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The high rate of endoreduplication in the repair deficient CHO mutant EM9 parallels a reduced level of methylated deoxycytidine in DNA

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ABSTRACT

It has been recently proposed that hypomethylation of DNA induced by 5-azacytidine (5-azaC) leads to reduced chromatid decatenation that ends up in endoreduplication, most likely due to a failure in topo II function [S. Mateos, I. Domínguez, N. Pastor, G. Cantero, F. Cortés, The DNA demethylating 5-azaC induces endoreduplication in cultured Chinese hamster cells, Mutat. Res. 578 (2005) 33–42]. The Chinese hamster mutant cell line EM9 has a high spontaneous frequency of endoreduplication as compared to its parental line AA8. In order to see if this is related to the degree of DNA methylation, we have investigated the basal levels of both endpoints in AA8 and EM9, as well as the effect of extensive 5-azaC-induced demethylation on the production of endoreduplication. Based on the correlation between the levels of DNA methylation and indices of endoreduplication we propose that genomic DNA hypomethylation in EM9 cell line is probably an important factor that bears significance in relation to the high basal level of endoreduplication observed in this cell line.

1. Introduction

After the passage of the replication fork during eukaryotic DNA synthesis, the double-stranded DNA molecules of sister chromatids inevitably become concatenated. Resolution of this DNA entanglement must occur as a pre-requisite for proper anaphase segregation [1,2]. The nuclear enzyme DNA topoisomerase II (topo II) is capable of breaking and resealing double-stranded DNA in a concerted fashion, and is the only known eukaryotic enzyme capable of decatenation [3,4]. This unique activity of DNA topo II in decatenating and unknotting is essential for segregating fully replicated daughter chromosomes. In most eukaryotic cells DNA replication and mitosis are coupled and mutually dependent, thus ensuring that they occur only once during each cell cycle. This coupling, however, is broken in several instances: the dependency of mitosis upon DNA synthesis is broken in meiosis, where two successive events of chromosome segregation occur without an intervening DNA synthesis; and conversely, the dependency of DNA synthesis upon mitosis is broken in endoreduplication, where multiple rounds of DNA replication occur in the absence of intervening mitoses, leading to the production of chromosomes with doubling series of chromatids [5,6]. Endoreduplication, a common symptom of disrupted cell cycle progression, more particularly progression through mitosis, becomes evident at metaphase by the presence of diplochromosomes, made up of four chromatids held together, instead of the normal two. In spite of being a normally rare event in animal cells, induction of endoreduplication has been reported in cells infected with simian virus 40 [7], in retinoblastoma negative cells expressing either the p21cip1/waf1 Cdk inhibitor [8] or the myogenic transcription factor MyoD [9], as well as in p21-nullizygous cells treated with DNA-damaging agents [10]. Also, different chemical and physical treatments have been reported to induce endoreduplication to different degrees, either through interference with cytoskeleton assembly [11,12] or as a result of DNA damage [13–16] (for a review see [17]). Focusing on topo II, inhibitors that interact with the enzyme as topoisomerase "poisons", i.e. chemicals that cause DNA strand breaks through stabilization of topo II covalently bound to DNA in the intermediate form so-called "cleavable complex" [18] as well as those considered as true catalytic inhibitors [19] are able to induce endoreduplication [20,21] due to prevention of decatenation of replicated chromosomes by topo II with the subsequent failure to complete a normal mitosis.

DNA topo II, a fundamental nuclear enzyme, cleaves the double-stranded DNA molecule at preferred sequences within

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its recognition/binding sites. In recent years considerable effort has been invested in our laboratory toward understanding the molecular mechanisms that regulate the role of this enzyme in chromosome segregation. An important contribution came from a recent investigation where we examined the possible relative importance of DNA substitution by halogenated nucleoside analogues of thymidine for topo II interaction with DNA and its ability to form cleavable complexes for the enzyme to carry out its function. Our observation was that all the thymidine analogues tested are able to induce endoreduplication to different degrees as a result of segregation failure, the yield of endoreduplication being parallel to the relative level of halogenated nucleoside substitution for thymidine in DNA achieved [22]. Also, our results indicated that all the analogues protect DNA from double-strand breaks induced by the topo II poison *m*-AMSA, such protection depending upon the relative percent of incorporation of a given thymidine analogue in DNA [23]. In order to continue our studies on the specificity of topo II for DNA sequence in mammalian cells, we have recently evaluated the possible influence of the methylated state of DNA on chromosome segregation [24]. The drug 5-azacytidine (5-azaC), which acts as a strong hypomethylating agent at the C5 position of cytidine after its incorporation into DNA, was used to induce extensive modification of the DNA sequence. Our results indicated that the presence of 5-azaC in DNA induces a dose-dependent increase in the yield of endoreduplicated cells that parallels the levels of hypomethylation observed [24]. Keeping this in mind, it is known that the Chinese hamster cell line EM9 is a repair deficient mutant that shows an elevated spontaneous yield of endoreduplication and a 10-fold higher baseline frequency of sister chromatid exchanges (SCEs) relative to the parental cell line AA8. Also, former reports have indicated an increase in endoreduplication associated with a deficiency in various DNA repair genes, including the XRCC3 gene, important in recombination processes [25]. As a continuation of our previous work, in the current study we have evaluated whether the high level of spontaneous endoreduplication in the mutant EM9 as compared to the parental cell line AA8 correlates with any possible difference between both cell lines in the degree of DNA methylation. For that purpose, we have investigated the basal levels of both endpoints in AA8 and EM9, as well as the effect of extensive 5-azaC-induced demethylation on the production of endoreduplication. Our data suggest that reduced methylcytosine content in global genomic DNA may be related to endoreduplication.

2. Materials and methods

2.1. Chemicals and enzymes

A stock solution of 5-azaC (Sigma) was prepared in distilled water (H_2O_D) and kept in 200 μl vials at −20 °C until use. Just before an experiment, a vial was thawed and then further diluted in medium in order to obtain the final concentration of 5-azaC desired. Budesonide was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). It was dissolved in dimethyl sulfoxide (DMSO), made up fresh for each experiment, and directly added to the culture medium. The maximum concentration of DMSO was 0.24%. Restriction endonucleases HpaII and MspI (Promega) were used according to the suppliers' recommended protocol in the activity buffer provided.

2.2. Cell culture

The parental Chinese hamster ovary AA8 cell line and the repair deficient EM9 mutant were purchased from the American Type Culture Collection (ATCC), USA. EM9 cells were originally derived from AA8 cells and are known to be single strand-break (SSB) repair deficient with a specific defect in XRCC1 [26,27]. They were grown as monolayers in McCoy's 5A medium with 10% fetal calf serum, 2 mM L-glutamine and the antibiotics, penicillin (50 U/ml) and streptomycin (50 μg/ml). Cells were cultured at 37 °C in 5% CO_2 in air.

2.3. Genomic DNA preparations

Untreated control cells or cells treated for 24 h with different doses of 5-azaC (from 0.025 to 15 μM) or budesonide (from 50 to 90 μM) were processed to obtain an extract of genomic DNA. Briefly, about 15×10^6 cells was collected in 10 ml PBS using a scraper and then centrifuged at 1200 rpm for 6 min. After centrifugation, the supernatant was discarded and the pellet was resuspended with 500 μl of lysis solution (10 mM Tris–HCl at pH 6, 25 mM EDTA, 100 mM NaCl, 0.5% SDS). Lysate of cells was obtained by passing the mixture at least 10 times through a sterile insulin syringe. Then, 500 μl of lysate per sample was incubated at 50 °C for 60 min in a water bath in the presence of proteinase K (100 μg/ml) and RNase (10 μg/ml). At the end of the incubation, DNA was extracted twice with phenol–chloroform–isoamyl alcohol mixture (25:24:1). The genomic DNA was then precipitated with 7.5 M ammonium acetate and with 100% ethanol. After centrifugation at 14,000 rpm for 15 min at 4 °C, the supernatant was carefully removed and the pellet rinsed with cold 70% ethanol. Subsequently, the DNA pellet was dried and diluted in 50 μl TE buffer at pH 7.4. DNA concentration was measured by spectrophotometry at 260 nm. Purity of DNA was assessed using the ratio of OD 260/280 with a ratio of 1.8–2.0 being considered of high purity [28]. Quantification of all DNA samples was performed using a Beckman DU-64 Spectrophotometer.

2.4. Genomic DNA digestion and electrophoresis

A total of 2 μg of genomic DNA per sample was digested overnight with approximately 10-fold excess of either HpaII or MspI restriction endonucleases according to the manufacturer's protocol (Promega). Also, a DNA aliquot per sample was incubated without restriction enzyme and served as background control. Briefly, the DNA digestion was performed in 20 μl reaction mixtures containing 2 μg of DNA, 10× buffer A (in the case of digestion with HpaII) containing 60 mM Tris–HCl (pH 7.5), 60 mM NaCl, 60 mM $MgCl_2$ and 10 mM DTT, or 10× buffer B (when MspI was used for the enzyme digestion) containing 60 mM Tris–HCl (pH 7.5), 500 mM NaCl, 60 mM $MgCl_2$ and 10 mM DTT, 0.1 mg/ml BSA. In all cases, 15 units of the restriction enzyme was used and H_2O_D was added to complete the final reaction volume. The reaction mixtures were incubated overnight at 37 °C and terminated by adding 2 μl of loading buffer consisting of 5% (v/v) Sarkosyl, 0.0025% bromophenol blue and 25% (v/v) glycerol. The mixtures were subjected to 1% (w/v) agarose-gel electrophoresis in TAE (Tris/acetate/EDTA) running buffer. The agarose gels were stained with ethidium bromide and images were captured using a UV transilluminator (Vilber Lourmat, France). The relative changes in methylation status in different samples were compared by densitometric analysis of the gels using the software program PCBAS 2.08e (IsotopenmeBgeräte GmbH, Germany). Densitometric values obtained after background subtraction from at least three independent experiments were pooled and the mean values were expressed as the percent of DNA migrated from the well. Statistical evaluation was done using Student's *t*-test for significance.

2.5. Induction of endoreduplication

Exponentially growing AA8 or EM9 cells were cultured for 24 h in the presence of a wide range of different concentrations of the cytidine analogue 5-azaC (0.025, 0.05, 0.075, 0.1, 0.5, 1, 2.5, and 15 μM) previously shown as efficiently inducing hypomethylation at the C5 position of cytidine after incorporation into DNA. On the other hand, reversal of DNA hypomethylation in EM9 exponential cells was produced after 24 h treatment with different concentrations (50, 60, 70, 80, and 90 μM) of the chemopreventive and methylating agent budesonide. 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 control. Colcemid (2×10^{-7} M) was finally added for 2 h 30 min to all the cultures for metaphase arrest. The flasks were gently 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 scored and classified as normal or as showing diplochromosomes. All the experiments were carried out in triplicate.

2.6. Cell proliferation assay

AA8 and EM9 cells in the exponential growth phase were harvested using trypsin–EDTA (Gibco BRL), and resuspended in medium. They were seeded at 5×10^3 cells/100 μl in 96-well microtitre plates (Nunc) and allowed 24 h to attach. Then they were incubated further for 48 h in the presence of the cytidine analogue 5-azaC. The concentration range tested (0.025 to 200 μM) was prepared in tissue culture medium from a 10^{-2} M 5-azaC stock solution. Following the recommendations of the National Cancer Institute (USA), the analysis of cytotoxic effects induced by the 5-azaC was carried out using a cell growth test, the sulforhodamine B (SRB) assay, as described previously [29,30]. Briefly, 50 μl/well of cold 50% trichloroacetic acid (TCA) (final concentration 10%) was added to the culture and incubated at 4 °C for 1 h, to precipitate proteins and to fix the cells. The supernatant was then discarded, and the plates were washed five times with deionized water and air-dried. The cells were then stained with 100 μl/well of 0.4% SRB dissolved in 1% acetic acid for 30 min at room temperature. Unbound SRB was removed by washing five times with 1% acetic acid, and then the plates were air-dried. The stained protein was solubilized in 100 μl/well of 10 mM unbuffered Tris base by shaking. The optical