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A quantitative PCR-based assay reveals that nucleotide excision repair plays a predominant role in the removal of DNA-protein crosslinks from plasmids transfected into mammalian cells

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ABSTRACT

DNA-protein crosslinks (DPCs) are complex DNA lesions that induce mutagenesis and cell death. DPCs are created by common antitumor drugs, reactive oxygen species, and endogenous aldehydes. Since these agents create other types of DNA damage in addition to DPCs, identification of the mechanisms of DPC repair is challenging. In this study, we created plasmid substrates containing site-specific DPC lesions, as well as plasmids harboring lesions that are selectively repaired by the base excision or nucleotide excision repair (NER) pathways. These substrates were transfected into mammalian cells and a quantitative real-time PCR assay employed to study their repair. This assay revealed that DPC lesions were rapidly repaired in wild-type human and Chinese hamster derived cells, as were plasmids harboring an oxoguanine residue (base excision repair substrate) or cholesterol lesion (NER substrate). Interestingly, the DPC substrate was repaired in human cells nearly three times as efficiently as in Chinese hamster cells (> 75% vs ~25% repair at 8 h post-transfection), while there was no significant species-specific difference in the efficiency with which the cholesterol lesion was repaired (~60% repair). Experiments revealed that both human and hamster cells deficient in NER due to mutations in the xeroderma pigmentosum A or D genes were five to ten-fold less able to repair the cholesterol and DPC lesions than were wild-type control clones, and that both the global genome and transcription-coupled sub-pathways of NER were capable of repairing DPCs. In addition, analyses using this PCR-based assay revealed that a 4 kDa peptide DNA crosslink was repaired nearly twice as efficiently as was a ~38 kDa DPC, suggesting that proteolytic degradation of crosslinked proteins occurs during DPC repair. These results highlight the utility of this PCR-based assay to study DNA repair and indicate that the NER machinery rapidly and efficiently repairs plasmid DPC lesions in mammalian cells.

1. Introduction

DNA-protein crosslinks (DPCs) are unusually bulky lesions formed upon covalent trapping of proteins on DNA strands [1]. These helix-distorting complexes are mutagenic, toxic, and are able to block essential cell processes such as transcription and replication [2,3]. Proteins of various sizes and functions are capable of becoming crosslinked to DNA *via* multiple mechanisms [4,5]. For example, endogenous DPCs can be formed by the trapping of repair proteins recruited to sites of DNA damage or as a byproduct of lipid peroxidation of reactive oxygen species in the blood [6–9]. Exogenous agents such as ionizing radiation, UV light, cigarette smoke, and chemotherapeutics such as cisplatin also create DPCs [5,10–15]. Despite their common occurrence and cytotoxic effects, the exact mechanism(s) by which DPCs are repaired is still not well understood.

In order to gain insight into the repair mechanism of DNA-protein crosslinks, DPC-forming agents have been used to assess hypersensitivity in repair mutants [11]. Results from these experiments have provided evidence for the roles of nucleotide excision repair (NER) and homologous recombination (HR) in DPC repair [16]. However, there are contradictory reports in the literature regarding the relative contributions of the two repair pathways. Specifically, genetic studies performed in *Escherichia coli* revealed that *uvrA* and *recA* mutants deficient in NER or HR were hypersensitive to the DPC-inducing agent formaldehyde [17,18]. However, only *recA* and not *uvrA* mutants were hypersensitive to DPCs induced by azacytidine [19,20]. In *Saccharomyces cerevisiae*, mutants deficient in NER, but not HR, were sensitive to formaldehyde [21,22]. Similarly, human cells from xeroderma pigmentosum patients possessing mutations in the NER pathway were sensitive to DPC-inducing agents [23,24].

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Since all known DPC-forming agents induce other types of DNA damage, such as DNA monoadducts and DNA-DNA cross-links, it is difficult to conclude if sensitivity to these drugs is influenced by lesions other than DPCs. In an effort to overcome this potential limitation, investigators have directly examined the kinetics of DPC formation and removal from wild-type and repair-deficient clones following exposure to DNA damaging agents [25]. However, these studies have yielded contradictory results. For example, Quievryn et al. failed to detect differences in the kinetics of formaldehyde-induced DPC removal between NER-deficient human fibroblasts and control [26]. Conversely, DPCs induced by nornitrogen mustard accumulated at higher rates in human cells deficient in the NER gene *XPA* compared to HR-deficient or wild-type clones [4].

To more directly assess the involvement of NER in DPC repair, Minko et al. incubated DPC-containing oligonucleotides with UvrABC nucleases from *Escherichia coli* and saw slower incision rates of DNA containing a 16 kDa protein compared to smaller DNA-peptide cross-links [27,28]. *In vitro* studies performed with human nucleases saw similar results where 4 and 12 amino acid peptide-crosslinks were recognized by the NER machinery but were unable to remove a 16 kDa protein-crosslink [29]. Nakano et al. later reported a size limit of 8–10 kDa for the excision of crosslinked proteins by NER in mammalian cells while Baker et al. saw NER-directed repair of a 38 kDa attached to plasmid DNA [30,31]. Currently, the role of NER in the repair of DPCs (specifically those consisting of proteins larger than 10 kDa) remains unclear. However, it is hypothesized that the decreased efficiency of NER to repair larger protein-crosslinks is caused by steric hindrance of damage recognition proteins and suggests that proteolytic degradation is necessary prior to repair. While it was originally proposed that the proteasome is responsible for proteolysis of full size DPCs, more recent studies have suggested that a different protease (Spartan in humans) is involved [32–37].

To clarify the role of NER in the repair of DPCs in mammalian cells, as well as to address more specific questions regarding how size and location influence DPC repair, we employed a PCR-based assay we term Strand-Specific Primer Extension-Quantitative Polymerase Chain Reaction (SSPE-qPCR). This assay is capable of quantifying the repair kinetics of a broad range of lesions present on plasmid DNA transfected into repair deficient and corrected mammalian cells. This assay provides significant advantages over previously utilized approaches in that it is rapid, highly quantitative, and extremely flexible. Importantly, this method directly measures repair activity, in contrast to other plasmid-based strategies that rely on indirect measures such as host-cell reactivation of gene function. Results from our initial analyses provide new insight into the ability of the global genome and transcription-coupled NER pathways to repair DPCs in hamster and human cells.

2. Material and methods

2.1. Materials

2.1.1. Chemicals and enzymes

Oligodeoxynucleotides (ODNs) containing 8-oxo-2′-deoxyguanosine (8-oxo-dG) or cholesterol modifications were obtained from Midland Certified Reagent (Midland, TX). All other ODNs were purchased from the University of Minnesota Genomic Center. Human oxoguanine glycosylase 1 (OGG1) was expressed and purified from BL21(DE3) bacteria (Thermo Fisher) using a pET-28a expression vector [38]. Single-stranded M13 vector and all enzymes were obtained from New England Biolabs (Beverly, MA) unless specified otherwise. Chemicals were purchased from Sigma Chemical (St. Louis, MO) unless indicated.

2.1.2. Cell lines

Chinese hamster lung fibroblast cell lines V79 (GM16136) and V-H1 (GM16141) were obtained from the Coriell Institute for Medical Research (Camden, NJ). V79 are wild-type cells from which the V-H1 clones were derived following an ethylnitrosourea-induced mutagenesis screen [39–41]. V-H1 cells belong to nucleotide excision repair complementation group 2 and lack a functional *XPD* gene [42]. These cells are deficient in the *ERCC2* gene, which codes for the *XPD* protein involved in the helicase activity that unwinds duplex DNA during nucleotide excision repair [42]. Chinese hamster lung fibroblast cells were cultured in Ham's F-12 modified essential Eagle's media (Life Technologies, Grand Island, NY) supplemented with 9% fetal bovine serum (Atlanta Biologics, Atlanta, GA). Chinese hamster ovary CHO-K1 cells were a kind gift from Professor Harry Orr (University of Minnesota). Immortalized human dermal fibroblasts from xeroderma pigmentosum patients with inactivating mutations in the NER *XPD* gene (GM08207) or *XPA* gene (GM04312), as well as gene-corrected clones (GM15877 and GM15876, respectively) derived from these lines were obtained from the Coriell institute. Human and CHO-K1 cells were cultured in Dulbecco's modified Eagle's media (Life Technologies, Grand Island, NY) supplemented with 9% fetal bovine serum. All cells were maintained in a humidified atmosphere of 5% carbon dioxide, 95% air, at 37 °C.

2.2. Methods

2.2.1. Taq polymerase extension assay

To confirm that Taq polymerase permanently stalls at abasic sites (and by extension, lesions attached to abasic sites) 200 pmol of the oligodeoxynucleotide M13-RSV-Zeo-8oxo (template) was annealed to 200 pmol of primer C (Table 1) in restriction enzyme buffer 2 (New England Biolabs) and water in a volume of 10 μL at 95 °C for 5 min and

Table 1
Oligodeoxynucleotides (ODNs) used is this study (5′ → 3′).

ODN	Sequence	Use
M13-8oxo	AGGGTTTTCCA(8-oxo-dG)TCACGACGTT	Primer Extension
M13-cholesterol	CCGGGTACCGAGCTCGAATTC(cholesterol)GTAATCTTGGTCATAGCTG	Primer Extension
M13-RSV-Zeo-8oxo (template)	ACTGGTCAACTTGGCCAT(8-oxo-dG)TTGGCC TTGGAGGTCGACACC	Primer Extension
M13-RSV-Zeo-8oxo (coding)	CACCTCCAAGGCCAACAT(8-oxo-dG)GCCAAG TTGACCAGTGCCGTT	Primer Extension
M13 Primer R	CGGCTCGTATGTTGTGTG	qPCR of M13 plasmid
M13 Primer L	GCTGCAAGGCGATTAAGT	qPCR of M13 plasmid
RSV-Zeo 1	TATCCGAGATCCGAGGAA	Topo 2.1 PCR Cloning Kit
RSV-Zeo 2	TATGGATCGTCGAGACTC	Topo 2.1 PCR Cloning Kit
M13-Zeo R Primer	ACGCCATTTGACCATTCAAA	qPCR of M13-Zeo plasmid
M13-Zeo L Primer	CCGGTCGGTCCAGAACTC	qPCR of M13-Zeo plasmid
Primer C	GGCCAACATGGCCAA	Taq extension assay
Primer Z	GGTGTGCACCTCCAA	Taq extension assay
Zeo F1	CAAGTTGACCAGTGCCGTTC	RT PCR
Zeo R1	TGATGAACAGGGTCACGTCG	RT PCR
Abasic complement	GTCGACCTCCAA	Taq extension assay
Abasic	ACTGGTCAACTTGGCCAT(abasic)TTGGCCTTGGAGGTCGAC	Taq extension assay
Abasic correct	ACTGGTCAACTTGGCCATGTTGGCCTTGGAGGTCGAC	Taq extension assay