Review Article

DNA crosslinking damage and cancer - a tale of friend and foe

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Abstract: Interstrand crosslinks (ICLs) represent a major challenge for DNA replication and transcription by preventing DNA strand separation. Cells deficient in ICL repair are hypersensitive to a variety of bifunctional alkylating agents and exhibit excessive genomic instability. Patients with deficient ICL repair, such as those with Fanconi anemia, are predisposed to a broad spectrum of cancers. The profound cellular toxicity of ICLs is exploited clinically in cancer chemotherapy. Therefore, understanding the mechanism of ICL repair and its impact on cancer development and treatment is very important. Studies of diseases with defective ICL repair, especially Fanconi anemia, have revealed unique ICL repair mechanisms in humans. In this review, we describe pathways and factors involved in ICL damage response and their implications in cancer development and treatment.

Key Words: Interstrand crosslink; DNA repair; cancer; Fanconi anemia

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Introduction

DNA crosslinking damage occurs when crosslinking agents covalently connect two nucleotide residues from the same DNA strand (intrastrand crosslink) or from opposite strands [interstrand crosslink (ICL)]. Intrastrand crosslinks can be readily removed by the nucleotide excision repair (NER) mechanism (1). An ICL, however, constitutes an absolute block to DNA strand separation, thus interrupting essential DNA metabolic processes such as replication and transcription. Left unrepaired, ICLs can be extremely toxic especially in dividing cells, stalling DNA replication and leading to cell death. As such, ICL-forming antitumor drugs including melphalan and cisplatin are among the most widely-used chemotherapeutic agents. The first clinical application of an ICL drug, nitrogen mustard, dated back to the 1940s (2).

Earlier understanding of ICL repair mechanism was obtained primarily from studies in model systems such as *Escherichia coli* and yeast (3). The availability of genetic mutants and well-defined biochemical assays made it possible to establish the first ICL repair pathway, which combines NER and homologous recombination. More recently, investigations in patients with Fanconi anemia (FA) revealed an important mechanism exclusive in higher eukaryotes (vertebrates). FA patient cells are hypersensitive to ICLs as demonstrated by reduced survival rates and elevated chromosomal abnormalities. Genomic instability of FA patients is closely correlated with cancer development. Understanding of the ICL repair mechanism and their roles in cancer development and treatment is extremely important for patients with ICL repair-related diseases and for ICL-based chemotherapeutics. In this review, we describe the basic concepts of ICL repair and their implications in cancer development and treatment.

Formation of interstrand crosslinks

Formation of DNA crosslinks relies on two independently reactive groups in a single alkylating molecule. When the two reactive groups react with two bases residing on opposing DNA strands, an ICL is formed. The covalently targeted sites on DNA are usually the N7 position of guanine or the exocyclic N2-amino group of guanine from nucleotide residues from the opposite strands (4).

ICL agents exist from naturally occurring as well

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as synthetic sources. Naturally occurring crosslinking agents include psoralens, mitomycin C, nitrous acids, etc. Mitomycin C was originally found in fungi with antibiotic activity mediated by its DNA crosslinking ability since bacteria are easily killed by a single unrepaired ICL (5). Psoralens are natural compounds derived from plants. Psoralens target specifically the TA sequences and react with the opposing thymines to form ICLs upon photo activation by UV radiation. Because of this unique feature, psoralens are effective ectopical treatment of psoriasis (6). Certain metabolites of alcohol, cigarette, and high fat diet, such as acetaldehyde and malondialdehyde, also act as DNA interstrand as well as DNA-protein crosslinkers. Recent studies demonstrated that ICL repair-deficient mice are sensitive to aldehyde (7-9), indicating that these endogenous crosslinking agents present an internal risk of genomic instability. Another endogenous interstrand crosslinking agent is nitrous acid, which is formed in the stomach during consumption of nitrite-containing food additives.

Synthetic ICL agents consist of a broad array of bi-functional alkylators such as nitrogen mustard, carmustine, platinum compounds, and diepoxybutane. Nitrogen mustard gas was used as a chemical weapon during World War I. The observation that white blood cell counts decreased drastically from nitrogen mustard exposure led to the exploratory application of this compound in cancer therapy. Nitrogen mustard was used as a chemotherapeutic agent for lymphoma and leukemia for a period of time (10,11). Since then, many more ICL agents have been used for cancer treatment, including derivatives of nitrogen mustard such as melphalan and cyclophosphamide, and platinum-containing drugs such as cisplatin and caboplatin.

As described above, human bodies are subjected to endogenous and therapeutic ICL exposure. As an exceedingly genotoxic and cytotoxic DNA lesion, one unrepaired DNA ICL could yield lethality in monocellular organisms whereas 20 to 40 unrepaired ICLs are fatal to mammalian cells (5,12). As a result, ICL repair mechanisms are essential in maintaining genomic integrity and cell viability.

ICL repair mechanisms

ICL repair mechanism is highly conserved in most unicellular organisms. Studies in *Escherichia coli* in the early 1970s demonstrated a recombination-dependent and error-free ICL repair pathway. In this model (*Figure 1*), NER factors initiate a strand-unhooking step by introducing dual incisions flanking the ICL lesion. Homologous recombination then fills the resulting gap by invading an undamaged chromosome. A subsequent round of NER reaction removes the remaining lesion and results in error-free repair of ICLs (13,14). This model, also called the Cole's model (13), is supported by both genetic and biochemical evidence (15-18). A similar repair mechanism also operates in yeast (19,20). A minor ICL response pathway was shown to be recombination-independent since the recombinase RecA is not required. It was shown that polymerase Pol β is involved in a lesion bypass process leading to error-prone ICL removal (21,22).

In higher eukaryote and particularly in vertebrate cells, a complex ICL processing mechanism has evolved to facilitate ICL damage response. The importance of this pathway is reflected by the hypersensitivity of FA patient cells to crosslinking agents. Essentially, two types of mechanisms for ICL removal have been observed in eukaryotes: recombination-dependent and recombination-independent.

The recombination-dependent ICL repair pathway, alternatively termed as replication-dependent ICL repair, functions during late S or G2 phases of the cell cycle, where the ICL-damage sites are adjacent to an undamaged sister chromatid. Initiation of this repair mechanism depends on stalling of the replication fork, and the lesion removal process includes translesion synthesis, homologous recombination, and NER. In a model (*Figure 2*) based on studies from *Xenopus laevis* egg extracts (23), two replication forks converge at the ICL site and are both blocked. Unhooking of ICL takes place when structure-specific nucleases such as XPF/ERCC1 make dual incisions flanking the crosslinked site on the same DNA strand. This step releases the covalent linkage between the complementary strands. The resulting gap is filled by translesion polymerases to bypass the remaining lesion and join the downstream Okazaki fragment. The restored duplex DNA, with the crosslinked oligonucleotides attached to one strand, is further repaired by NER factors. This fully repaired sister chromatid is subsequently utilized to remove the double strand break on the sister chromatid via a classical homologous recombination pathway.

The FA pathway is involved in ICL repair from the initial recognition of stalled forks to the final step of homologous recombination (24). Two important steps seem to require the FA mechanism in the recombinational ICL repair. First, an active FA pathway is crucial for the recruitment of incision factors, as two of the potential incision factors, SLX4 and FAN1, depend on monoubiquitinated FANCD2 for their loading to ICL site via ubiquitin-binding domains (25,26). Secondly, FA pathway may tether the secondary NER incision with the ensuing homologous recombination

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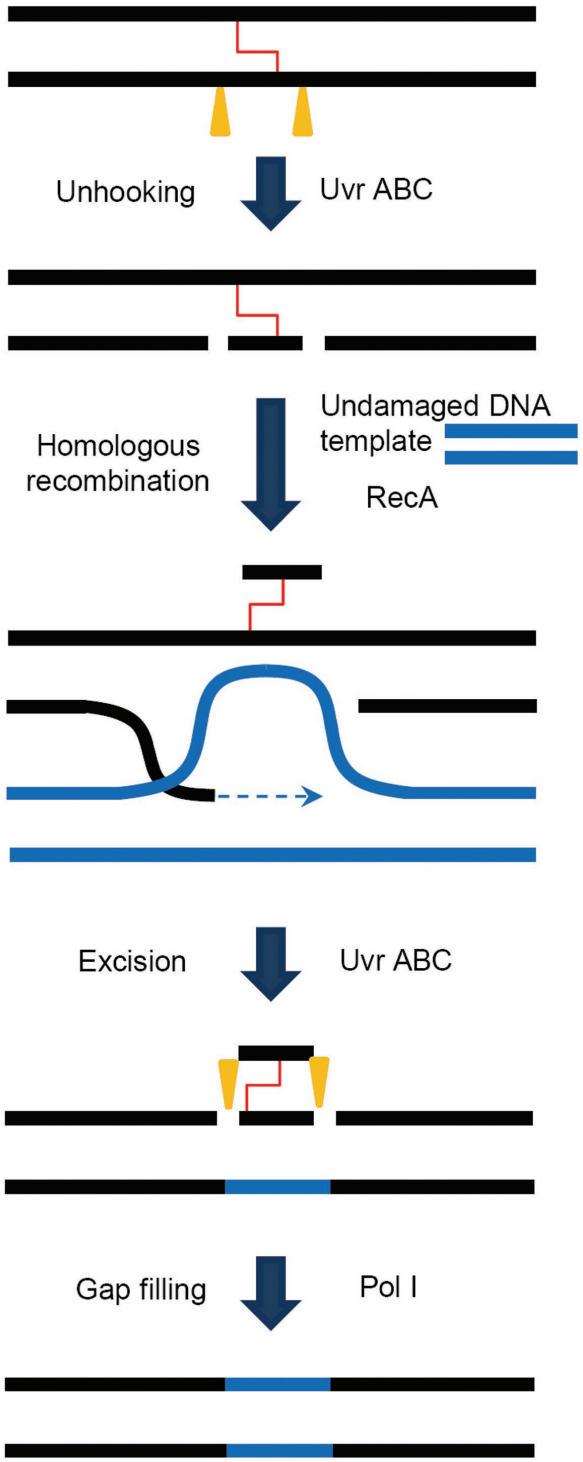

Figure 1 Interstrand crosslink repair pathway in prokaryotes. This pathway includes nucleotide excision repair and homologous recombination, which is error free (indicated as blue DNA patches). Note, this model is adapted from the one proposed by Cole. *et al.* in 1973 (13)

to restore the disconnected sister chromatid (27).

The recombination-independent ICL repair mechanism, also called mutagenic ICL repair, mainly occurs in cells during the G1 phase (*Figure 3*). Initiation of recombination-independent ICL repair depends on the native damage recognition proteins such as XPC and utilizes the NER dual incision to achieve the unhooking step (28,29). NER nucleases, XPF/ERCC1 and XPG provide the 5' and 3' incision, respectively. Repair synthesis of the resulting gap is aided by lesion bypass DNA polymerase ζ (30). The involvement of lesion-bypass polymerases in the recombination-independent ICL repair dictates the mutagenic nature of this pathway, which also underlines why cancer patients under ICL agent treatment are prone to chemotherapy-induced secondary malignancies (31,32).

Factors in ICL repair pathways

As described in the previous model (*Figure 2*), proteins involved in ICL repair include but are not limited to the 15 known FA genes (A, B, C, D1, D2, E, F, G, I, J, L, M, N, O, and P) (33-35). Essential recombination factors such as RAD51, structure-specific endonucleases such as XPF/ERCC1 and MUS81/EME1, Holliday junction processing factors, and translesion DNA polymerases are also parts of the orchestrated process during ICL repair.

FA is a rare human genetic disease characterized by pancytopenia, a broad spectrum of developmental abnormalities, and a high risk of cancer (36). Each FA subtype is associated with a distinct gene encoding a corresponding FA protein. Cells derived from FA patients exhibit high levels of chromosomal breakage and formation of radial chromosomes (37,38), indicating that ICL repair-deficient cells have high levels of genomic instability. In the classical FA pathway, FA core complex (consisting of A, G, FAAP20, C, E, F, B, L, and FAAP100) has E3 ubiquitin ligase activity with the catalytic function attributed to the RING domain-containing FANCL protein. The major function of the core complex is to execute DNA damage-induced monoubiquitination of the FANCI/D2 complex (39). The activated FANCI/D2 complex is suggested to recruit downstream effectors, including nucleases, translesion polymerases, and homologous recombination factors to repair ICLs (25,26,40). The exact role of FANCD2 monoubiquitination remains unclear.

FANCM is a DEAH domain helicase with ATP-dependent DNA translocase activity (41,42). FANCM forms a complex with FAAP24 and with the MHF1/MHF2 histone-fold complex (43,44). FANCM is important but not essential

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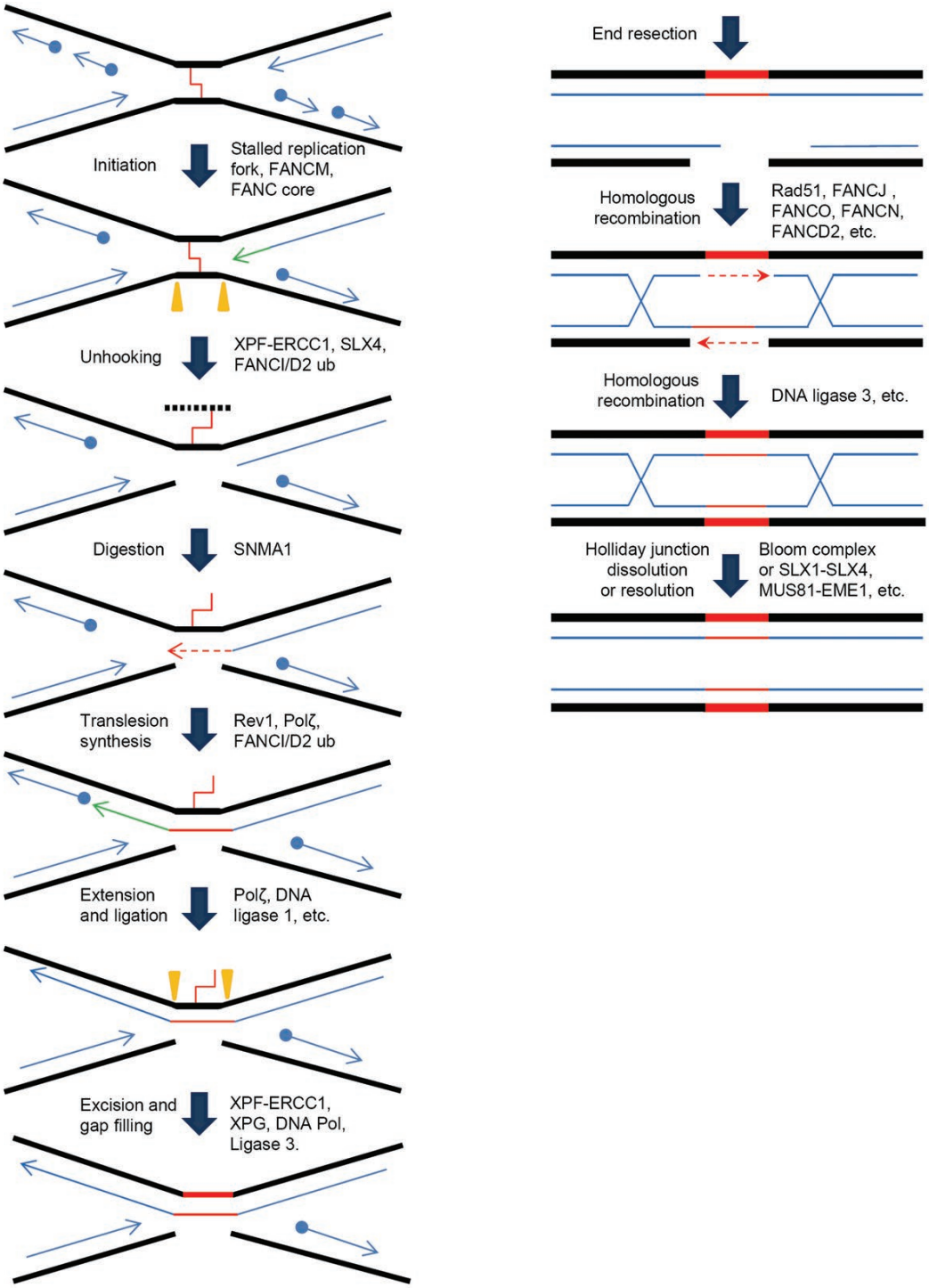

Figure 2 Recombination-dependent interstrand crosslink repair pathway in vertebrates. This pathway includes initiation, unhooking, SNMA1 digestion, translesion synthesis, nucleotide excision, gap filling, and homologous recombination. Note, this model is adapted from the one proposed by Raschle, M. *et al*. in 2008 (23). In their study, however, SNM1A digestion is not required. Instead, as the green-arrowed strands indicated, the new strand is extended to one base ahead of the lesion site before excision occurs. After unhooking, Rev1 inserts a cytosine into the position across the lesion on the complementary strand. Then Pol ζ, the key translesion polymerase, processes the DNA synthesis beyond the lesion site

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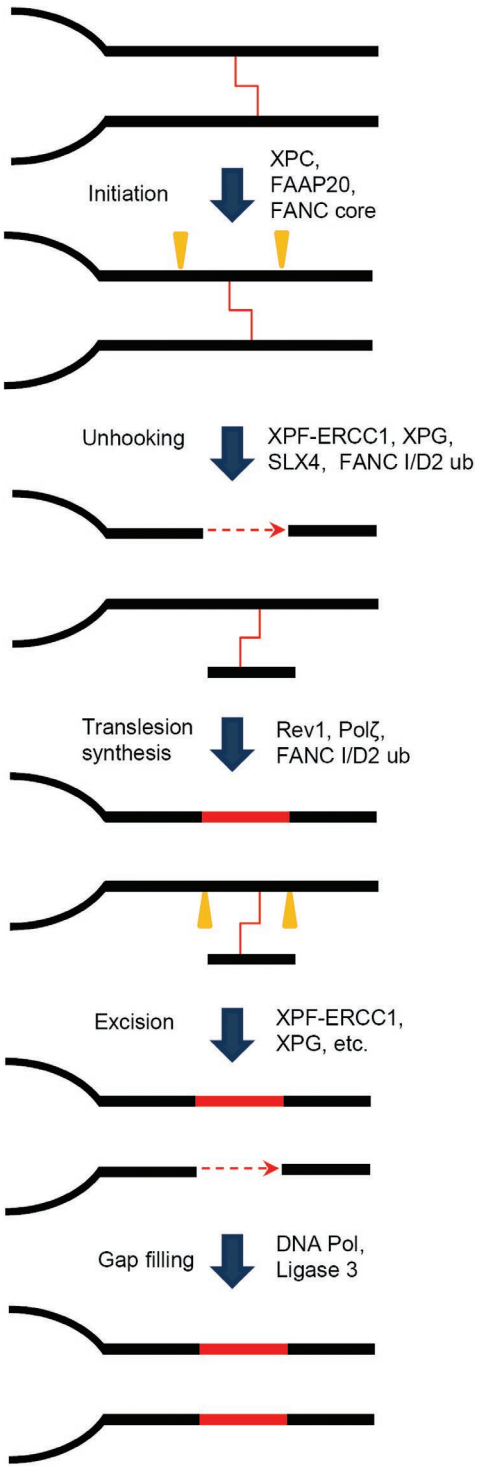

Figure 3 Recombination-independent interstrand crosslink repair pathway in vertebrates. This pathway includes initiation, unhooking, translesion synthesis, nucleotide excision, and gap filling. Note, this model is adapted from the one proposed by Zheng H. *et al.* in 2003 (28)

in the activation of the FA pathway (45). Biochemical studies suggest that the FANCM/FAAP24 complex stabilizes and remodels stalled DNA replication forks (44,46,47). FAAP24 complex is found to play a role in ATR-mediated checkpoint activation (48-50), whereas FANCM is shown to be involved in recombination-independent ICL repair by promoting PCNA ubiquitination thus facilitating the recruitment of NER incision factors to the ICL sites (45).

The FA gene group consisting of FANCD1 (BRCA2), FANCN, FANCJ, and FANCO are also previously established recombination factors that connect FA with breast/ovarian cancer susceptibility. Mutations of both alleles of these genes lead to a corresponding subset of FA, whereas mutation of one allele causes breast cancer predisposition. The recombination factors are likely to act downstream of ICL processing, especially when DNA double strand break forms (51,52). FANCO, also named RAD51C, is a paralogs of RAD51 (35,53). RAD51C forms complexes with RAD51B, RAD51D, XRCC2, and XRCC3 (54,55). One of the major roles of these paralogs is the recruitment and regulation of the recombinase RAD51 onto single-stranded DNA (54). Cells deficient in any of the RAD51 paralogs are sensitive to ICLs and double strand breaks because of the resulting deficit in homologous recombination (56).

Three heterodimeric structure-specific endonucleases involved in ICL repair are XPF/ERCC1, SLX1/SLX4, and MUS81/EME1. SLX4 is found to be mutated in the FANCP complementation group (34,57,58). SLX4 and SLX1 form a heterodimeric nuclease that functions as a Holliday junction resolvase in ICL repair (59-62). During ICL repair, SLX4 also serves as a scaffold protein to assemble a multi-activity nuclease complex involving XPF/ERCC1 and MUS81/EME1. XPF/ERCC1 functions in both NER and ICL repair (63,64). The NER activity of XPF/ERCC1 is SLX4 independent, which was supported by studies demonstrating that FANCP patient cells were not sensitive to UV radiation (57,65). A recent study also demonstrated that SLX4-dependent XPF/ERCC1 activity in replication-dependent ICL repair is to complete the unhooking during ICL repair (65). The unhooked oligonucleotides might be digested by another nuclease, SNM1A (66), which provide a promising alternative to the more difficult lesion bypass synthesis step.

In addition to the heterodimeric nucleases, the newly discovered nuclease FAN1 plays a moderate role in ICL repair. FAN1 is recruited to the ICL sites by the ubiquitinated FANCD2 via an ubiquitin-binding zinc finger domain in FAN1 (25). The other important domain of FAN1, the virus-type replication-repair nuclease domain,

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displays 5'-3' exonuclease activity and structure-specific 5'-flap endonuclease activity (25,67-69). These activities enable FAN1 to excise the exposed DNA ends as well as the stalled DNA replication structures.

Translesion DNA polymerases are important components of ICL repair. Normal replicative DNA polymerases are usually blocked ahead of the ICL site. Studies in *Xenopus laevis* egg extracts demonstrated that the translesion polymerases including Y-family polymerase Rev1 and the B-family polymerase Pol ζ (Rev3/Rev7) have essential roles in the complete removal of ICLs. In this models, the ICL repair intermediate utilizes replisome remodeling machinery to extend the stalled DNA strand to one base ahead of the ICL site (23). Upon unhooking, the deoxycytidyl transferase of Rev1 inserts a cytosine into the position across the ICL lesion on the complementary strand (70,71), followed by Pol ζ to extend the unpaired strand.

Interstrand crosslinking damage and development of cancer

Unrepaired or misrepaired DNA ICLs are major sources of genomic instability. ICL-inducing agents are known to be potent carcinogens. Nitrogen mustard gas exposure, aside from acute impacts, is known to cause high incidence of cancer especially leukemia (72). The onset of acute myeloid leukemia is much higher in cancer patients treated with ICL agents than those without (31,32). Clinical observations clearly indicate that administration of ICL drug has accumulative effect and the leukemogenicity of these agents is dose-dependent. However, the risk of developing leukemia is justifiable considering the benefit of chemotherapy for advanced primary malignancies.

Studies in animal models in recent years suggest that acetaldehyde, an endogenous metabolic product from alcohol, was carcinogenic (73). For example, rats exposed to acetaldehyde vapor had increased risk of squamous cell carcinoma in the respiratory epithelium, including nasal and laryngeal carcinomas. Epidemiological studies also support this notion as alcohol consumption has been linked to increased tumor incidents (74,75). More recently, mice defective in both FA pathways and Aldehyde Dehydrogenase 2 are found to be embryonic lethal (8,9). The types of DNA damage, as a result of accumulating aldehyde, may be both DNA crosslinks and DNA-protein crosslinks.

Mutation of ICL repair genes resulting failure in ICL repair, mimics ICL agent exposure. FA patients in particular exhibit high risk of hematopoietic malignancies, including myelodysplastic syndrome and acute myeloid leukemia, which account for 52% of tumors in FA patients by the age of 40 (76). The risk of all cancers, solid tumors, and acute myeloid leukemia is 50-fold, 48-fold, and 800 fold higher, respectively, in FA patients than in the general population (77). Of the solid tumors diagnosed in FA patients, head and neck squamous cell carcinoma is the most common (700-fold increase in risk) followed by esophageal cancer and gynecological cancers. The FA patients with FANCN or FANCD1 mutations usually exhibit early onset of cancer during childhood, contributing to the early mortality (78-81). FA patients defective in the homologous recombination process, (FANCD1, FANCN, and FANCJ) are more prone to breast cancer (82-84), whereas FANCO mutation carriers are more susceptible to ovarian cancer (53,85). The varying spectrum of cancer susceptibility and time of tumor onsets from different groups of FA patients underline distinct functions of FA genes during ICL removal.

Two potential mechanisms may also account for ICL-related cancers in FA patients. First, unrepaired DNA ICLs is a strong apoptosis inducer in hematopoietic cells (86), the resulting selection pressure and an increased requirement of clonogenicity for the remaining hematopoietic stem cells proved to be a key factor in the heightened risk of hematopoietic dysplasia and cancer in FA patients (87). Second, FA patients are known to have an increased risk to human papilloma virus or other oncogenic viral infection, which increases the risk of head and neck squamous cell carcinoma and other solid tumors (88). Current model suggests that the typical extension of G2 phase in FA cells (from unrepaired ICLs) increases the susceptibility to human papilloma virus infection. About 85% of head and neck squamous cell carcinoma in FA patients are positive for human papilloma virus (89).

ICL and cancer therapy

Because DNA ICLs are profoundly cytotoxic and are especially effective in killing dividing cells, ICL-inducing agents are widely used for cancer treatment, especially for solid tumors. These agents include but are not limited to nitrogen mustards, platinums, mitomycin C, and psoralens.

Clinical use of nitrogen mustards dates back about 70 years. Two of these agents, cyclophosphamide and melphalan, are still administrated as front line treatments for many forms of leukemia and myeloma. Cyclophosphamide, also known as cytophosphane with trade names including Cytoxan, Endoxan, Neosar, Procytox, and Revimmune, is routinely used in treatment of lymphoma and some types of leukemia (90)

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and in phase 3 clinical trials for treatment of node-positive breast cancer (91). The adverse effects of cyclophosphamide administrated at high doses include neutropenia and acute myeloid leukemia, which is the major limiting factor (92). The original clinical use of melphalan, also known as L-Phenylalanine Mustard and the trade name Alkeran, was melanoma treatment. Later, it proved to be more effective in treating myeloma (93,94). Currently, melphalan is a standard regimen for multiple myeloma (95). It is occasionally used in ovarian cancer and melanoma, though. The primary side effect of this agent is bone marrow suppression.

Other nitrogen mustard derivatives used in chemotherapy include chlorambucil (Leukeran) and ifosfamide (Ifex). Chlorambucil is primarily used for chronic lymphocytic leukemia, but replaced with Fludarabine in pediatric patients for the management of neural and bone marrow toxicity (96). Ifosfamide is used for various cancers, including testicular, lung, and breast cancer with similar side effects. Interestingly, the ifosfamide metabolite chloroacetaldehyde has chemical properties similar to those of acetaldehyde and chloral hydrate, which may explain its encephalopathy complications (97).

Platinum compounds are another class of DNA ICL-inducing agent. Cisplatin is widely used in the treatment of various solid tumors including lung cancer, ovarian cancer, lymphoma, and testicular cancer. The cure rate for testicular cancer increased from 10% to 81% with the use of adjuvant therapy with cisplatin (98). The main side effects of treatment with cisplatin are nephrotoxicity, neurotoxicity, and bone marrow suppression. Carboplatin is a second-generation platinum drug with less severe side effects in the kidney. Platinum agents developed more recently include oxaliplatin, satraplatin, picoplatin, nedaplatin, and triplatin.

In addition to the nitrogen mustard- and platinum-based drugs, mitomycin C and psoralens are also common ICL-based anticancer drugs. Mitomycin C is often used to treat esophageal, breast, and bladder cancer. The main toxic effect of intravenous mitomycin C is bone marrow suppression. Psoralens have an advantage over other ICL-based chemotherapy in the treatment of skin cancer in that psoralen-mediated ICLs are formed upon UV photo activation. Psoralens are given topically to treat cutaneous T-cell lymphoma within a defined surface area. The most common side effect of treatment with psoralens is dermatitis, which has appeared in long-term follow-up studies (99). Furthermore, psoralen exposure-induced secondary cancer is also reported from psoriasis treatment, presumably from mutations arisen from mutagenic ICL repair (100,101).

Cancers bearing BRCA2 mutations, such as breast and ovarian cancer, respond better to ICL-based chemotherapy than do cancers without these mutations. The main reason is that cancer cells with deficient homologous recombination mechanism are deficient in the later stage of ICL repair and thus sensitive to this chemotherapy. Therefore, genetic mutations affecting ICL repair actually provide tumor selectivity for ICL-based therapy. For example, platinum-based chemotherapy has improved survival rates in patients with ovarian cancer carrying BRCA2 mutations over those in patients with sporadic ovarian cancer (102,103). However, solid tumors in FA patients are mostly treated with radiation and surgery rather than ICL agents, because somatic cells in FA patients are overly sensitive to ICL agents, even much reduced ICL exposure can be fatal.

Given the effectiveness of ICL-based chemotherapy, future translational research effort may be applied in two directions. First, improvement of toxicity profile will extend the clinical benefit of ICL drugs. Reduced or reversible side effects will allow patients to tolerate prolonged treatment (104). Second, sensitization of cancer cells to ICL drugs could be another strategy to achieve additional tumor control. As the mechanisms of ICL repair emerge, key repair factors can be legitimate targets for sensitization. Although ICL-based chemotherapeutic drugs have been used since 70 years ago, much potential exists for future development and refinement of this classical regimen.

Conclusions

DNA ICLs is a complex and severely genotoxic lesion. Cellular mechanisms dealing with ICLs have proven to be complicated and await further investigation. On the one hand, failure in the proper repair of ICLs is likely a significant source of genomic instability and hence cancer development. The manifestations of FA, especially its cancer risk, have fully demonstrated this notion. On the other hand, the profound cytotoxicity of ICL-inducing agents yielded a major class of cancer chemotherapeutic drugs. This class of bifunctional alkylating drugs has continuously evolved with increasing variety and efficacy. Future studies of cellular mechanism of ICL response are expected to advance cancer etiology as well as therapy.

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