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Mutagenic Repair of DNA Interstrand Crosslinks

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Abstract

Formation of DNA interstrand crosslinks (ICLs) in chromosomal DNA imposes acute obstruction of all essential DNA functions. For nearly a century, bifunctional alkylators, also known as DNA crosslinkers, have been an important class of cancer chemotherapeutic regimens. The mechanisms of ICL repair remains largely elusive. Here, we review a eukaryotic mutagenic ICL repair pathway discovered by work from several laboratories. This repair pathway, alternatively termed recombination-independent ICL repair, involves the incision activities of the nucleotide excision repair (NER) mechanism and lesion bypass polymerase(s). Repair of the ICL is initiated by dual incisions flanking the ICL on one strand of the double helix; the resulting gap is filled in by lesion bypass polymerases. The remaining lesion is subsequently removed by a second round of NER reaction. The mutagenic repair of ICL likely interacts with other cellular mechanisms such as the Fanconi anemia pathway and recombinational repair of ICLs. These aspects will also be discussed.

Introduction

DNA interstrand cross-link (ICL) is one of the most severe cytotoxic lesions. It is formed when a bifunctional alkylating molecule reacts with bases on opposing strands of the DNA double helix. The covalently jointed DNA helix block strand separation, which is absolutely essential for normal DNA metabolisms such as replication, recombination, and transcription. Repair of an ICL lesion requires that the covalent joining of the DNA helices be reversed in some fashion. The two opposing bases affected by the crosslinking moiety renders error-free repair unfeasible in the absence of undamaged homologous sequence. Thus, ICLs lesions are the most severe form of DNA damage, their removal poses a major challenge to the cells. In unicellular organisms, one DNA ICL is sufficient to kill repair-deficient mutant cells, while some 40 unrepaired ICLs in mammalian repair-deficient cell are lethal (Lawley and Phillips 1996; Magana-Schwencke et al. 1982).

The severe cytotoxicity of ICL-inducing agents has been applied to cancer treatment for nearly 70 years (Gilman and Philips 1946). From the early use of nitrogen mustard as the first antitumor drug to the newly reemerged bifunctional alkylator bendamustine (Eichbaum et al. 2009; Glode and Jarkowski 2009; Kahl et al. 2009; von Minckwitz et al. 2009), DNA crosslinking agents have continued to play an important role in the treatment of many types of malignancies. Understanding of the repair mechanisms involved in ICL removal will not only define crucial cellular processes in the maintenance of genetic integrity, but also facilitate better application and development of this critical class of chemotherapeutic regimen.

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Investigation of ICL repair in prokaryote was carried extensively in the early 70s using *Escherichia coli* as a model system. Cole and colleagues suggested a model in which the combined actions of NER and homologous recombination achieve a complete error-free removal of an ICL (Cole 1971; Cole 1973; Cole et al. 1976; Cole and Sinden 1975). In this model (Fig. 1A.), the NER incision activities introduce dual incisions flanking the crosslinked base on the same strand; the resulting gap is resynthesized using undamaged homologous sequence through homologous recombination. Biochemical and genetic evidence seem to support this model. *E. coli* Mutants in homologous recombination and NER (*recA* and *uvr*, respectively) are hypersensitive to ICLs. A gapped intermediate produced by NER processing was identified (Cole et al. 1976) and could initiate recombination with an undamaged homologous donor (Cheng et al. 1991; Cheng et al. 1988; Rupp et al. 1971; Sladek et al. 1989). In yeast, genetic evidence also supports a similar mechanism as NER and HR mutants both exhibit hypersensitivity to DNA ICLs (Miller et al. 1982; Simon et al. 2000). Although recombination-dependent mechanisms seem to play a major role in ICL removal, studies in *E. coli* suggested a PolB-dependent mechanism for the repair of nitrogen mustard-based crosslinks (Berardini et al. 1999; Berardini et al. 1997). As this repair mechanism is independent of RecA, it excludes the involvement of homologous recombination. This is the first hint that a recombination-independent repair mechanism could lead to the removal of ICLs.

In mammalian cells, deficiencies in recombination factors such as Brca2 and the Rad51 recombinase family cause profound cellular sensitivity to crosslinking damage. The exact role of homologous recombination in ICL repair remains obscured in higher eukaryotes. The role for homologous recombination might be two-fold. When cell cycle stages are in favor of homologous recombination, processing of ICLs may give rise to recombinogenic intermediate(s) capable of initiating homology search and subsequent recombination events, which depends on the presence of undamaged homologous donor sequence. On the other hand, replication forks stalled by ICLs may lead to DNA double strand breaks either as a potential repair intermediate or as a product of fork collapse. It is conceivable that restoration of replication forks may require homologous recombination factors to reengage the sister chromatid bearing the DSB. In either case, however, the NER mechanism is unlikely to play a major role as does in the *E. coli* system.

In mammalian cells, recent investigation has uncovered a mutagenic ICL repair mechanism that involves NER incision and translesion DNA synthesis (TLS) (Sarkar et al. 2006; Shen et al. 2006; Wang et al. 2001; Zheng et al. 2003). This repair mechanism likely operates during G1 or nondividing cells when homologous recombination is less frequent and may account for the base substitutions induced by ICLs. This article will focus on this mutagenic mammalian repair pathway.

Recognition and NER-dependent processing of ICLs

The bifunctional alkylating agent family consists of a large variety of distinct compounds. Interstrand cross-links formed with different compounds exhibit divergent nucleotide sequence preferences with varying degrees of double helix distortion. However, nucleotide residues involved in an ICL are incapable of normal H-bonding in the double helix and often lead to local unwinding, so DNA damage recognition proteins that exhibit affinity to helix distortion or mismatches may recognize ICLs in the genome. Indeed, lesion binding proteins from distinct repair mechanisms, such as XPA and XPC proteins from the NER pathway and MutS complex from the mismatch repair pathway, were all found to exhibit affinities to ICLs biochemically (Thoma et al. 2005; Vasquez et al. 2002; Zhao et al. 2009).

In order to study ICL repair *in vivo*, we site-specifically placed a psoralen-or mitomycin C crosslink into a reporter in between a mammalian promoter and a downstream reporter (EGFP or luciferase), the original intention was to recapitulate the Cole's model in mammalian cells. Given that filling in of the gapped intermediate in the Cole's model requires undamaged homologous sequence, the expected result was that the crosslinked reporter would not be able to express unless a promoter-less plasmid bearing undamaged sequence homologous to the crosslinked region is supplied. Establishing the Cole's model in mammalian cells would allow a platform for the study of ICL-induced homologous recombination. The actual result, however, showed that the crosslinked reporter was able to express at a level far exceeding the expected background in the absence of an undamaged homologous donor (Wang et al. 2001). Judging from the amount of EGFP expression in repair proficient cells, it appeared that at least 30% of the crosslinked plasmid transfected into the cells was repaired and consequently able to express the EGFP gene. If no repair had occurred, the background EGFP expression would be less than 1%, because the purity of the crosslinked plasmid is routinely greater than 99%. Such a result indicates that mammalian cells have the ability to remove ICLs in the absence of undamaged homologous sequences. This functional outcome served as the foundation of investigating a recombination-independent ICL repair mechanism.

Using the crosslinked reporter plasmid, mammalian DNA repair mutants displaying crosslinking sensitivity were screened. Among the first group of mutants, the XPA and ERCC1 mutants showed significantly diminished removal of ICLs, which suggests that NER incision may be involved in the initial processing of the crosslink. Subsequent mutant analyses confirmed that the entire NER incision components, the damage recognition/binding protein XPC and XPA, the TFIIH helicase complex and the excinucleases XPG and ECRR1/XPF, were required to support the recombination-independent repair of ICLs formed by either mitomycin C or photoreactive psoralen (Zheng et al. 2003). The requirement for the complete NER incision apparatus for recombination-independent ICL repair suggests that the incision activities are involved in an initial unhooking step. Most likely, the dual incisions are placed, as in the canonical fashion, at either side of the crosslink on the same strand, leading to the generation of a gapped intermediate with the incised oligonucleotide still attached to the opposing strand through the crosslink moiety. This process is able to unhook the two covalently joined DNA strands (Fig. 1B, first incision step)

Translesion repair synthesis of the ICL repair intermediate

In the absence of undamaged homologous donor sequence, recombination unlikely provides a template for the resynthesis of the gapped intermediate generated by the NER incision. Normal repair synthesis polymerases such as Pol δ (Nishida et al. 1988) are unable to replicate through a modified base. Thus, a possible mechanism is to employ a lesion bypass DNA polymerase to replicate over the still adducted base when regular repair synthesis is stalled. Yeast mutants of lesion bypass DNA polymerases, particularly the Rev3/Pso1 mutant, display strong crosslink hypersensitivity (Cassier-Chauvat and Moustacchi 1988). A conceivable role for Rev3, which encode the catalytic subunit of lesion bypass polymerase ζ (Pol ζ) in mammals (Esposito et al. 2000; Gibbs et al. 1998; Lin et al. 1999), would be to carry out TLS over the crosslinker-adducted base in the gapped intermediate. Testing of mouse and DT40 Rev3 knockout cells confirmed that Rev3 is the principal lesion bypass polymerase for the mutagenic repair of ICLs in MEF cells and chicken lymphoblastoid cells, respectively. The Rev3-associated protein Rev1 is also shown to have a role in the recombination-independent ICL repair (Shen et al. 2006).

Recently, Polk was examined for its ability in lesion bypass synthesis of potential intermediates derived from a MMC-mimicking ICL (Minko et al. 2008). Interestingly, Polk not only catalyzed the incorporation opposite the crosslinked base with relatively high accuracy (97%), but also replicated beyond the lesion. The coupled lesion bypass and extension activity of Polk may circumvent the requirement of two separate catalytic activities (Rev1 and Rev3) to bypass the crosslinked gapped intermediate. Consistently, mouse cells lacking Polk exhibited a noticeable decrease in NER repair synthesis (Ogi and Lehmann 2006), suggesting that Polk may have a role in dealing with non-canonical lesions. Thus, Polk is a worthy candidate as a lesion bypass polymerase in ICL repair.

Proliferating Cell Nuclear Antigen (PCNA) is an essential processivity factor for the replicative DNA polymerases. Early studies in bacteria showed that the DNA polymerases processivity clamp, beta-clamp, can promote TLS in *E. coli* (Pham et al. 2001; Tang et al. 2000; Tang et al. 1999; Wagner et al. 2000). Similarly in yeast, TLS is promoted by monoubiquitination of PCNA at K164 (Stelter and Ulrich 2003); possibly by stimulating the catalytic activity of pol eta and Rev1 (Garg and Burgers 2005).

In mammalian cells, PCNA is mono-ubiquitinated after DNA damage at K164. This modification has been demonstrated to mediate polymerase switching when replication encounters a damaged base (Kannouche et al. 2004). Structural biology and structure-function studies recently further solidified the ubiquitination-dependent interaction between PCNA and lesion bypass DNA polymerases (Bienko et al. 2005; Hishiki et al. 2008).

Similar to DNA replication encountering a monoadduct lesion, repair synthesis of the gapped intermediate during ICL removal also encounters the crosslinked base. Since normal NER repair synthesis employs the same replication factors, such as RFC, RPA, PCNA, Pol δ as in replicative synthesis, a similar mechanism may be in place to recruit lesion bypass polymerases to replicate through the remaining lesion for gap fill-in. To test the hypothesis that PCNA monoubiquitination is important for recombination-independent repair of ICLs, Shen *et al* analyzed ICL repair in a DT40 mutant where the endogenous wild type PCNA is replaced by a human PCNA K164R mutant with which DNA damage-induced monoubiquitination of PCNA was abolished (Shen et al. 2006). The result confirmed that lack of PCNA monoubiquitination decreased the ICL repair activity accompanied by a drastically reduced mutation rate. Thus a model for the recombination-independent ICL repair is postulated as in Fig. 1B. In this model, the NER incision activity initiates the processing of an ICL by introducing two nicks flanking the ICL on the same strand, the resulting gapped intermediate is resynthesized by lesion bypass polymerase Pol ζ . Recent cell biology studies generated additional support for this mechanism. Muniandy *et al* investigated the removal of laser-localized psoralen crosslinks. They found that XPC along with other NER factors are rapidly recruited to the crosslinked area and removal of the crosslinks strongly depends on XPC (Muniandy et al. 2009), suggesting NER processing is an essential early step in ICL removal in the recombination-independent repair mechanism.

Recombination-Independent ICL Repair and Mutagenesis

The involvement of TLS in recombination-independent ICL removal dictates that this repair mechanism is not error-free by nature. First the lesion bypass polymerases are intrinsically of low fidelity (Plosky and Woodgate 2004; Prakash et al. 2005). Secondly, the remaining lesion at the gapped intermediate is incapable of normal base pairing and would be subjected to frequent base substitutions if used as a template base. Indeed, photoreactive psoralen-based ICLs result in approximately 10% frequency of base substitutions at positions opposite to the crosslinked bases on both strands (Wang et al. 2001). For ICLs mediated by mitomycin C, the base substitution rate is around 20% (Zheng et al. 2003). Furthermore, mutations were

found adjacent to the crosslinked nucleotide residues, which could be a further indication of participation of lesion bypass polymerase due to their much reduced fidelity. In yeast, repair of ICL were also found to be mutagenic (McHugh et al. 1999; Ruhland and Brendel 1979; Sarkar et al. 2006). Thus, recombination-independent ICL repair appears to be an error-prone mechanism. Consistently, clinical application of UVA + psoralen in psoriasis patients is known to cause treatment-induced skin cancers (Stern and Laird 1994; Stern and Lange 1988). Bifunctional alkylator therapy-induced hematologic malignancies are also known to be the most serious long-term complications of patients successfully treated for their primary disease (Levine and Bloomfield 1992). The mutagenesis impact from recombination-independent removal of ICLs may provide a mechanistic base for the development of treatment-induced secondary cancers.

Recombination-Independent ICL Repair and Fanconi anemia

Cells derived from the cancer-prone Fanconi anemia patient are characteristically sensitive to DNA crosslink agents. Thirteen subtypes of FA have been identified and each has been attributed to mutations in a distinct gene (Wang 2007). Currently, FA proteins are grouped into three complexes based on their roles and lack thereof in FANCD2 monoubiquitination. The FA core complex, consisting of FANCA-C, E-G, L, M, and two FANC associated proteins, is required for damage-induced FANCD2 monoubiquitination (Ciccia et al. 2007; Wang 2007). FA I/D2 heterodimeric complex are subjected to damage-induced monoubiquitination through the E3 ligase activity of the FANC core complex (Matsushita et al. 2005; Smogorzewska et al. 2007; Taniguchi et al. 2002). FANCD1, FANCI, and FANCF are considered homologous recombination factors, but they are not involved in the monoubiquitination of the FA I/D2 complex. To date the mechanisms of how FA proteins and FANCD2 monoubiquitination protect cells from DNA damage remains unclear. Our recent work at the molecular level have shown that FA proteins are directly recruited to the site of DNA interstrand crosslinks and the recruitment of recombination-related FA proteins are controlled by the presence of replication fork stalled at the site of an ICL (Shen et al. 2009). Interestingly, enrichment of FA core components and the FANC I/D2 complex is not affected by whether the ICL is encountered by replication. This indicates that function of the FA core and FANC I/D2 complex are able to recognize ICL or a processed intermediate.

FANCD2 mutant cells exhibited a moderate reduction in recombination-independent ICL repair (Shen et al. 2009). Although the exact functions of FA protein in recombination-independent ICL repair is unclear, one plausible role might be to recruit lesion bypass polymerase to carry the translesion repair synthesis. There are several preliminary indications for such a working model. The human FANCD2 protein and YFP-tagged REV1 were shown to colocalize in nuclear foci following inhibition of DNA synthesis (Niedzwiedz et al. 2004). Importantly, damage-induced Rev1 foci are rendered defective by the lack of FA core complex components (Mirchandani et al. 2008). More recently, biochemical evidence suggests that the FANC I/D2 complex is required for the incision as well as lesion bypass synthesis of ICLs at a stalled replication fork (Knipscheer et al. 2009). Collectively, it supports a general model that part of the FA function is to promote TLS by loading lesion bypass polymerases. It also implies that there may be a certain degree of functional redundancy between PCNA monoubiquitination-mediated polymerase switching. Alternatively, different types of lesions, even the chemical distinctions among different subtypes of ICLs, may differ in the choice of mechanisms for the recruitment of lesion bypass polymerases.

Evolutionary conservation and cell cycle-dependent function of recombination-independent ICL repair

The recombination-independent ICL repair functions in the absence of an undamaged homologous sequence donor. For nondividing cells and cells in the G1, or early S phases of the mitotic cycle, homologous recombination between sister chromosomes seems to be exceedingly rare in the mammalian system (Stark and Jasin 2003). Thus, alternative mechanisms such as recombination-independent ICL repair likely to operate in mammalian cells to remove ICL when homologous recombination is less frequent. An elegant work from Sarkar *et al* not only showed that the NER and lesion bypass-based recombination ICL repair is conserved in budding yeast, they also demonstrated that this pathway is the only mechanism for cell survival against DNA crosslinking agents in stationary cells (McHugh and Sarkar 2006; Sarkar et al. 2006). Using haploid yeast strains, they found that defects in this mechanism render G0 phase cells hypersensitive to the killing of DNA crosslinkers, indicating that the function of recombination-independent repair acts in cells where homologous recombination is not available and suggesting the importance of this pathway in G1 phase cells in the mammalian systems. Consistently, cell biology studies indicated that the NER-based ICL removal occurs in G1 phase cells (Muniandy et al. 2009), further suggesting that cells utilize the NER/lesion bypass-based mutagenic repair mechanism when conditions for homologous recombination are less favorable.

Conclusion remarks

Work from several groups during the past decade has identified recombination-independent ICL repair as a conserved mechanism in eukaryotes. There are two key steps in this pathway. The first step utilizes NER incision activities to uncouple the crosslink, converting the covalently joined DNA helix into a gapped intermediate with the incised oligo patch attached to the crosslinked base in the single stranded portion. The second step is gap resynthesis, accomplished by polymerase switching from normal repair synthesis polymerase to a lesion bypass polymerase. After the gap resynthesis, the remaining lesion is of monoadduct nature and is expected to be removed by a second round of NER reaction. The TLS step dictates that this pathway is error-prone and may account for ICL-induced mutagenesis. Although the recombination-independent ICL repair is a relatively defined mechanism in eukaryotes, it is clearly not the only mechanism to remove the ICLs. As a strong DNA replication blocking lesion, detection and repair of ICLs seems to be coupled with resolving stalled replication forks. This complex event is expected to involve Fanconi anemia proteins and structure-specific endonuclease activities such as Slx4, Slx1, and Mus81-Eme1 (Hanada et al. 2006; Raschle et al. 2008; Svendsen et al. 2009). Giving the lack of *bona fide* Fanconi anemia homologues in yeasts, much work remains in defining their roles in ICL repair and in DNA damage response in general. Future studies may reveal even more unexpected complexity of ICL repair in higher eukaryotes.

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Figure 1. Models and their characteristics of ICL repair in *E. coli* and eukaryotes. **A.** The Cole's model depicting homology-dependent error-free repair of ICLs. **B.** Recombination-independent ICL repair. “●” indicates crosslinking moiety. Green arrows indicate positions of NER incision. DNA patches in green depicting error-free repair synthesis. DNA patches in red depicting error-prone repair synthesis.