SDS Precipitation of Protein-DNA Complexes. Nuclei were lysed in 2% SDS-10 mM EDTA-10 mM Tris (pH 8.0). The lysates were then subjected to the KCl/SDS assay, the same day as the lystate was obtained. The precipitation procedure, using KCl and SDS, was as described in Ref. 18.

3 The abbreviation used is: SDS, sodium dodecyl sulfate.
RESULTS

To analyze the importance of different enzymes for the various stages of in vivo DNA synthesis, one needs to be able to distinguish between different replication intermediates. This is difficult because of the large size of the mammalian genome.

To partly overcome problems resulting from the large size of mammalian genome, we have developed a procedure of cell lysis in dilute alkali at 0°C to partly denature the DNA. During the alkaline treatment, macromolecules are removed from the DNA, and the base pair structure is disrupted. However, the DNA strands cannot separate before enough time has elapsed to allow unwinding of the DNA. The unwinding is initiated at gaps (and/or alkali-labile regions) in the DNA present, e.g., at the DNA replication forks (1, 15). The amount of DNA that may be unwound at each gap has been estimated as 20 kilobases. When the number of gaps is increased by, e.g., X-irradiation, a higher amount of DNA is denatured.

When the solution is neutralized, the high-molecular-weight DNA is renatured and forms double-stranded DNA. DNA replication intermediates smaller than 20 kilobases, which are released from the DNA during the unwinding, remain in solution as single-stranded DNA molecules (1, 2). The fragments can then be separated from the high-molecular-weight DNA by agarose gel electrophoresis. The process can be visualized as a selective melting of active replicons, facilitating the analysis of replication intermediates.

Untreated Cells. When cells are pulsed with \(^{3}H\)thymidine for 45 s, lysed in dilute alkali, and the DNA then separated in 0.75% agarose gels, one detects high-molecular-weight DNA (double-stranded) and single-stranded 10-kilobase DNA replication intermediates and Okazaki fragments (Fig. 1A) (2).

When cells are pulsed with thymidine for 45 s and incubated in fresh medium for 30 min one can detect high-molecular-weight DNA and some 10-kilobase DNA, but no Okazaki fragments. The data show that Okazaki fragments have a shorter half-life than 10-kilobase DNA replication intermediates. The Okazaki fragments (present in the replication forks) are joined to give rise to 10-kilobase DNA (located outside the fork), which later gives rise to high-molecular-weight DNA (2).

Treatment with Etoposide. Drug treatments are carried out for either 5 min or 30 min. Cells incubated with the drug for 5 min are pulsed with \(^{3}H\)thymidine for 45 s at the beginning or at the end of treatment (Fig. 1A). The gel electrophoretic separation shows high-molecular-weight DNA and DNA migrating as Okazaki fragments as well as another population which is smaller than 10 kilobases. One can detect a small amount of 10-kilobase DNA intermediates. The results are similar irrespective of whether the pulse labeling was performed at the beginning or at the end of treatment.

In cells pulsed with \(^{3}H\)thymidine during the last 45 s of a 30-min drug treatment, one can detect essentially the same picture as in cells treated with drug for 5 min, i.e., high-molecular-weight DNA, Okazaki fragments, and the new population of fragments. However, now one cannot detect 10-kilobase DNA intermediates (Fig. 1B).

Treatment with Teniposide. Teniposide is an analogue to etoposide which similarly inhibits DNA topoisomerase II (13, 14). In parallel to the experiments described above for etoposide, we have performed incubations with teniposide according to the same protocols. Fig. 2 shows that teniposide prevents the appearance of the 10-kilobase DNA replication intermediate. The other data obtained are essentially the same as described above for etoposide (not shown).

Steady-State Labeled Cells. Etoposide is known to cause fragmentation of DNA upon isolation. It is possible that the new population of DNA fragments that we detect is formed because of this. Therefore we treated cells with steady-state labeled DNA with etoposide. The gel electrophoretic separation now showed high-molecular-weight DNA and DNA fragments at Slices 29 to 38, which is the location of the new population of DNA molecules detected during the pulse labeling (Fig. 3).

Hence the data show that DNA topoisomerase II inhibitors allow the formation of short DNA replication intermediates but prevent their joining to large intermediates (10-kilobase DNA).

Next we performed experiments with different concentrations of the drug (10 to 100 µg/ml). Fig. 4 shows that, at low drug concentrations (10 µg/ml), one can detect a certain amount of 10-kilobase DNA molecules. One needs to raise the concentration of drug to 100 µg/ml in order to completely prevent the detection of 10-kilobase DNA replication intermediates.

KC1/SDS Precipitation of Protein-DNA Complexes. The importance of DNA topoisomerase II was also examined with
ALTERED FORMATION OF DNA IN ETOPOSIDE (TENIPOSIDE)-TREATED CELLS

Fig. 3. Steady-state labeled cells. Melanoma cells with steady-state labeled DNA were treated with etoposide (100 µg/ml) for 60 min (O). Cells not treated with drug (●). The cells were lysed in dilute alkali, and the DNA was separated in 0.75% agarose gels. The numerals across the top (25, 10, and 2) denote the size (in kilobases, kb) and location of single-stranded DNA markers.

Fig. 4. Incubations with increasing concentration of etoposide. Cells were treated with etoposide for 30 min at 10 µg/ml (○), 50 µg/ml (●), or 100 µg/ml (▲). Pulse labeling of the cells with thymidine was for 45 s at the end of the treatment. The cells were lysed in dilute alkali, and the DNA was separated in 0.75% agarose gels. The numerals across the top (25, 10, and 2) denote the size (in kilobases, kb) and location of single-stranded DNA markers.

KCI/SDS precipitations using pulse-labeled DNA (labeled for 45 s with thymidine) and crude nuclear extracts from etoposide-treated cells. This approach is based on the fact that mammalian DNA topoisomerase form a "cleavable complex" with DNA. Treatment of the complex with protein-binding detergents induces DNA breakage and the covalent linkage of topoisomerases to the ends of the broken DNA strands. The level of topoisomerase-DNA complexes can be determined using the KCI/SDS precipitation assay (18).

Fig. 5 shows that, with increasing duration of the VP-16 treatment, more and more protein-DNA complexes are formed. These data and the data of Figs. 1 and 2 indicate that DNA-topoisomerase II is involved in the joining of Okazaki fragments to 10-kilobase DNA intermediates.

The Post-Elongation Stage. Ten-kilobase DNA is believed to give rise to the post-elongation stage. We use the following approach to delineate the stage existing between the joining of large replication intermediates (10-kilobase DNA) and the appearance of mature chromatin (1).

Cells are lysed in dilute alkali. When the solution is neutralized, the DNA strands larger than 20 kilobases renature and form double-stranded large DNA. The DNA fragments smaller than 20 kilobases remain in solution as single-stranded DNA. To remove the single-stranded DNA, we treat the sample with the enzyme nuclease S1, which digests single-stranded but not double-stranded DNA. The remaining double-stranded DNA is then separated in agarose gel electrophoresis.

When cells with steady-state labeled DNA (mature chromatin) are analyzed, the results of the electrophoretic separation show small labeled double-stranded DNA fragments (1). The denatured-renatured mature chromatin DNA therefore contains short stretches of single-stranded DNA which allows nuclease S1 to break up the DNA (Fig. 6).

However, when cells are labeled with short pulses of [3H]-thymidine only large double-stranded DNA fragments (larger than 20 kilobases) can be detected. This newly ligated DNA is not fragmented by nuclease S1 like the mature chromatin DNA and represents a distinct stage in the DNA-synthetic pathway. We call this stage the post-elongation stage (1) (Fig. 6). With continued incubation of the cells in fresh medium, the post-elongation stage DNA is converted to mature chromatin DNA (which can be fragmented by nuclease S1).

The ability of nuclease S1 to fragment DNA changes when the nucleosome repeat length is altered. This is done by incubating isolated nuclei in salt solutions. By incubating the nuclei in KCl one can reduce the repeat length from 190 to 195 base pairs to 165 to 170 base pairs and finally to 140 to 145 base pairs (16, 17).

Fig. 7 shows the electrophoretic pattern of DNA from nuclei with steady-state labeled DNA fragments that are treated with
ALTERED FORMATION OF DNA IN ETOPSIDE (TENIPOSIDE)-TREATED CELLS

The fragmentation of DNA by nuclease S1 is reduced when the repeat length is 165 to 170 base pairs (Fig. 7). Moreover when the repeat length is 140 to 145 base pairs, the level of DNA fragmentation is reduced still further. Now about half of the label appears as fragments, and half as high-molecular-weight DNA (Fig. 7).

The Post-Elongation Stage in Cells Treated with Etoposide. Cells treated with etoposide for 30 min were pulsed with [3H]thymidine during the last 45 s. The cells were lysed in dilute alkali, and the DNA was digested with nuclease S1, and then separated in 0.75% agarose gels. Control cells were pulsed with thymidine for 45 s.

Fig. 8A shows that, in the control cells, one can detect only the high-molecular-weight DNA typical of the post-elongation stage. In cells treated with the drug, the label appears in both the high-molecular-weight DNA and the double-stranded DNA fragments which are generated from the mature chromatin DNA by digestion with nuclease S1. Hence the drugs alter the mechanisms of DNA synthesis.

In another experiment, cells were washed free of the drug and incubated in fresh medium for 3 min or 15 min. When the DNA was then separated in agarose gels, more and more DNA fragments appeared with time (Fig. 8B). Already after a postincubation for 3 min, all label appeared in the DNA fragments.

Fig. 7. Incubation of isolated nuclei in 0.3 M and 0.6 M KCl solutions. Nuclei, isolated from cells with prelabeled DNA, were lysed without prior salt incubation (○) (nucleosome repeat length, 190 to 195 base pairs) and after incubation in 0.3 M KCl (●) (nucleosome repeat length, 165 to 170 base pairs) or 0.6 M KCl (×) (nucleosome repeat length, 140 to 145 base pairs). The DNA was digested with nuclease S1, and then separated in a 0.75% agarose gel. The arrow denotes a 2-kilobase single-stranded DNA marker used to standardize the gel separation.

various concentrations of KCl, lysed in dilute alkali, and treated with nuclease S1. When the nucleosome repeat length is 190 to 195 base pairs, the DNA is completely fragmented by nuclease S1.
This is much earlier than in control cells pulsed with thymidine but not treated with the drug (1, 19).

Furthermore we performed experiments with different concentrations of the drug (50 to 250 μg/ml). Fig. 8C shows that, by increasing the drug concentration, one can reduce the amount of labeled post-elongation stage DNA. At 250 μg/ml one can detect only the double-stranded DNA fragments typical of mature chromatin.

As shown in Fig. 1, treatment with etoposide prevents the appearance of 10-kilobase DNA. It seems very likely that the depletion of 10-kilobase DNA results in the absence of the post-elongation stage.

Pulse Labeling of 10-Kilobase DNA before the Addition of Etoposide. Cells were pulsed with [3H]thymidine for 10 min with etoposide added during the last 5 min. This protocol should allow the detection of 10-kilobase DNA in drug-treated cells. In agreement Fig. 9A shows a gel separation with labeled 10-kilobase DNA present.

The cells were then assayed for the high-molecular-weight DNA typical of the post-elongation stage. Fig. 9B shows that now one can detect the post-elongation stage even when the cells are treated with doses of drug up to 250 μg/ml.

Hence the drug does not inhibit the formation of the high-molecular-weight DNA typical of the post-elongation stage when the cells are not depleted of 10-kilobase DNA intermediates.

DISCUSSION

The two drugs etoposide and teniposide interfere with DNA-topoisomerase II. We show here that, in mammalian cells, the inhibition of DNA topoiso-merase II alters the formation of DNA replication intermediates. We can detect the short Okazaki fragments but not the large 10-kilobase DNA replication intermediates. The results are essentially the same irrespective of which drug was used. The data are also supported by the results of KCI/SDS precipitations of protein-pulse-labeled DNA complexes.

It is known that in control cells, the pulse-labeled DNA appears in 10-kilobase fragments and then passes through a distinct stage, termed the post-elongation stage, before being incorporated into mature chromatin. When a pretreatment with etoposide (teniposide) prevents the appearance of 10-kilobase DNA, we cannot detect the post-elongation stage. However, when one reverses the protocol in order to allow the formation of 10-kilobase DNA in the drug-treated cells, one can then detect the post-elongation stage. Hence the data lend further support to our earlier conclusion that the post-elongation stage is formed by the joining of large DNA replication intermediates.

The movement of the replication fork during DNA synthesis causes unwinding of the parental DNA strands. This unwinding of the parental strands generates positive superhelical stress, which must be relieved to allow continued fork movement. This topic is usually called the swivel problem (20) and has mainly been analyzed in organisms with closed circular DNA. Similar topological problems are, however, encountered in the linear mammalian DNA due to their extreme length coupled with the existence of multiple replicons. Furthermore the chromatin fiber of interphase chromosomes is topologically constrained into loops.

DNA topoisomerase II can serve as a swivel by either acting on the unreplicated DNA ahead of the replication fork or on the daughter DNA strands behind the replication fork. It has been shown recently in rat prostatic adenocarcinoma cells that newly replicated DNA is associated with DNA topoisomerase II (21). In the analysis of the rat cells, one was not able to examine defined DNA replication intermediates, as we have done here.

Analysis of yeast cells with conditional mutants has shown that the enzyme is required for chromosome segregation, at least in lower eukaryotes (7–9). The connection of the enzyme with DNA synthesis is also seen in the chromosome formation in SV 40, which in the final stages involves the production of catenated dimers (22–25). Such structures can only be separated by enzymes like type II DNA topoisomerases.

REFERENCES


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