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DNA–protein crosslinks processed by nucleotide excision repair and homologous recombination with base and strand preference in *E. coli* model system

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ABSTRACT

Bis-electrophiles including dibromoethane and epibromohydrin can react with O^6-alkylguanine-DNA alkyltransferase (AGT) and form AGT-DNA crosslinks *in vitro* and *in vivo*. The presence of human AGT (hAGT) paradoxically increases the mutagenicity and cytotoxicity of bis-electrophiles in cells. Here we establish a bacterial system to study the repair mechanism and cellular responses to DNA–protein crosslinks (DPCs) *in vivo*. Results show that both nucleotide excision repair (NER) and homologous recombination (HR) pathways can process hAGT-DNA crosslinks with HR playing a dominant role. Mutation spectra show that HR has no strand preference but NER favors processing of the DPCs in the transcribed strand; UvrA, UvrB and Mfd can interfere with small size DPCs but only UvrA can interfere with large size DPCs in the transcribed strand processed by HR. Further, we found that DPCs at TA deoxynucleotide sites are very inefficiently processed by NER and the presence of NER can interfere with these DNA lesions processed by HR. These data indicate that NER and HR can process DPCs cooperatively and competitively and NER processes DPCs with base and strand preference. Therefore, the formation of hAGT-DNA crosslinks can be a plausible and specific system to study the repair mechanism and effects of DPCs precisely *in vivo*.

1. Introduction

DNA–protein crosslinks (DPCs) are common DNA lesions generated by endogenous and exogenous agents, including the enzymes topoisomerase I, topoisomerase II and DNA polymerase β, endogenous aldehyde metabolites, physical factors such as ionizing radiation and UV light, chemical agents including formaldehyde and transition metals, and bifunctional chemotherapeutic drugs such as nitrogen mustards and platinum compounds [1–4]. The bulky nature of DPCs blocks normal physiological processes such as replication, transcription, DNA repair and chromatin remodeling [1]. Since all known DPC-inducing agents can produce a variety of DNA lesions in addition to DPCs, it is difficult to precisely evaluate the cellular effects of DPCs as well as the DNA repair mechanisms required to process DPCs *in vivo* [2]. In order to understand the mechanism of DPC formation and repair, several *in vitro* model systems have been developed [5–8]. Repair studies with these systems suggested that NER could efficiently remove small size DNA-peptide crosslinks [9,10]. Genetic and biochemical studies have shown that both NER and HR could remove DPCs and both pathways play different roles. In bacteria, NER repairs DPCs that contain proteins which are less than 15 kDa whereas oversized DPCs are processed by HR [11]. In mammalian cells, NER does not contribute to the repair of DPCs unless the proteins are < 10 kDa whereas HR plays a pivotal role to process DPCs [9,12]. However, previous model systems cannot study the heterogeneity of DNA repair of DPCs at DNA base level and the precise effect of DPCs *in vivo*.

The repair protein O^6-alkylguanine-DNA alkyltransferase (AGT or MGMT) protects the cells from the genotoxic effects caused by endogenous and exogenous alkylating agents [13,14]. Unexpectedly, AGT can enhance the cytotoxic and mutagenic effects induced by dibromoethane (DBE) in prokaryotic and eukaryotic cells [15–18]. Some bis-electrophiles including DBE and

Abbreviations: AGT (MGMT), O^6-alkylguanine-DNA alkyltransferase; hAGT, human wild type AGT; NER, nucleotide excision repair; TC-NER, transcription-coupled NER; GC-NER, global genome-coupled NER; BER, base excision repair; DSB, double-strand break; RNAP, RNA polymerase; HR, homologous recombination; TLS, translesion synthesis; GSH, glutathione; PMSF, phenylmethylsulfonyl fluoride; PCR, polymerase chain reaction; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; MW, Molecular Weight; DPC, DNA–protein crosslink; Oligo, oligodeoxyribonucleotide; DTT, dithiothreitol; DBE, 1,2-dibromoethane; EBH, epibromohydrin; BDO, 1,3-butadiene diepoxide; *E. coli*, Escherichia coli; Rif^{res}, rifampicin resistance; rpoB, gene encoding β-subunit of RNA polymerase in *E. coli*; HPRT, hypoxanthine phosphoribosyltransferase; CHO, Chinese hamster ovary.

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epibromohydrin (EBH) are environmental toxicants that have been used globally as pesticides, solvents and chemical intermediates in agriculture and industry [18,19]. It has been shown that DBE can react directly with human AGT (hAGT) to form a highly reactive half-mustard intermediate and then hAGT can facilitate the binding to DNA and form hAGT-DNA crosslinks [18,20]. *In vitro* experiments showed that the order of bases forming covalent hAGT-oligodeoxyribonucleotide (Oligo) complexes is G > T > C > A and the predominant crosslinked site is N^7-G [18,20,21]. Other epoxide compounds of bis-electrophiles such as EBH and 1,3-butadiene diepoxide (BDO) can produce AGT-DNA adducts *via* two mechanisms: (a) compounds react with AGT to form intermediates and then intermediates react with DNA to form AGT-DNA adducts; or (b) compounds react with DNA to form intermediates and then these intermediates react with AGT to form AGT-DNA adducts [21–23]. The presence of hAGT can increase the mutagenicity and cytotoxicity of EBH and BDO in *E. coli* cells [22,23]. Human histones and glyceraldehyde 3-phosphate dehydrogenase are also identified as candidate proteins that can form DPCs with bis-electrophiles-diepoxybutane but not DBE as determined in a global proteomic screen besides AGT and glutathione (GSH). However, neither of them will enhance mutagenesis *in vivo* [24,25]. AGT is very likely the only nuclear protein that can enhance mutagenesis induced by DBE and EBH *in vivo*. The formation of hAGT-DNA crosslinks in cells may provide a good model to study the cellular response and precise repair mechanism of DPCs *in vivo*. Previous studies based on UV-induced cyclobutane pyrimidine dimer repaired by NER have discovered that the repair mechanism of NER and transcription-coupled NER (TC-NER) which prefers to repair the transcribed DNA strand [26]. While a study in *Escherichia coli (E. coli)* suggested that TC-NER is not involved in the repair mechanism of DPCs [27]. It is still unknown whether NER processes DPCs with strand preference. As DPCs are dominantly formed at GC pairs and processed by both HR and NER pathways in our model system, it may provide some novel data that has not been shown in previous studies. We generated several cell strains *via* knockout of the UvrA, UvrB, RecA and Mfd genes in GWR109 cells that harbor a deletion of the endogenous AGT genes Ogt and Ada. These cell strains were treated with DBE and EBH. Our data show that both NER and HR can remove hAGT-DNA crosslinks yet HR plays a dominant role. Mutation spectra show that NER cannot efficiently remove DPCs at TA sites and the presence of NER can obviously prevent DPCs at TA sites and transcribed strand processed by HR. This study indicates that NER can remove DNA lesions with base and strand preference and NER can compete with HR to remove DPCs.

2. Materials and methods

2.1. Materials

Oligodeoxyribonucleotides and all the primers were synthesized and purified by the Macromolecular Core Facility, Hershey Medical Center. *E. coli* XL1-blue bacterial strain was purchased from Stratagene (La Jolla, CA). Wild type *E. coli* and *E. coli* bacteriophage P1 were obtained from ATCC (Manassas, VA). QIAquick PCR Purification kit, QIAprep Spin Miniprep kit, Blood& Cell Culture DNA Maxi kit, the pQE30 plasmid and Anti-penta His antibody were obtained from Qiagen (Chatsworth, CA). Pfx DNA polymerase was purchased from Invitrogen (Carlsbad, CA). Ampicillin, kanamycin, tetracycline, chloramphenicol, isopropyl β-D-thiogalactopyranoside, L-arabinose, DBE, EBH, PMSF, rifampicin and most other biochemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO). KOD DNA polymerase was purchased from Novagen (Madison, WI). HRP linked anti-mouse IgG and anti-rabbit IgG were purchased from Cell signal technology (Danvers, MA). Plasmids pKD46 and pKD3 were provided by Dr. B. Wanner (Department of Biological Sciences, Purdue University, West Lafayette, IN).

2.2. Bacterial strains and media

The bacterial strains GWR109, FC326, FC218, CJM1 as well as CJM2 with the deletion of the endogenous AGT genes *Ada* and *Ogt* were generously provided by Dr. L. Samson (Biological Engineering Division and Center for Environmental Health Sciences, MIT, Cambridge, MA) [28,29]. The BW25113 cell strain lacking exonuclease V of the RecBCD pathway was provided by Dr. B. Wanner [30]. CJM1 and CJM2 with inactive *UvrB* gene were derived from FC326 and FC218 respectively. FC326 and FC218 cells with a chromosomal deletion of the lactose operon carry mutated lacZ sequences at codon 461(GAG) on an F' episome. These *E. coli* cells with the mutated lacZ alleles are lac- and cannot grow in M9 minimum medium with lactose as a sole carbon source; however, the lac- cells can be reverted to the lac+ cells (wild type) *via* AT to GC (FC326: AAG to GAG) and GC to AT (FC218: GGG to GAG) mutations respectively and the mutated cells can grow in the medium with lactose as the sole carbon source [28,29]. Cell mediums were prepared as described previously [29,31].

2.3. Knockout UvrA, UvrB, RecA and Mfd in GWR109 cell strain

Gene disruption was carried out as described previously [30]. Plasmid pKD3 containing chloamphenicol (Cam)-resistance gene and FLP recognition target site was used as template. Primers UvrA-P1: UvrA-P2, UvrB-P1: UvrB-P2, RecA-P1: RecA-P2, and Mfd-P1: Mfd-P2 containing FLP sequence, ribosome binding site and targeted genes sequence were used for PCR to obtain UvrA, UvrB, RecA and Mfd PCR products respectively and their sequence is listed in Supplementary Table S1. PCR was carried out using KOD DNA polymerase and the PCR products were digested by Dpn I for 1 h at 37 °C and purified with QIAquick Gel Extraction kit. PCR products were electroporated into competent BW25113 cells and transformants were selected in LB plates containing ampicillin (Amp) and Cam. BW25113 cells with targeted gene disruption were used as donor strain for P1 transduction.

P1 transduction was performed using the protocol as described previously with modifications [32] and http://biology4.wustl.edu/levin/protocols.php. Positive colonies were isolated and verified by PCR and UV sensitivity assay.

2.4. PCR verification of gene knockout

The correct gene disruption for all the mutants was confirmed by PCR. The isolated positive colonies were suspended in 50 μl H_2O, 5 μl portions were taken from them and used for 50 μl PCR reaction mixture. Two pairs of primers were used for each set of disrupted genes. The first pair of primers were named TP1-1 and TP1-2 and their sequence was designed to recognize DNA outside of the modification of the knockout genes. The PCR reaction can produce the correct fragments of targeted genes and replaced sequences. The second pair of primers were named TP2-1 and TP2-2 and their sequence lies within the modified sequence of knockout genes. So if the targeted genes were knocked out, then the correct size PCR product cannot be obtained. The sequence of primers is listed in Supplementary Table S1. 1% Agarose gel with ethidium bromide was used to identify the PCR products.

2.5. Determination of AGT expression

The plasmids pQE30 or pQE-hAGT (which has an N-terminal $(His)_6$-tag replacing the terminal M- with the sequence MRGS$(H)_6$GS-) [33] with pREP4 were co-transformed into cell