Mutation Research 664 (2009) 48–54

Contents lists available at ScienceDirect

Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis

journal homepage: www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

Quantitative PCR analysis of diepoxybutane and epihalohydrin damage to nuclear versus mitochondrial DNA

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ARTICLE INFO

Article history:
Received 26 September 2008
Received in revised form 4 December 2008
Accepted 6 February 2009
Available online 21 February 2009

Keywords:
Diepoxybutane
Quantitative PCR
DNA damage
Cytotoxicity
Alkylating agent
Apoptosis

ABSTRACT

The bifunctional alkylating agents diepoxybutane (DEB) and epichlorohydrin (ECH) are linked to the elevated incidence of certain cancers among workers in the synthetic polymer industry. Both compounds form interstrand cross-links within duplex DNA, an activity suggested to contribute to their cytotoxicity. To assess the DNA targeting of these compounds *in vivo*, we assayed for damage within chicken erythroprogenitor cells at three different sites: one within mitochondrial DNA, one within expressed nuclear DNA, and one within unexpressed nuclear DNA. We determined the degree of damage at each site via a quantitative polymerase chain reaction, which compares amplification of control, untreated DNA to that from cells exposed to the agent in question. We found that ECH and the related compound epibromohydrin preferentially target nuclear DNA relative to mitochondrial DNA, whereas DEB reacts similarly with the two genomes. Decreased reactivity of the mitochondrial genome could contribute to the reduced apoptotic potential of ECH relative to DEB. Additionally, formation of lesions by all agents occurred at comparable levels for unexpressed and expressed nuclear loci, suggesting that alkylation is unaffected by the degree of chromatin condensation.

1. Introduction

Bifunctional alkylating agents have played a central role in cancer chemotherapy since the introduction of nitrogen mustards into clinical settings over 50 years ago [1]. The profound cytotoxicity of these agents has been attributed to formation of covalent DNA interstrand cross-links that disrupt normal replication and transcription [2]. While DNA cross-linkers remain widely used as anticancer agents, their use is associated with subsequent development of hematopathologies such as myelodysplastic syndrome and acute myeloid leukemia [3–5]. Occupational exposure to cross-linkers, or agents that are metabolized to cross-linkers, can also increase cancer risk. For example, workplace exposure to 1,3-butadiene (BD) has been linked to increased incidence of hematopoietic and lymphatic cancers [6–9], and exposure to epichlorohydrin (ECH) appears to increase risk of lung cancer [10,11]. We are investigating the mechanisms by which these high-volume industrial chemicals exert their biological effects. While the molecular determinants of the ultimate effect of a cross-linking agent remain to be elucidated, genomic targets are likely to play a role in carcinogenicity versus antitumor potential.

The metabolites of BD include monoepoxides and a diepoxide, diepoxybutane (DEB). DEB is 100-fold more mutagenic and cytotoxic than the monoepoxide metabolites, suggesting that the biological effects of BD exposure may arise principally from the formation of DNA interstrand cross-links [12,13]. DEB reacts with synthetic DNA oligomers to form interstrand cross-links preferentially at 5′-GNC sites [14]. Although the 5′-GNC consensus sequence of DEB is preserved in defined-sequence nucleosomal core particles [15], such model systems may not be completely representative of the sites targeted in the genome. High-level chromatin structure modulates the DNA binding of many mutagens, carcinogens, and anticancer drugs *in vivo* [16]. Indeed, chromatin structure has been proposed to explain the different DEB products observed with free and cellular DNA [17]. Furthermore, the relative contributions of mitochondrial DNA (mtDNA) damage versus nuclear DNA (nDNA) damage to the effects of DEB and other cross-linkers are unknown. Mutations in mtDNA are linked to human diseases such as cancer, the normal process of aging, and the triggering of apoptosis [18–20].

Our goal in this study was to assess DNA damage *in vivo* by DEB, the industrial cross-linker ECH, and the related compound epibromohydrin (EBH). Like DEB, ECH and EBH form cross-links between deoxyguanosine residues, although they are less stringent in their

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doi:10.1016/j.mrfmmm.2009.02.004

sequence requirements, cross-linking 5′-GC and 5′-GNC sites about equally [21]. We used the technique of quantitative polymerase chain reaction [QPCR], in which the presence of DNA lesions can reduce the amount of PCR product relative to an unmodified template by inhibiting Taq polymerase [22]. The lesion frequency at the locus of amplification can be determined through a Poisson analysis of the amplification of the damaged template versus the undamaged template [23]. QPCR has been used to assess DNA damage induced by a wide variety of genotoxins [24]. DEB and the epihalohydrins principally alkylate at the N7 position of deoxyguanosine residues [14,21], leading to heat-sensitive adducts that are likely to undergo cleavage under thermal cycling conditions [25]. QPCR was therefore used to detect total damage, including both monoadducts and cross-links, in this study.

We used QPCR to monitor reactivity of DEB, ECH, and EBH within 6C2 (erythro-progenitor) chicken cells at loci differing in their degree of chromatin condensation. Each locus was approximately 400 base pairs (bp) in length. One locus was within mtDNA (a portion of the cytochrome *b* gene), one was within open, expressed nDNA (a portion of the folate receptor [FR] gene), and one was within highly condensed, unexpressed nDNA (near the β-globin locus). MtDNA lacks any associated proteins, whereas nDNA interacts with histones to form chromatin [26]. Chemical modification of histones, a control mechanism for gene expression [27], modulates histone–DNA interactions and chromatin structure [28] and thereby has the potential to affect reactivity with external agents.

We found that the lesion frequencies for DEB were comparable for all loci, whereas the lesion frequencies for the epihalohydrins were three to four times higher for the nuclear loci than for the mitochondrial one. These findings suggest that DEB partitions about equally between the two genomes *in vivo* but that ECH and EBH target nuclear DNA preferentially. Decreased targeting of the mitochondrial genome by ECH could contribute to its reduced apoptotic potential relative to DEB. Furthermore, chromatin condensation appeared to have no significant influence on nDNA alkylation by the agents examined.

2. Materials and methods

2.1. Cell culture

6C2 (erythro-progenitor) chicken cells were grown in Richter's modified Eagle's medium supplemented with 10% fetal bovine serum, 2% chicken serum, 1 mM HEPES, 50 μM β-mercaptoethanol, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained at 37 °C in 5% CO_2.

2.2. Cytotoxicity assays

The cytotoxicities of DEB and ECH (both as racemic mixtures from Sigma–Aldrich) were determined in 6C2 cells via propidium iodide exclusion [29,30]. (*Caution*: DEB, ECH, and EBH are suspect carcinogens and must be handled appropriately.) Briefly, confluent cells were plated at a 1:5 dilution with MEM Richter's modification with L-glutamine and grown for at least 18 h (37 °C, 5% CO_2) to ensure entry into the log phase of growth. Cells were treated with 0, 2.5, 10, 25, 100, or 250 mM DEB or ECH, and 1 mL aliquots were removed after 15, 30, and 60 min. Treated cells were centrifuged at 250 × *g* for 5 min, and the pellets were washed with PBS (137 mM NaCl, 10 mM phosphate, and 2.7 mM KCl [pH 7.4]), centrifuged again, and suspended in 1 mL PBS. 10 μL propidium iodide (from a 50-ng/mL stock in PBS) were added, and samples were covered with aluminum foil and incubated at room temperature for 10–15 min. Cell viability was determined via flow cytometry (BD Biosciences FACScalibur with CellQuest software), with propidium iodide emission indicating cell death [29]. The viable fraction was plotted versus concentration, and the Solver tool in Excel was used to optimize the values of *a* and *b* in the equation $y = 1/[1 + [x/a]^b]$ to achieve the best-fit dose response curve, with *a* being the LD_{50} (the dose lethal to 50% of the cells). Data were obtained in duplicate or triplicate, and SolverAid [31] was used to determine the standard error for the LD_{50} value at each time point.

2.3. Treatment with cross-linkers

Cells at 85–95% confluence were passaged into a total volume of 5 mL of fresh medium 24 h prior to treatment with cross-linker. To establish appropriate reaction conditions, $(1–5) \times 10^6$ cells were incubated with varying concentrations of DEB (2.5–250 mM) for varying times (15–180 min) at 37 °C in 5% CO_2. After initial experiments with DEB, a concentration of 250 mM was used in subsequent QPCR experiments for all cross-linkers. ECH and EBH incubation times ranged from 15 to 240 min (previous work suggested that longer incubation times would be required for these agents than for DEB [21]). After incubation, cells were transferred to 15 mL conical tubes and the cell culture plates were washed with 5 mL of PBS, which was subsequently added to the tubes containing the cells. Cells were centrifuged at 500 × *g* for 5 min, and the resulting cell pellets were washed with 10 mL of PBS. Cells were pelleted again by centrifugation as above and stored at −20 °C until DNA isolation.

2.4. DNA isolation

Cell pellets were re-suspended in 300 μL digestion buffer (100 mM NaCl, 10 mM Tris–Cl, pH 8, 25 mM EDTA, 0.5% SDS, 0.1 mg/mL proteinase K) and incubated at 50 °C for ∼15 h. Samples were extracted with an equal volume of phenol/chloroform/isoamyl alcohol followed by ethanol precipitation with 2.5 M ammonium acetate and 10 μg of glycogen. DNA pellets were washed with 70% ethanol, dried, and re-suspended in 100 μL water. DNA was subsequently ethanol precipitated with 0.3 M sodium acetate, washed with 70% ethanol, dried, and re-suspended in 100 μL water. DNA was quantitated using a NanoDrop® ND-1000 UV-Vis spectrophotometer.

2.5. PCR primer design

All primer pairs amplified circa 400 bp products. Mitochondrial PCR primers were derived from the universal primers H15149 and L14841 [32] but were designed using the program OLIGO (Molecular Biology Insights) to be exactly complementary to the chicken genome. The sequences of these primers were as follows: 5′-CTCCCAGCCCCATCCAACATCTCTGCTTGATGAAA and 5′-TAACGGTGGCCCCTCAGAATGATATTTGGCCCCA. Primers for the expressed and unexpressed loci were designed based on genomic data and published maps of the region in the vicinity of the β-globin domain [33,34]. For the expressed region, we used a portion of the folate receptor gene, which is located upstream of the β-globin locus and is expressed in the 6C2 cell line [34]. The primer sequences for this locus were 5′-AAAGTACTACGCCTGGAAGAAGAGA and 5′-ATTCAGAAATGGATCATGAACAAAC. For the unexpressed locus, we probed a region of highly condensed chromatin at the 3′ terminus of the β-globin gene [33]; primer sequences were 5′-AGTACTGCCGTGTGTTTGCTC and 5′-TACAGCCCTCTCAGCAAGTAA. Amplification of the correct regions was confirmed through sequencing of the products (Supporting Information) and comparison with the *Gallus gallus* genomic data via BLAST [35].

2.6. Quantitative PCR

Initial experiments were performed to determine the linear range of amplification for each locus by varying the amount of template DNA in each PCR reaction. Subsequent PCR reactions (25 μL) contained 1.25 units of Taq DNA polymerase, 1× Taq buffer B (Fisher Bioreagents), 2.5 mM $MgCl_2$ (3.0 mM for the FR locus), 0.2 mM each dNTP, 0.5 μM each primer, 1 μCi [α-^{32}P]dATP, and 2 ng template DNA. Nuclear DNA was amplified via a "touchdown" protocol [36] that consisted of the following thermal cycling sequence: 5 min at 95 °C; 10 cycles of 30 s at 95 °C; 30 s at 65 °C (decreased by 1 °C each cycle); 30 s at 72 °C; 20 cycles of 30 s at 95 °C; 30 s at 55 °C; 30 s at 72 °C; 10 min at 72 °C. Mitochondrial DNA was amplified via a "two-step" protocol: 5 min at 95 °C; 25 cycles of 30 s at 95 °C and 30 s at 70 °C; 10 min at 72 °C. Following thermal cycling, 5 μL loading dye (0.25% xylene cyanole in 40% sucrose) was added to each PCR reaction. A 10-μL aliquot of each PCR reaction was analyzed via 8% native polyacrylamide gel electrophoresis (PAGE) at 200 V for circa 1.5 h at 4 °C after pre-running for 1.5 h. Gels were subsequently dried, exposed to phosphorimager screens, and quantitated using a Storm 840 phosphorimager (Molecular Dynamics).

2.7. Poisson analysis of QPCR products

Lesion frequencies at various loci were calculated through a Poisson expression analysis. Lesions per strand were calculated as the negative natural logarithm of the ratio of the intensity of the QPCR product from the damaged DNA to the intensity of the QPCR product from undamaged DNA [23]. This value was then converted to lesions/kbp by correcting for the length of each PCR product (unexpressed locus, 398 bp; expressed locus, 451 bp; mitochondrial locus, 376 bp). Values for lesions/kbp were plotted versus treatment time, with lesion formation frequency calculated from the slope of the best-fit line for the linear part of the curve [24]. Data from at least three trials were averaged.

2.8. Assessment of repair of ECH lesions

Cells at 85–95% confluence were passaged into a total volume of 5 mL of fresh media and incubated at 37 °C and 5% CO_2 for at least 18 h to ensure entry into the log phase of growth. Samples were then treated with 250 mM of ECH for 4 h at 37 °C and