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The nature of DNA plays a role in chromosome segregation: endoreduplication in halogen-substituted chromosomes

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Abstract

AA8 Chinese hamster ovary cells were treated with halogenated nucleosides analogues of thymidine, namely CldU, 5-iodo-2′-deoxyuridine (IdU), and 5-bromo-2′-deoxyuridine (BrdU), following different experimental protocols. The purpose was to see whether incorporation of exogenous pyrimidine analogues into DNA could interfere with normal chromosome segregation. The endpoint chosen was endoreduplication, that arises after aberrant mitosis when daughter chromatids segregation fails. Treatment with any of the halogenated nucleosides for two consecutive cell cycles resulted in endoreduplication, with a highest yield for CldU, intermediate for IdU, and lowest for BrdU. The frequency of endoreduplicated cells paralleled in all cases the level of analogue substitution into DNA. Our results seem to support that thymidine analogue substitution into DNA is responsible for the triggering of endoreduplication. Besides, the lack of any effect on endoreduplication when CldU was present for only one S-period strongly suggest that it is the nature of template, and not nascent DNA, that plays a major role in chromosome segregation. Taking into account that topoisomerase II cleaves DNA at preferred sequences within its recognition/binding sites, the likely involvement of the enzyme is discussed.

Keywords: DNA segregation; Sequence specificity; Endoreduplication; DNA topoisomerases

1. Introduction

DNA topoisomerases are conserved nuclear enzymes that catalyze a variety of fundamental topological changes of DNA during many cellular processes such as replication, transcription and recombination through transient cleavage of the molecule, strand passing and religation (for a review, see [1]). According to their catalytic mechanisms, two classes of topoisomerases have been described so far. While type I topoisomerase breaks and rejoins one DNA strand at a time, type II enzyme is able to do so with the two strands that make up duplex DNA. Since topoisomerase-induced breaks in DNA are transient intermediates in the strand passage reaction, they are normally present at low steady-state levels and hence well tolerated by the cell as a necessary, though otherwise potentially dangerous process that proves mutagenic and even lethal when the enzyme is poisoned [2,3].

An interesting differential feature is that while both type I and type II enzymes are proficient in relaxing supercoiled DNA in order to relieve torsional tension generated during replication and transcription, only topoisomerase II is able to decatenate intertwined DNA molecules. This unique decatenating as well as unknotting activity of DNA topoisomerase II is essential for segregating replicated daughter chromosomes

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[4–10]. Apart from its important functional roles in chromosome condensation and segregation, topoisomerase II is a basic structural protein highly present in the nuclear matrix and chromosome scaffold [11].

The enzyme's catalytic cycle of topoisomerization of DNA begins with the binding of the homodimer to its double-stranded substrate. While binding does not seem to require any cofactor, the presence of divalent cations has been reported to stimulate it [12]. As to the double helix properties influencing such an interaction topoisomerase II-DNA, both nucleotide sequence and topology seem to play a role. Topoisomerase II cleaves DNA at preferred sequences within its recognition/binding sites, but there is no report on high specificity [13–16], in such a way that the laws that rule the nucleic acid specificity of topoisomerase II are as yet rather obscure.

Concerning the influence of the topological structure of DNA on binding and cleavage by topoisomerase II, on the other hand, it has been reported that the enzyme interacts preferentially with supercoiled DNA over relaxed molecules [3]. This behavior of topoisomerase II provides a plausible explanation to its strong interaction with supercoiled DNA as well as to its release of its relaxed reaction product.

Diplochromosomes, made up of four chromatids held together, instead of the normal two, are the visible mitotic manifestation of the rare, although sometimes spontaneous phenomenon of endoreduplication. This consists on two successive rounds of DNA replication without intervening mitosis, i.e. segregation of daughter chromatids [10,17].

A variety of agents either by interfering with cytoskeleton assembly [18,19] or by damaging DNA [20–23] have been reported to induce endoreduplication to different degrees. More recently, agents that interact with topoisomerase II have been used to provide further evidence that the enzyme is required for separation of daughter chromosomes. Both topoisomerase "poisons", i.e. chemicals that cause DNA strand breaks through stabilization of topoisomerase II covalently bound to DNA in the intermediate form so-called cleavable-complex [2] as well as those considered as true catalytic inhibitors [24] are able to induce endoreduplication [10,25,26] due to prevention of decatenation of replicated chromosomes by topoisomerase II with the subsequent failure to complete a normal mitosis.

In the present investigation, we have analyzed the possible influence of DNA substitution by halogenated nucleoside analogues of thymidine on chromosome segregation. Our observation was that all the thymidine analogues tested are able to induce endoreduplication to different degrees, while only their presence in template DNA strand(s) seems to result in the triggering of the endoreduplication process as a result of segregation failure. These results are discussed in terms of the possible major role of template DNA for topoisomerase II function in chromosome segregation.

2. Materials and methods

2.1. Cell culture

The Chinese hamster ovary fibroblast cell line AA8, purchased from American Type Culture Collection (ATCC, USA), was used in our experiments.

Cells were grown as monolayers in McCoy's 5A medium (Bio Whittaker, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and the antibiotics penicillin (50 U/ml) and streptomycin (50 μg/ml). Cells were cultured in a dark environment at 37 °C in an atmosphere containing 5% CO_2. On regular testing, cell cultures were found to be free from mycoplasma.

2.2. Preparation of nuclear extracts

Exponentially growing AA8 cells were processed to obtain extracts of nuclear proteins in order to determine later on the topoisomerase II activity (see Section 2.3). The procedure was basically that described by Heartlein et al. [27]. Approximately 1×10^7 cells were suspended in 1 ml of 0.32 M sucrose, 0.01 M Tris–HCl pH 7.5, 0.05 M $MgCl_2$, 1% Triton X-100 and thoroughly vortexed to lyse the cells. Nuclear pellets were obtained by centrifugation at 2,000 rpm (Eppendorf centrifuge), for 5 min at 4 °C. Nuclei were then washed in 1 ml of nucleus wash buffer (5 mM potassium phosphate buffer, pH 7.5, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 1 mM β-mercaptoethanol, and 0.5 mM dithiothreitol (DTT)). The nuclei were then pelleted as described above and resuspended in 50 μl of nucleus wash buffer, and 50 μl of 4 mM EDTA was added.

Following incubation at 0 °C for 15 min, the nuclei were lysed by adding 100 μl of 2 M NaCl, 20 mM Tris–HCl pH 7.5, 10 mM β-mercaptoethanol, 1 mM PMSF. Following a 15 min incubation at 0 °C, 50 μl of 18% polyethylene glycol (PEG-6000) in 1 M NaCl, 50 mM Tris–HCl pH 7.5, 10 mM β-mercaptoethanol, and 1 mM PMSF were added. The suspension was incubated for a further 40 min at 0 °C. The supernatant from a 30 min centrifugation at 12,500 rpm at 4 °C was then collected. Total protein concentration in each extract was determined in a Beckman DU-64 spectrophotometer by the Bradford protein assay [28] and extracts were kept at −80 °C for no longer than 1 month.

2.3. Topoisomerase II activity in nuclear extracts

Topo II activity in nuclear extracts was assayed using a TopoGen (Columbus, OH, USA) assay kit based upon decatenation of kinetoplast DNA (kDNA). The amount of nuclear extract protein from AA8 cells used in each assay was 100 ng. Reaction products were resolved using agarose gel electrophoresis of DNA. After incubation for 1 h with either 10 or 20 μM CldU at 37 °C the samples were loaded onto 1% agarose gels and subjected to electrophoresis for 2.5 h at 100 V (topo II assay). Finally, gels were stained with 0.5 μg/ml ethidium bromide, destained (30 min) in distilled water and photographed.

2.4. Induction of endoreduplication

Actively growing AA8 cells were cultured for either 12, or 24 h in the presence of 5-chloro-2′-deoxyuridine (CldU) together with 100 μM deoxycytidine (dC) and 1 μM fluorodeoxyuridine (FdU), this latter to control the relative incorporation of CldU and deoxythymidine (dT). Variations in the relative proportion of exogenous nucleosides provided to culture along with the DNA synthesis inhibitor FdU in turn results in variations in their relative levels of incorporation into DNA [29,30]. Using this methodology, the level of CldU incorporation into DNA was established to be in the range of 5–100%, for comparison with unsubstituted controls. In a different set of experiments, cells were given either CldU, 5-iodo-2′-deoxyuridine (IdU) or 5-bromo-2′-deoxyuridine (BrdU) for 24 h, under the experimental conditions to achieve 100 and 50% substitution of the corresponding halogenated nucleosides into DNA.

After treatment the cultures were thoroughly washed and maintained in fresh medium for 18 h to allow them to recover. Cultures that did not receive any treatment served as controls. Colcemid (2×10^{-7} M) was finally added for 2 h 30′ to all the cultures for metaphase arrest. The flasks were shaken to dislodge the mitotic cells, which were collected by centrifugation, treated with 0.075 M KCl for 2 min (hypotonic treatment), fixed in methanol:acetic acid (3:1) and dropped onto clean glass microscope slides. The slides were stained with 3% Giemsa in phosphate buffer pH 6.8 and mounted in DPX. Two thousand metaphases per culture were counted and classified as normal or as having diplochromosomes. Thymidine analogues substitution into DNA was always assessed by the fluorescent plus Giemsa (FPG) staining method as reported elsewhere [29]. All the experiments were carried out in triplicate.

3. Results

3.1. CldU substitution into DNA and endoreduplication

First, we analyzed the possible influence of the halogenated nucleoside CldU after its incorporation into DNA for thymidine on a proper chromosome segregation. To measure missegregation leading to aberrant mitosis, endoreduplication was the endpoint selected [10,17]. In order to assess the possible role, if any, played by either parental or daughter DNA strands for the induction of endoreduplication, two basic experimental protocols were followed (Fig. 1).

CldU substitution into DNA took place for either one, or two consecutive S-periods before allowing the cells to recover for an additional cell cycle during which endoreduplication (if any) might occur. The observation was that endoreduplication was effectively induced, but the induction of endoreduplication was dependent upon the experimental design, in such a way that only cells grown in the presence of CldU for two subsequent S-periods showed metaphases with the characteristic diplochromosomes (Fig. 2). Differential staining of CldU-substituted third mitosis (M3) chromosomes [31] indicated that analogue

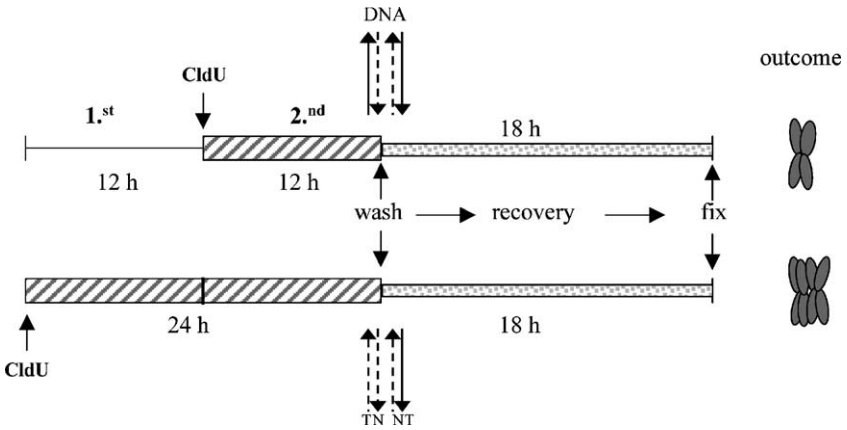

Fig. 1. Schematic diagrams of the experiments performed to analyze the relationship between CldU substitution into DNA and endoreduplication. Dashed lines indicate CldU substitution in either nascent (N) or template (T) DNA. As can be seen, only when the halogenated nucleoside was present in template DNA cells were bound to endoreduplicate, provided that they were given a recovery period for them to pass through an additional S-phase.

substitution into DNA actually took place for two consecutive rounds of DNA replication, followed by an additional S-period in absence of CldU (Fig. 2). In a different experiment, we allowed CldU to be present only in the template (no CldU in any of the nascent DNA at all) and our observation was that it was enough to induce endoreduplication, given the necessary recovery time (data not shown).

Fig. 3 shows that the induction of endoreduplication was clearly dependent on the degree of CldU substitution into DNA, with the higher effect observed for 100% CldU substitution, and decreasing stepwise as CldU incorporation into DNA reached lower values.

3.2. Comparison between different halogenated nucleosides

Next, based on the previous observation on the induction of endoreduplication by CldU, our purpose was to assess the possible effectiveness of different

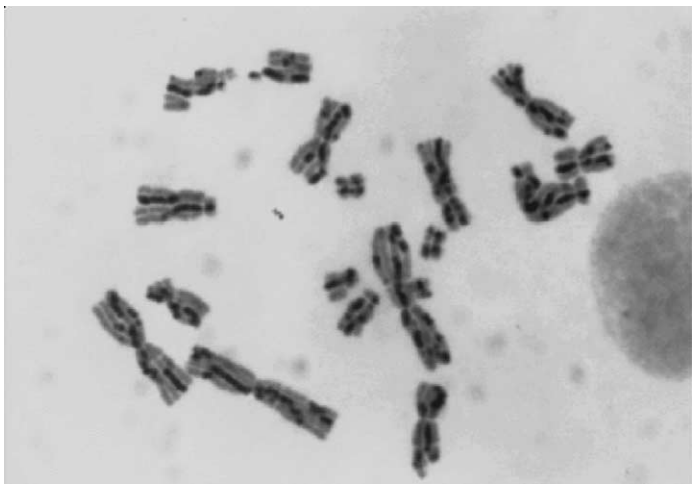

Fig. 2. CldU-substituted third mitosis (M3) diplochromosomes showing the differential Giemsa staining indicative that analogue substitution into DNA has taken place for two consecutive rounds of DNA replication, followed by an additional S-period in absence of CldU (during which endoreduplication took place).

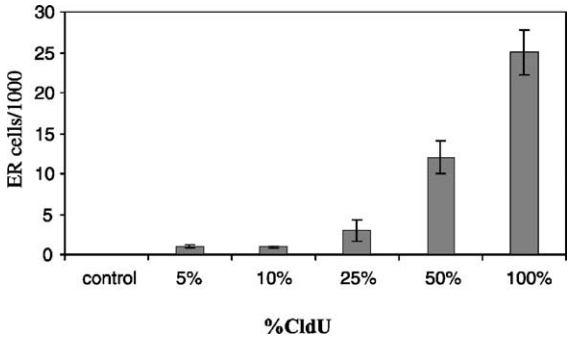

Fig. 3. CldU substitution into DNA for two cell cycles and endoreduplication. As can be seen, CldU substitution for thymidine results in the induction of endoreduplication in a fashion clearly dependent upon the degree of incorporation of the halogenated nucleoside into DNA. Bars indicate standard errors from three experiments.

halogenated nucleosides, namely CldU, IdU and BrdU to induce endoreduplication after their incorporation into DNA for two consecutive cell cycles. AA8 cells were given either CldU, IdU or BrdU under the experimental conditions necessary to achieve in each case either 100 or 50% of analogue substitution into DNA, assessed by differential staining of chromosomes [30], and the corresponding data on endoreduplication are shown in Fig. 4. As can be seen, though with different efficiency depending upon the thymidine analogue, all the halogenated nucleosides tested were able to interfere with the normal development of mitosis and hence endoreduplicated metaphases were readily observed. The yield of endoreduplicated cells was highest for CldU, lowest for BrdU and intermediate for IdU. In good agreement with our previous observations for CldU (see above) on the other hand, for all the halogenated pyrimidines the frequency of endoreduplicated cells correlated well with the level of analogue incorporation into DNA (Fig. 4).

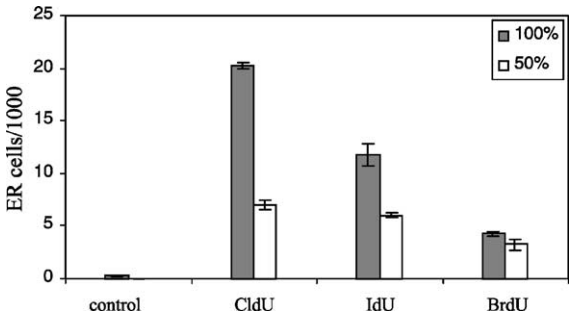

Fig. 4. Comparison between different halogenated nucleosides in their effectiveness to induce endoreduplication. DNA was either fully (100%) or partly (50%) halogen-substituted. As can be seen, all the thymidine analogues tested induced endoreduplication, with a highest yield for CldU, intermediate for IdU, and lowest for BrdU. Bars indicate standard errors from three experiments.

3.3. Lack of direct interaction between CldU and topoisomerase II

In order to see whether or not there was a direct interaction between DNA topoisomerase II and the different halogenated nucleosides (free in the medium) that might be responsible for the apparent loss of function of the enzyme leading to endoreduplication, catalytic activity of topoisomerase II was analyzed *in vitro* after incubation with CldU for comparison with untreated controls. Cell extracts were incubated with either 10 μM (the dose used in our cytogenetic experiments) or 20 μM CldU and the decatenating activity was assessed using catenated kinetoplast DNA as a substrate.

The result was that the ability of the CldU-treated extracts to decatenate kDNA and release double-stranded circular DNA monomers was similar to that observed for untreating control extracts (not shown).

4. Discussion

It is widely accepted that topoisomerase II plays a major role for segregating replicated daughter chromatids before anaphase [4,32,33]. Indeed, we have recently reported a very efficient induction of endoreduplication by the topoisomerase II specific inhibitor ICRF-193, a bis-dioxopiperazine [26].

Though it is generally agreed upon that nucleotide sequence plays a role, the rules that determine the nucleic acid specificity of topoisomerase II are as yet far from being completely elucidated. It has been reported that topoisomerase II cleaves DNA at preferred sequences within its recognition/binding sites, but there is no report on high specificity [13–16].

In order to study the sequence specificity of double-strand DNA cleavage by *Drosophila* topoisomerase II, the frequencies of the nucleotides and dinucleotides in the region near the site of phosphodiester

bond breakage was analyzed [34] and revealed a non-random distribution. The consensus sequence derived was 5′-GT.A/TAY decrease ATT.AT..G-3′ where a dot means no preferred nucleotide, and Y stands for pyrimidine [34]. On the other hand, DNase I footprint analysis has revealed that *Drosophila* topoisomerase II can protect a region in both strands of the duplex DNA, with the cleavage site located near the center of the protected region [35], and it has been proposed that the strong DNA cleavage sites of *Drosophila* topoisomerase II [34] likely correspond to specific DNA-binding sites of the enzyme [13,36].

The interaction between calf thymus topoisomerase II and DNA was also characterized by means of a transcription assay [37] and it was concluded that topoisomerase II binds to a region of DNA located symmetrically around the enzyme-mediated cleavage site.

We have observed the induction of endoreduplication in AA8 Chinese hamster cells treated with different halogenated nucleosides, namely CldU, IdU and BrdU for two consecutive cell cycles, while treatment for just one cell cycle did not yield any endoreduplication. This latter is a rare phenomenon that results from failure in chromosome segregation leading to aberrant mitosis without proper anaphase, and the subsequent re-replication of non-split chromosomes that finally show up as diplochromosomes made up of four chromatids in the next mitosis [10,17].

Although the observation that treatment of the cells for 12 h (roughly one cell cycle) with CldU did not result in endoreduplication seemed to indicate otherwise, we have considered the unlikely hypothesis of a possible direct interaction between the exogenous halogenated nucleoside and topoisomerase II. According to our expectations, we have found a lack of interaction that could result in a loss of decatenating activity of topoisomerase II that in turn might hamper proper chromosome segregation.

In our opinion, even though the possible involvement of other proteins cannot be ruled out at present, our observations seem to favor the likely hypothesis that the nature of DNA might play a role for the recognition/binding of topoisomerase II and its subsequent cleavage of the fully replicated molecule for chromosome segregation. It has been reported that eukaryotic topoisomerase II preferentially cleaves alternating purine–pyrimidine repeats within the consensus sequence, and additionally, GT, AC and AT repeats were better substrates for cleavage than GC repeats [14,37]. Furthermore, the distribution of DNA cleavage sites induced by topoisomerase II in the presence or absence of enzyme poisons were mapped in the simian virus 40 genome [16] and the finding was that strong sites tended to occur within A/T runs such as those that have been associated with binding to the nuclear scaffold [16].

We chose three halogenated nucleosides that are readily incorporated into DNA as thymidine analogues and are able to induce sister chromatid exchanges [38,39] as well as chromosomal aberrations [40] to see whether or not there was an effect on chromosome segregation. Our observation was that all the halogenated nucleosides tested were able to induce endoreduplication to different degrees, in such a way that the yield of endoreduplicated cells was highest for CldU, intermediate for IdU and lowest for BrdU. Besides, the frequency of cells showing diplochromosomes paralleled the relative percentage established concerning the halogenated pyrimidine:deoxythymidine incorporation into DNA.

Taken as a whole, our results seem to support the idea that the presence of anomalous bases such as halogenated pyrimidines in DNA results in a defective function of topoisomerase II in chromosome segregation that eventually leads to aberrant mitosis and the subsequent endoreduplication [26]. Besides, the observation that analogue incorporation for only one S-period, i.e. only in nascent DNA, does not result in endoreduplication, contrasting with the increased yield of endoreduplication when incorporation takes place for two consecutive rounds of replication, seems to point to the importance of template DNA for chromosome segregation to proceed normally.

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