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Short Communication

SELECTIVE DAMAGE TO THE ACTIVE X CHROMOSOME BY CAMPTOTHECIN AND AMSACRINE AS DETERMINED BY AN ALLELE-SPECIFIC ALKALINE UNWINDING ASSAY

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Abstract—Previous studies with MCF-7 cells demonstrated that several agents induce greater strand breakage in active genes than in nontranscribed centromeric regions. To better assess the effects of gene activity and inactivity, an allele-specific DNA strand break assay was developed, which allowed direct comparison of damage at a specific genetic locus on the active and inactive X chromosomes. The ZP lymphoblastoid cell line is heterozygous at the glucose-6-phosphate dehydrogenase (G6PD) locus, and the unexpressed (A) allele on the inactive X chromosome contains a *Fok*I restriction site that is lacking in the expressed (B) allele on the active X. ZP cells were treated with camptothecin or amsacrine, and subjected to alkaline-induced DNA unwinding. Following detergent lysis and digestion of single-stranded DNA with S1 nuclease, the remaining double-stranded DNA was isolated and subjected to polymerase chain reaction (PCR) with primers that flank the polymorphic *Fok*I site, with [α-^{32}P]dCTP being added in the last PCR cycle. The resulting labeled PCR product was cleaved with *Fok*I to assess the A/B allele ratio in the double-stranded DNA fraction. Treatment with camptothecin and amsacrine increased the apparent A/B ratio by factors of 2–3 and 1.5–2 respectively, indicating that the active B allele is preferentially damaged by these agents.

Key words: gene-specific damage; X chromosome inactivation; DNA repair; topoisomerase I; topoisomerase II; allele-specific PCR

Numerous approaches have been used to investigate heterogeneity of DNA structure and function, and its possible effects on damage and repair [1]. Experiments employing the alkaline unwinding/Southern blotting assay for locus-specific strand breaks have shown that, in MCF-7 cells, γ-radiation, teniposide (VM-26) and amsacrine [4′-(9-acridinylamino)methanesulfon-*m*-anisidide; m-AMSA] induce more DNA breakage in the genomic regions encompassing both the active c-*myc* and the inactive β-globin gene, than in nontranscribed α-satellite DNA [2–4]. In an effort to assess intragenomic differences in DNA damage and repair without the possible complications of sequence heterogeneity, we have exploited mammalian X chromosome inactivation to compare strand breakage and rejoining at the same X-linked locus in active and inactive contexts.

The human ZP lymphoblastoid cell line [5] was established from normal lymphocytes of a female G6PD§ A/B heterozygote; the variant A allele of G6PD differs from the wild-type B allele by a single base substitution at position 376 in exon 5 (numbering according to cDNA), which produces a new *Fok*I restriction site. While both alleles were expressed in the initial population of lymphocytes, SV40 immortalization resulted in a cell line that, within 10 passages, produced only protein of the electrophoretically distinguishable B allele. Thus, in all the immortalized cells the A allele must be on the inactive X chromosome, making the ZP cell line ideal for examining damage and repair at a single gene in both an active and an inactive state.

Materials and Methods

ZP cells (gift from Barbara Migeon, Johns Hopkins University) were maintained in 25-mL T-flasks in RPMI medium containing 15% fetal bovine serum. For each experiment, approximately 10^8 cells in 80 mL of medium (10 mL/flask) were labeled for 48 hr with 0.1 μCi/mL of [*methyl*-^{3}H]thymidine (70 Ci/mmol; ICN). The cells were combined, centrifuged, washed and incubated in fresh medium for 24 hr. The cells were then combined again, centrifuged, resuspended in 160 mL of fresh medium, split into 20-mL aliquots, and treated with various concentrations of amsacrine, camptothecin or vehicle (DMSO) for 1 hr at 37°. After treatment, the cells were centrifuged at 4°, washed, suspended at 4° in 0.5 mL of phosphate-buffered saline, and then subjected to alkaline unwinding (pH 12) for 10 min (see Ref. 2 for details) at 22°. Following unwinding, cell lysates were adjusted to pH 5 and treated with S1 nuclease to digest single-stranded DNA. The fraction of undigested double-stranded DNA remaining was determined as acid-precipitable radioactivity. The DNA was then isolated, and a 93-bp segment (bp 328–420) of the G6PD gene, containing the *Fok*I polymorphism, was amplified by PCR to quantitate the relative amount of each allele present. Each 100-μL reaction was performed with 1 μg of genomic DNA, 0.15 μg each of the primers (5′-GGCCAGTACGATGATGCAGC-3′ and 5′-CAGGTAGAAGAGGCGGTTGG-3′) and other standard components from a Perkin-Elmer GeneAmp kit. The thermal profile consisted of 30 cycles of 1 min at 94°, 15 sec at 59° and 1 min at 70°, followed by a final 7-min extension at 70°. Reactions were halted during the last denaturation step and held at 94° while 10 μCi of [α-^{32}P]dCTP (3000 Ci/mmol; New England Nuclear) was added to label only the last strand polymerized. This procedure avoids the formation of labeled heteroduplexes that would be

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§ Abbreviations: G6PD, glucose-6-phosphate dehydrogenase; and PCR, polymerase chain reaction.

resistant to *Fok*I cleavage, resulting in overestimation of the amount of the uncleavable B allele. The reaction mixture was extracted with phenol:chloroform (1:1) and electrophoresed on a 12% nondenaturing polyacrylamide gel. The appropriate 93-bp band was excised, eluted, precipitated, and treated for 2.5 hr with 3 units of *Fok*I, in a 25-μL reaction that also contained 0.3 μg of unlabeled pUC19 DNA. Following extraction with phenol:chloroform (1:1) to remove *Fok*I, 5μL of the reaction mixture was subjected to agarose gel electrophoresis in order to verify that all five *Fok*I sites in pUC19 were completely cleaved, and the remainder was again run on a 12% polyacrylamide gel. The extent of cleavage of the labeled 93-bp fragment into 63- and 30-bp fragments, which should reflect the relative abundance of the A allele in the DNA remaining double-stranded after alkaline unwinding, was assessed by Phosphorimage analysis.

Results and Discussion

As expected, camptothecin treatment resulted in a marked concentration-dependent acceleration of alkali-induced DNA unwinding in SV40-transformed ZP lymphocytes, as determined from the S1 nuclease susceptibility of cellular [^{3}H]DNA following alkaline treatment (Fig. 1). This effect was presumably due to camptothecin-induced trapping of transient topoisomerase I-induced single-strand breaks [5].

To compare damage at the active and inactive G6PD loci, a 93-bp G6PD fragment containing the *Fok*I polymorphism was amplified by PCR, and the isolated, ^{32}P-labeled fragment was treated with *Fok*I to assess the relative abundance of A and B alleles. Phosphorimage analysis (Fig. 2) showed that in all samples a portion of the fragment was converted to the expected 30- and 63-bp fragments. For samples not subjected to alkaline unwinding, drug treatment had no effect on the apparent A/B allele ratio, which assumed a constant value of 0.7–0.8. It is uncertain why this ratio deviated from the expected value of exactly 1.0, but the discrepancy could have resulted from slight allele-specific differences in efficiency of isolation, amplification or final autoradiographic detection of DNA, or from incomplete cleavage of the A allele at the *Fok*I site (although the internal control DNA was always completely cleaved and further *Fok*I treatment did not increase the A/B ratio). In any case, however, the uniformity of the ratio suggests that any such effects are reproducible and thus should not appreciably impact comparisons between experimental samples.

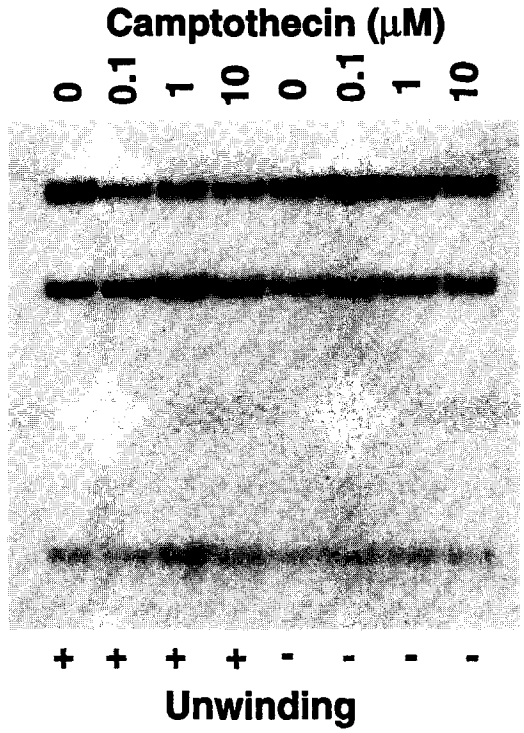

Fig. 2. Phosphorimage of a polyacrylamide gel used for the determination of allele ratios. The upper band represents the uncleavable B allele on the active X, and the two lower bands represent the products of cleavage of the inactive A allele at the polymorphic *Fok*I site. These lower bands show a relative increase in the drug-treated cells (compare lanes 1 and 3), indicating preferential damage to the active B allele.

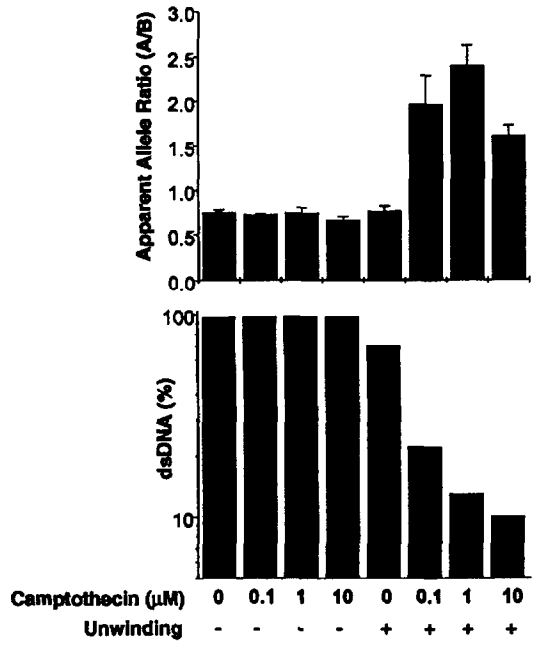

Fig. 1. Fraction of double-stranded DNA remaining following camptothecin treatment and alkaline unwinding (bottom panel), and apparent G6PD allele ratios (A/B) in the double-stranded fraction (top panel). Error bars show the standard deviation of three independent PCR assays.

The A/B ratio was also unchanged for untreated samples subjected to alkaline unwinding. However, for drug-treated samples subjected to unwinding, the A/B (cleaved/uncleaved) ratio was consistently higher, reaching a maximum of 1.5–2.5, or 2–3 times the control value, at the intermediate concentration of 1 μM (Figs. 1–3). From these data, we infer that the B allele, on the active X chromosome, sustained substantially more camptothecin-induced damage than the inactive A allele, and was thus preferentially depleted by the alkaline unwinding and S1 nuclease digestion procedure. Using equations derived previously [2], we estimate from these data and from the overall damage levels that the difference in damage susceptibility of the two alleles was approximately 2-fold. However, this is probably a minimum estimate, since any heterogeneity in sensitivity among cells in the population would tend to attenuate observed differences between alleles. The fact that the difference between alleles is greater at 1 μM than at 5–10 μM could also be explained by the existence of a cell population that sustains little damage, in either allele, at any drug concentration; alternatively, it is possible that damage to the active allele reaches a plateau at 1 μM, whereas damage to the inactive allele is still concentration dependent up to 5–10 μM.

It is also important to note that the alkaline unwinding assay assesses damage over very large domains of DNA, on the order of 500 kb for a 10-min unwinding period [2]. The results thus indicate that the DNA domain containing the transcriptionally inert G6PD locus on the inactive X chromosome is significantly

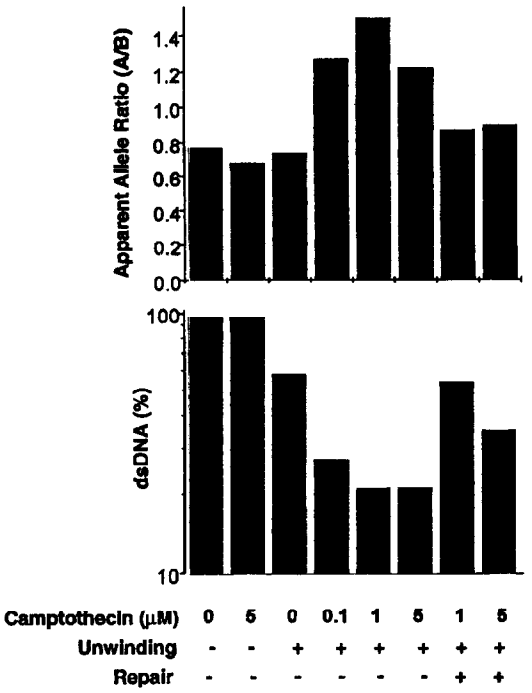

Fig. 3. Reversal of the effects of camptothecin-induced damage on the double-stranded DNA fraction (bottom panel) and the A/B allele ratio (top panel), during a 2-hr repair period following treatment.

less sensitive to camptothecin-induced damage than is the corresponding domain on the active X chromosome containing an active G6PD locus. This difference in sensitivity is presumably attributable, at least in part, to the topoisomerase I activity directly associated with G6PD transcription [6], although increased accessibility of surrounding DNA to topoisomerase I and/or camptothecin, due to a less condensed chromatin structure in the active X, could also be a contributing factor. It is likely that the increased sensitivity to camptothecin extends to the active X chromosome as a whole.

As the strand breaks were reversed upon incubation in drug-free medium, the A/B ratio was restored to near control values (Fig. 3), suggesting that reversal of camptothecin-induced breaks occurs with similar kinetics on the active and inactive X chromosomes.

Similar results were obtained for cells treated with the topoisomerase II inhibitor amsacrine (*m*-AMSA), at concentrations of 1–10 μM (results not shown). In this case, however, the increase in the A/B ratio was smaller, approximately 1.5- to 2-fold, perhaps due to the absence of a component of hypersensitivity specifically associated with transcription; topoisomerase II activity is more strongly linked with replication than with transcription [6].

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