Multiple Repair Pathways Mediate Tolerance to Chemotherapeutic Cross-linking Agents in Vertebrate Cells

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Abstract

Cross-linking agents that induce DNA interstrand cross-links (ICL) are widely used in anticancer chemotherapy. Yeast genetic studies show that nucleotide excision repair (NER), Rad6/Rad18-dependent postreplication repair, homologous recombination, and cell cycle checkpoint pathway are involved in ICL repair. To study the contribution of DNA damage response pathways in tolerance to cross-linking agents in vertebrates, we made a panel of gene-disrupted clones from chicken DT40 cells, each defective in a particular DNA repair or checkpoint pathway, and measured the sensitivities to cross-linking agents, including *cis*-diammine-dichloroplatinum (II) (cisplatin), mitomycin C, and melphalan. We found that cells harboring defects in translesion DNA synthesis (TLS), Fanconi anemia complementation groups (FANC), or homologous recombination displayed marked hypersensitivity to all the cross-linking agents, whereas NER seemed to play only a minor role. This effect of replication-dependent repair pathways is distinctively different from the situation in yeast, where NER seems to play a major role in dealing with ICL. Cells deficient in Rev3, the catalytic subunit of TLS polymerase Polζ, showed the highest sensitivity to cisplatin followed by *fanc-c*. Furthermore, epistasis analysis revealed that these two mutants work in the same pathway. Our genetic comprehensive study reveals a critical role for DNA repair pathways that release DNA replication block at ICLs in cellular tolerance to cross-linking agents and could be directly exploited in designing an effective chemotherapy. (Cancer Res 2005; 65(24): 11704-11)

Introduction

cis-Diamminedichloroplatinum (II) (cisplatin) can form intrastrand cross-links, interstrand cross-links (ICL), DNA-protein cross-links, and monoadducts with DNA (reviewed in ref. 1). Among these lesions, ICLs are believed to be the main determinant of the toxicity of cross-linking agents presumably due to the difficulty of eliminating ICLs. However, intrastrand cross-links also seem to contribute to cisplatin toxicity (2). The toxic effects of ICLs as well as other types of cross-links are thought to be a result of blocked transcription and DNA replication (reviewed in refs. 3, 4). In particular, replication fork blocks in cycling cells might be crucial for cytotoxicity of ICLs because of the resulting single-strand breaks (SSB) and double-strand breaks (DSB). A single unrepaired DSB can stimulate the DNA damage checkpoint and trigger apoptosis in chicken B-cell line DT40 as well as in mammalian cells.

Comprehensive study of yeast mutants reveals that nucleotide excision repair (NER), homologous recombination, and postreplication repair (PRR) play a major role in repairing DNA damage caused by cross-linking agents (5–7). Cisplatin adducts are eliminated by the NER pathway, which uses Xpc/Hr23B and Xpa to detect the lesions. These proteins recruit two endonucleases, Ercc1-Xpf and Xpg, leading to incision at the 5′ and 3′ sides of a DNA lesion and to removal of the lesion. PRR helps restarting replication stalled at lesions that are not repaired before the S phase of cell cycle. This pathway includes two major branches: translesion DNA synthesis (TLS) and homologous recombination (8, 9). TLS can fill the strand gap by employing the Rad6/Rad18 complex, an ubiquitin-conjugating enzyme, and specialized TLS polymerases, such as Polη, Polκ, and Polζ (8). Although Rad18 is required for the function of each TLS polymerase in yeast, this may not the case in vertebrate cells, as Rad18-deficient DT40 cells have a less severe phenotype than do Polζ-deficient DT40 cells (9, 10). Homologous recombination can fill the strand gap by using the other intact sister DNA as a template (reviewed in refs. 11, 12) or could be required to repair replication-induced DSB, and nonhomologous end-joining (NHEJ) might also be employed to religate DNA strands that are broken as a result of replication fork stalling. Finally, RecQ helicases, such as yeast Sgs1 and mammalian Bloom helicase (BLM), also help stabilizing stalled forks by suppressing "promiscuous" recombination (13).

Although the primary structure of genes involved in DNA repair and checkpoint pathways are well conserved in the eukaryotes, the relative role for each pathway is distinctly different between yeast and mammalian cells. A good example is the differential contribution of homologous recombination and NHEJ to ionizing radiation (IR)–induced DSB repair, with homologous recombination

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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being the dominant pathway in yeast and with NHEJ being of major importance in mammalian cells (14). In addition, the vertebrate repair machinery seems to be more complex than its yeast counterpart. There are several homologous recombination genes that are present in vertebrates but not in the budding yeast. Such genes include *XRCC2*, *XRCC3*, and tumor suppressor genes for familial breast and ovarian cancer, *BRCA1* and *BRCA2*. Furthermore, Fanconi anemia (FA) complementation group (FANC) proteins and poly(ADP-ribose) polymerases (PARP) are also present only in higher eukaryotes. The mutations in *FANC* genes are responsible for the highly cancer-prone genetic disease FA, and the relevant mutants show hypersensitivity specifically to cross-linking agents (reviewed in ref. 15). There seems to be a cross-talk between FA and BRCA2 molecules, as some of the FA patients are found to carry mutations in the *BRCA2* gene (16). Parp-1 is rapidly activated by SSB and DSB and can modify various components of DNA replication and repair machinery by poly(ADP-ribosyl)ation (17). The role for damage checkpoints in cellular survival following DNA damage also seems to be different between yeast and vertebrate cells. In multicellular organisms, the damage checkpoint not only transiently arrests the cell cycle, as it is the case in yeast, but also triggers apoptosis. Thus, vertebrate damage checkpoints have both positive and negative effects on cellular survival. Moreover, recent studies suggest that mammalian and chicken damage checkpoint proteins can directly facilitate DNA repair, including stimulation of Artemis-dependent DSB repair by ATM (18) and the phosphorylation of Rad51 by Chk1 (19) as well as the contribution of Nbs1 to homologous recombination (20).

The ultimate goal of chemotherapy is to selectively eliminate cancer cells. To predict the effectiveness of each chemotherapeutic treatment, one needs to understand the correlation between vulnerability to chemotherapeutic agents and activity of each cellular DNA damage response pathway. To this end, pioneering studies in yeast might be a starting point but are surely not sufficient to allow a systematic assessment of the effects of chemotherapeutic agents on mutants in different DNA damage response pathways that are relevant to humans. Such a genetic approach using a vertebrate cell line has been difficult to perform because of the lack of a good cellular model system for reverse genetic studies. Due to its high gene targeting efficiency and its stable karyotype, the chicken B lymphocyte line DT40 provides such a unique collection of isogenic mutant clones of DNA repair and damage checkpoint pathways (12). Moreover, because DT40 cells do not carry functional p53 and proliferate an extremely high rate, DT40 and malignant cancer cells may share comparable characteristics in cellular responses to cross-linking agents. Here, we describe for the first time a systematic analysis of the effects of DNA cross-linking agents on this panel of vertebrate mutant cell lines. Our study reveals an important role for Polζ presumably in both TLS- and Fanc-dependent cross-link repair to render the cells resistant to cisplatin.

Materials and Methods

Cell lines and cell culture. All mutants were generated from DT40 cells. The following mutants were kindly provided: *NBS1* gene-disrupted DT40 mutant cells (*nbs1*; Dr. Tauchi, Ibaraki University, Ibaraki, Japan, and Dr. Komatsu, Kyoto University, Kyoto, Japan), *fen1* and *ligase IV* (Dr. Koyama, Yokohama City University, Yokohama, Japan), *blm* and *wrn* (Dr. Matsumoto, AGENE Research Institute, Kanagawa, Japan), *blm/ku70* and *blm/rad54* Drs. Seki and Enomoto (Tohoku University, Tohoku, Japan), and *atm* by Dr. Yamamoto (Kanazawa University, Kanazawa, Japan). Disruption of the gene in each mutant was confirmed by the disappearance of either target mRNA or protein (references in Table 1 and data not shown). *rev3/fanc-c* double mutants were generated by introducing a *fanc-c* knockout construct containing a puromycin-resistant cassette (21) into *rev3* mutant (10). Note that FANC-C is encoded in a sex chromosome. Cells were cultured as described previously (10).

Colony formation assay. Cells (1×10^5) were incubated for 1 hour with complete medium containing cisplatin (Nihon-Kayaku, Tokyo, Japan), mitomycin C (Mitomycin Kyowa S, Kyowa Hakko, Tokyo, Japan), melphalan (Alkeran, GlaxoSmithKline, Tokyo, Japan), *trans*-diamminedichloroplatinum (II) (transplatin; Sigma, St. Louis, MO), methylmethan sulfonate (MMS; Nakalai, Kyoto, Japan), or vincristine (Oncovin, Nihon-Kayaku). Serially diluted cells were plated in triplicate onto 1.5% (w/v) methylcellulose semisolid medium as described previously (22).

Measurement of cisplatin sensitivity in liquid culture. Cells (5×10^6) were incubated with complete medium containing cisplatin for 1 hour. The cells were washed and incubated for ~10 days in cisplatin-free complete medium. Using flow cytometry, the number of cells was monitored (22) daily up to 10 days, when a population of the cells started to grow exponentially. This exponential growth curve was extrapolated to a zero time by a numerical fit to calculate the percentage of the cells that reacquired proliferation capability after the exposure to cisplatin. Alternatively, cells were continuously exposed to various concentrations of cisplatin for 2 days, and the number of cells was measured at 48 hours. The sensitivity was calculated by dividing the numbers of cells treated with cisplatin by those of untreated cells.

Karyotype analysis. For karyotype analysis, cells were treated with 5 μmol/L cisplatin for 1 hour, washed twice with PBS, and incubated with complete medium containing 0.1 μg/mL colcemid for another 3 hours. Cells were then fixed and metaphase spreads were prepared as described previously (23).

Results

Experimental design. To study the contribution of each DNA damage response pathway to the repair of cross-linking agents in vertebrate cells, we have made a panel of isogenic DT40 mutants, each defective in a particular DNA repair or checkpoint pathway (Table 1). When gene-disrupted clones from DT40 and murine embryonic stem cells were studied previously, their sensitivity to cross-linking agents was not necessarily evaluated under the same experimental condition. In this study, to understand the relative contribution of each repair pathway, we examined cellular response to cross-linking agents using colony survival assay after 1-hour exposure. The following three cross-linking agents were studied, cisplatin (Fig. 1), melphalan, a derivative of nitrogen mustard (Fig. 2*A*), and mitomycin C, a natural antitumor antibiotic (Fig. 2*B*) and transplatin (Fig. 2*C*). The sensitivity of each mutant was assessed by D_1 values (i.e., the dose that reduces the cell survival to 1%). Furthermore, to accurately compare the sensitivity of each clone, we normalized the sensitivities of mutant cells according to the D_1 values of their parental wild-type DT40 clone, because three parental clones used in different laboratories showed up to ±18% variation in the D_1 value. To examine the cause of cell death following cisplatin treatment, we scored chromosomal aberrations following 1-hour exposure of representative repair-deficient mutants to cisplatin.

Sensitivity profiles of repair and damage checkpoint-defective mutants to cross-linking agents. Figure 1 shows the sensitivity profile of all analyzed mutants to cisplatin. Cells deficient in *rev3, fanc-c*, and *rad18* displayed the highest sensitivity in this order. The *xrcc2, xrcc3, brca2*^{truncated} (involved in homologous recombination), *brca1, atm* (checkpoint), *snm1a, snm1b*

Table 1. Repair and checkpoint genes mutated in the analyzed DT40 clones

Gene	Function	Reference
RAD52	Homologous recombination	43
RAD54	Homologous recombination	44
XRCC2	Homologous recombination, promotion of Rad51 assembly	25
XRCC3	Homologous recombination, promotion of Rad51 assembly, resolution of the Holliday junction	25
BRCA1	Homologous recombination, damage checkpoint, transcription-coupled BER, regulation of transcription	*
BRCA2	Homologous recombination, promotion of Rad51 assembly. Note that BRC3 *brca2*^{truncated} mutant cells were analyzed.	45
NBS1	Homologous recombination, damage checkpoint, stabilization of Mre11/Rad50 complex. Note that the analyzed cells may express a small amount of NH_2-terminal truncated NBS1.	20
ATM	Damage checkpoint control	42
KU70	Initial step of NHEJ DSB repair	22
LIGASE IV	Last step of NHEJ DSB repair	39
SNM1a	Exonuclease, cross-link repair †	36
SNM1b	Cross-link repair †	36
ARTEMIS	Exonuclease, hairpin opening at DSBs	36
RAD18	Regulation of TLS, ubiquitin E3 ligase	9
RAD30	TLS	46
POLκ	TLS	23
POLβ	BER	‡
REV3	TLS, homologous recombination (the catalytic subunit of polζ)	10
POLQ	TLS, BER	§
XPA	NER	23
XPG	NER, transcription-coupled BER	47
XPF	NER, cross-link repair †	‖
MSH3	Mismatch repair	¶
BLM	RecQ helicase responsible for Bloom syndrome	27, 48
WRN	RecQ helicase responsible for Werner syndrome	49
FANC-C	Homologous recombination, damage response to ICLs	21
FANC-G	Homologous recombination, damage response to ICLs	50
PARP-1	Poly(ADP-ribosyl)ation, repair of DNA SSB and DSB	**
FEN1	BER, processing of 5′ flap during DNA replication	51

*R. Martin et al., submitted for publication.
†A defect of the indicated genes causes hypersensitivity to cross-linking agents in either yeast or mammalian cells. However, the nature of cross-link repair is unclear in mammalian cells.
‡Tano et al., submitted for publication.
§M.Y. et al., submitted for publication.
‖K.K. et al., in preparation.
¶Unpublished data.
**H.H. et al., submitted for publication.

(ICL repair), *rad30* (TLS), *xpa, xpg* (NER), *blm, wrn* (RecQ helicase), *msh3* (mismatch repair), and *parp-1* [base excision repair (BER), and SSB repair] mutants also showed elevated sensitivity to cisplatin. To confirm that the sensitivity to killing by cisplatin is caused by DNA cross-linking, we exposed representative cisplatin-sensitive mutants to other cross-linking agents, melphalan and mitomycin C. The sensitivity profiles to the three different cross-linking agents were very similar (Fig. 2*A* and *B*), including the order of sensitivity among *rev3, fanc-c*, and *rad18* cells. These observations revealed the critical roles of TLS and FANC pathway in processing DNA damages induced by a variety of cross-linking agents.

To further study the role for TLS in cellular tolerance to cisplatin, some hypersensitive mutants were exposed to transplatin. Transplatin has a much lower cytotoxicity than cisplatin presumably due to a slow conversion rate of transplatin monoadducts into ICLs [at least 10 times slower ($t_{1/2}$ > 17 hours) than that of cisplatin] and due to lack of the toxic d(GpG) intrastrand cross-links (24). We treated cells with transplatin only for 1 hour, so that most lesions should be monofunctional adducts. In agreement with the cisplatin sensitivity profile, *rev3* and *rad18* cells were more sensitive to killing by transplatin than any other cross-linker-sensitive mutants (Fig. 2*C*). To further support the notion that *rev3* and *rad18* play a critical role in bypassing monoadducts, we exposed the mutants to MMS, a monofunctional alkylating agent. *rev3* and *rad18* were sensitive to MMS (Fig. 2*D*), whereas *fanc-c* did not show a significant sensitivity (see Discussion). Thus, Rev3 and Rad18 may play an important role in the tolerance to platinum monoadducts in addition to tolerance to ICLs.

Besides a defect in DNA repair, the observed sensitivity of *rev3* and *rad18* mutants to cross-linking agents and MMS could also reflect compromised cell growth in these mutants (e.g., spontaneous cell death or low plating efficiency). However, *rad18* cells show normal growth property and 100% plating efficiency (9). To further verify that the sensitivity is due to DNA repair defects in the mutant cells, we measured the sensitivity of representative mutants to the anticancer agent vincristine, which kills cancer cells by disrupting microtubules but does not form covalent lesions on DNA. *rev3* and *rad18* were not hypersensitive to vincristine (Supplementary Data), suggesting that the sensitivity to cross-linking agents is indeed due to compromised DNA repair and does not reflect altered cell growth properties.

To further confirm if the cisplatin toxicity is mediated by DNA insults, we scored chromosome aberrations of representative mutants after cisplatin treatment. As expected, cells sensitive to cisplatin showed high levels of chromosomal aberrations, suggesting that unrepaired DNA damage is a main cause of cell death. *rev3* showed highest levels of aberrations followed by *fanc-c, xrcc2*, and *rad18* (Table 2). We found that TLS mutants exhibited predominantly chromatid-type breaks, where one of the sisters is broken. This observation is consistent with the role for TLS in releasing replication block in a sister chromatid. Interestingly, *xrcc2* exhibited higher percentage of chromosome-type breaks, where two sisters are broken at the same site. This might be the consequence of collapsed recombination structures, such as Holliday junctions.

Role for excision repair in cross-link repair. One of the major differences in cross-link repair between yeast and mammal is the extent of contribution by NER. All NER mutants in yeast show a relatively severe sensitivity to cross-linking agents. Thus, NER may be essential for initiation of ICL repair presumably by introducing

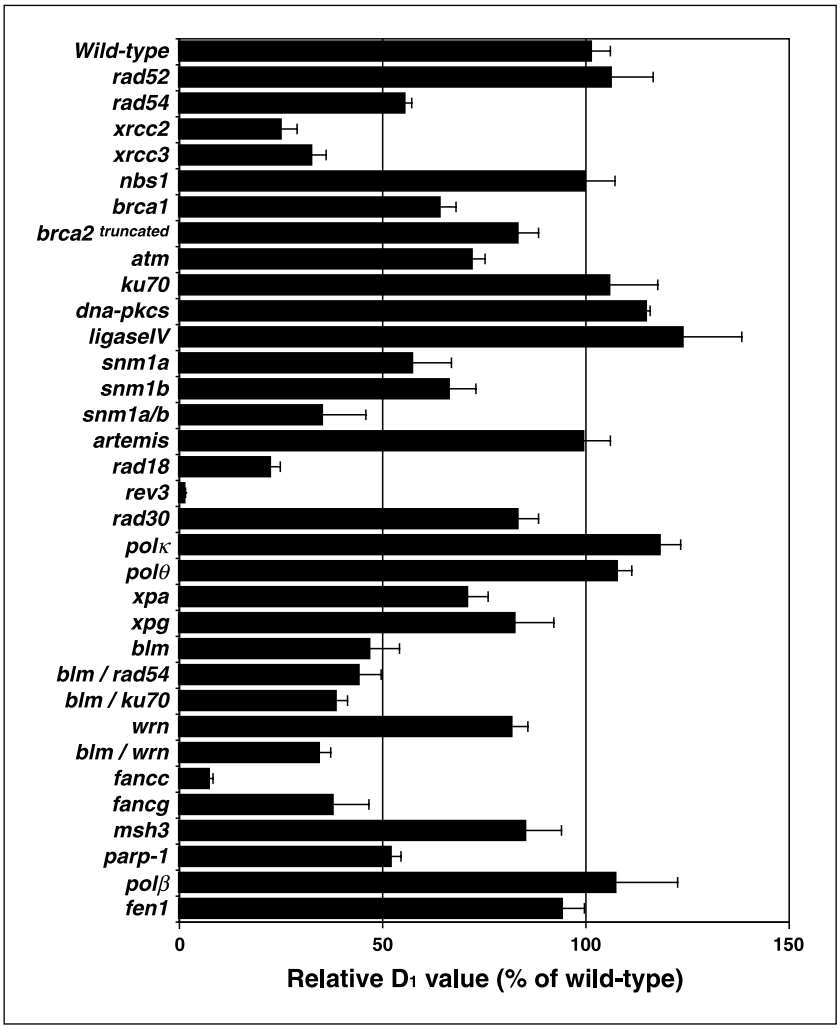

Figure 1. Toxicity profile of cisplatin on a panel of the repair-deficient DT40 mutants. Each D_1 value was calculated from three independent colony survival assays and normalized according to the D_1 value of parental wild-type cells. Note that *brca2*^{truncated} denotes cells that carry BRC3-truncated mutation of the *BRCA2* gene.

incisions in one strand and thereby unhooking ICLs. However, rodent Chinese hamster ovary (CHO) cell mutants defective in NER show only a mild sensitivity to cross-linking agents. In agreement with data from the CHO cell mutants, DT40 deficient in Xpa and Xpg exhibited only mild sensitivity to cisplatin (Fig. 1). On the other hand, we failed to detect any sensitivity of *xpa* to melphalan or mitomycin C (Fig. 2*A* and *B*). Thus, NER may have a minor role in cross-link repair in higher eukaryotic cells when compared with yeast. We were not able to evaluate the role for XPF, because *xpf*-null mutation seems to be lethal in DT40 cells.[6] We also assessed the involvement of BER by studying cells deficient in Polβ and Fen1. As expected, a defect in BER did not result in hypersensitivity to cisplatin. On the other hand, cells deficient in Parp-1, which is involved in a variety of DNA repair pathways, including BER, SSB, stabilization of replication forks, and perhaps DSB repair, showed high sensitivity to cisplatin as well as IR.[7] Taken together, these results suggest that the two excision repair pathways play a minor role, if any, in tolerance to cross-linking agents in chicken as well as in mammalian cells.

Role for homologous recombination in cross-link repair. According to yeast genetic studies, homologous recombination–mediated DSB repair plays a critical role in cross-link repair. Most of the DT40 mutants defective in homologous recombination showed a significant increase in sensitivities to cisplatin, melphalan, and mitomycin C (Figs. 1 and 2*A* and *B*). Interestingly, the analyzed homologous recombination mutants displayed marked variation in cellular response to cisplatin. Cells deficient in Rad51 paralogues (*xrcc2, xrcc3, rad51b, rad51c*, and *rad51d*; Fig. 1; refs. 25, 26) consistently showed prominent hypersensitivity; *brca1, brca2*^{truncated}, and *rad54* showed only mild sensitivity; and *nbs1* and *rad52* showed no increase (Fig. 1). In contrast, previous data indicate that Rad54 and Nbs1 have a critical role in DSB repair following X-rays. These findings reveal that vertebrate homologous recombination may consist of different subpathways that are employed at different types of damage.

Cells deficient in Blm and Werner DNA helicase (WRN), both of which are members of the RecQ helicase family, were also less resistant to killing by cisplatin. This moderate sensitivity of *blm* may be attributed to its increased frequency of aberrant homologous recombination associated with crossover as manifested by dramatic elevation of sister chromatid exchange (SCE) in *blm* mutants (27, 28). Paradoxically, although Blm and Rad54

[6] K. Kikuchi et al., in preparation.
[7] H. Hochegger et al., in preparation.

have opposite effect on the level of SCE, the sensitivity of *blm, rad54*, and *blm/rad54* double mutants were similar. This result implies complex functional interactions between Blm and Rad54; for example, enhancement of homologous recombination in *blm* and decrease in homologous recombination efficiency in *rad54* may be balanced in homologous recombination–dependent cross-link repair. The *wrn* mutant also showed mild sensitivity, whereas *blm/wrn* double mutations had an additive effect on the sensitivity. Thus, the two helicases may differentially contribute to cross-link repair. Recent data suggest that BLM can associate with FA complex (21, 29) and is recruited to damaged chromatin with the FA protein FancD2 (21), whereas WRN may be directly involved in homologous recombination (reviewed in ref. 30).

Interrelation of Rev3- and Fanc-c–dependent interstrand cross-link repair pathway. The highest sensitivity of *rev3* and *fanc-c* mutants led us to investigate the relation of these pathways in cross-link repair as has been done previously (31). We disrupted the *FANC-C* gene in *rev3* cells and found in agreement with the previous study that the double mutant was not more sensitive than the *rev3* single mutant (Fig. 3, *top right*). Whereas Niedzwiedz et al. evaluated cisplatin sensitivity by counting cells during continuous exposure of cells to the agent for ~48 hours in liquid culture, we employed additional two methods because cellular viability at a short period might not correlate with the colony survival (32). As shown in Fig. 3, we verified this result by using pulse exposure to cisplatin and monitoring survival by both colony formation assay (*top left*) and growth recovery in liquid culture (*bottom*).

Nonhomologous end-joining contributes only little to cross-link repair and its component, Ku, may interfere with other cross-link repair pathways. Although NHEJ is preferentially used to repair IR-induced DSBs in mammalian cells, mutations in NHEJ have little effect on the sensitivity to cross-linking agents (33). Similarly, we found that all the NHEJ mutants, including *ku70, ligase IV, dna-pkcs*, and *artemis* did not show hypersensitivity to killing by cisplatin (Fig. 1). The gene family that contains the Artemis endonuclease includes two additional paralogue genes, *SNM1A* and *SNM1B*, whose yeast homologue is SNM1 specifically involved in cross-link repair (34–36). The *snm1a* and *snm1b* DT40 mutants showed moderate and slight, respectively, sensitivities to cisplatin, and their double mutations had an additive effect as published before (36).

During analysis, we noticed that a defect in Ku70, which binds DSBs and initiates NHEJ, had opposite effects on the cisplatin sensitivity. The cisplatin sensitivity of *rad54/ku70* was higher than that of either mutant clone, whereas a defect in Ku70 in *blm* and *rev3* had little effect (Figs. 1 and 4). Remarkably, deletion of the *KU70* gene in *parp-1* reversed the cisplatin hypersensitivity nearly to wild-type level (Fig. 4, *top right*) This suppression is reminiscent of the higher efficiency of DSB-induced homologous recombination and resulting IR tolerance caused by the deletion of the *KU70* gene in DT40 and mammalian cells (37–39). The present data shed light on complex cross-talk between Ku, homologous recombination, and Parp-1 in cellular response to replication block at cisplatin cross-links.

Discussion

Here, we describe the contribution of multiple DNA repair and damage response pathway in cross-link repair in vertebrate cells. The sensitivity profiles of chicken DT40 cells revealed that the contribution to cross-link repair of each repair pathway seems to be different between yeast and vertebrates. Clearly, in DT40 cells, PRR, homologous recombination, and FANC pathways are mainly responsible for cross-link repair, whereas NHEJ, NER, and BER play only a minor role. As previous studies suggested, our data show that PRR is closely connected with the FANC pathway in ICL repair.

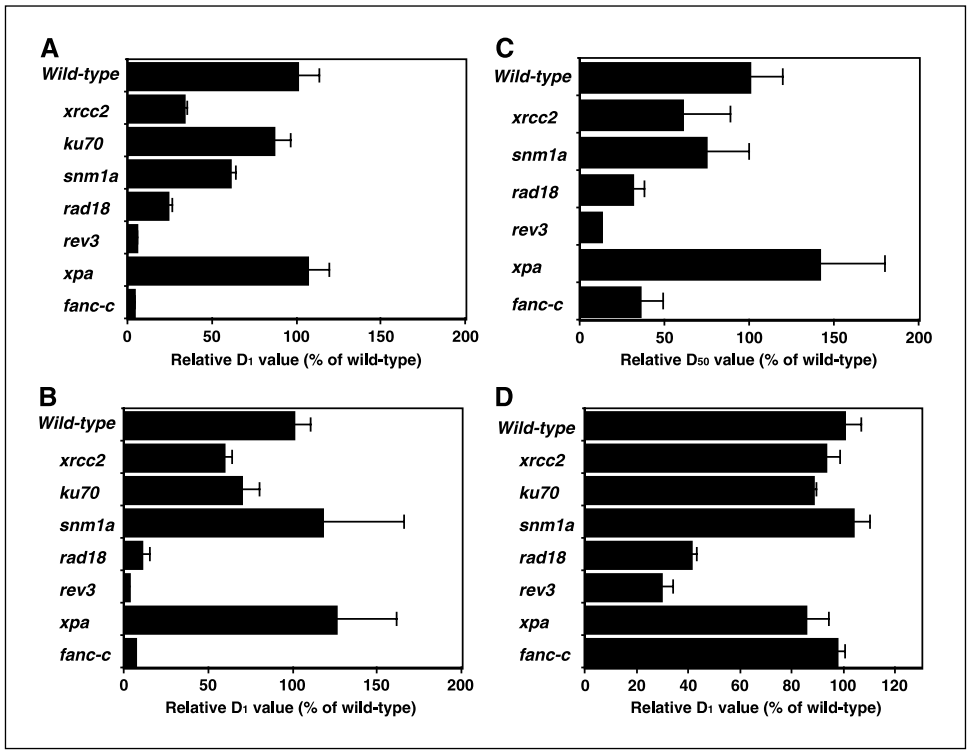

Figure 2. Toxicity profiles of cross-linking and alkylating reagents on a representative subset of the DT40 mutant panel. Melphalan (*A*), mitomycin C (*B*), transplatin (*C*), and MMS (*D*). D_1 and D_{50} values were calculated from three independent colony survival assays and normalized according to the D_1 and D_{50} values, respectively, of parental wild-type or heterozygous mutants.

Table 2. Cisplatin-induced chromosomal aberrations

Cells	Chromatid-type breaks	Chromosome-type breaks	Total (per cell)
Wild-type	3	2	5 (0.1)
ku70	6	1	7 (0.14)
xrcc2	7	13	20 (0.4)
rad18	8	3	11 (0.22)
rev3	29	12	41 (0.82)
xpa	3	1	4 (0.08)
fanc-c	17	5	22 (0.44)

NOTE: Cells were treated with 5 μmol/L cisplatin for 1 hour. Fifty metaphase spreads were analyzed. The numbers of aberrations per 50 cells are shown.

We also reveal dual actions of Ku70, which can partially substitute for the lack of Rad54-dependent homologous recombination but also interferes with cross-link repair as shown in the example of the *parp-1/ku70* double mutant.

Rev3 contributes to cellular tolerance to cisplatin presumably through Fanc-dependent homologous recombination pathway. Of all mutants tested in this study, Rev3, the catalytic subunit of Polζ, showed the highest sensitivity to cisplatin. This finding implies several possible explanations. Firstly, Rev3 might simply work as a TLS polymerase, allowing replication to continue through the DNA cross-link. Biochemically, this is hard to imagine especially for ICLs, and to date, such an activity has not been observed *in vitro*. Secondly, Rev3 may be involved in other repair pathways, such as homologous recombination. Both *rev3* and *rad18* mutants show decreased gene targeting frequencies and might be defective in specific homologous recombination reactions (9, 10). Thirdly, the finding that *rev3* and *rev3/fanc-c* to cisplatin showed very similar sensitivity implies that these two genes might act in the same pathway (Fig. 3; ref. 31). Thus, vertebrate Rev3 might have acquired a new function, besides its role in TLS, in dealing with ICL in combination with the FANC group of proteins. Indeed, Fanc- and Rev3-deficient DT40 cells have generally very different phenotypes; however, they seem to be epistatic in their sensitivity to cisplatin tolerance. Considering that vertebrate *rev3* is more sensitive to cisplatin than *rad18* or *fanc* mutants, Rev3-dependent response to cisplatin in vertebrate may be independently regulated by two ubiquitin-associated pathways (i.e., FA/BRCA and Rad6/Rad18 pathways).

It is unclear whether Fanc-dependent activation of Polζ facilitates TLS past monoadduct and intrastrand cross-link or facilitates ICL repair. We favor the idea that the Fanc-dependent pathway mainly affects ICL repair but not TLS for the following reason. Defective TLS can be substituted at least partially by more frequent usage of homologous recombination, as spontaneous SCE is increased in TLS mutant cells, including *polκ, polη*, and *polζ*.

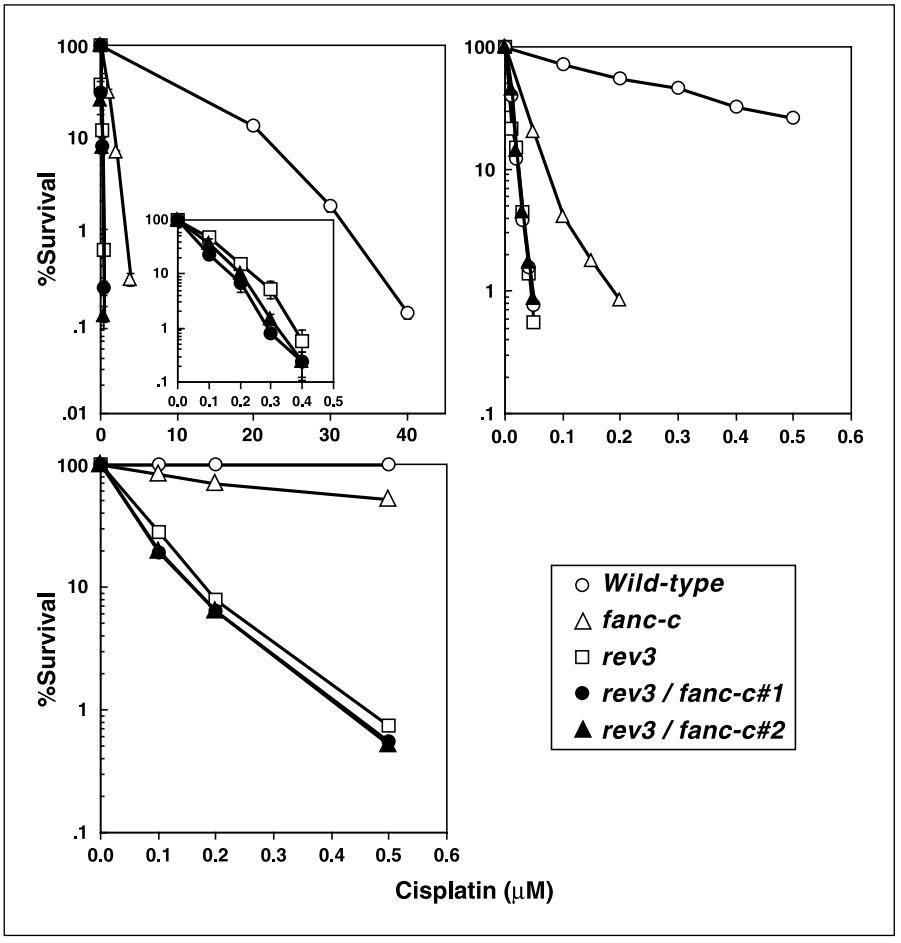

Figure 3. Similar cisplatin sensitivity between *rev3* and *rev3/fanc-c* cells. Sensitivity was assessed by colony survival assay in semisolid medium (*top left*) or by measuring the survival fractions in liquid culture (*bottom left*) after 1-hour pulse exposure to cisplatin. Alternatively, cells were continuously exposed to cisplatin in liquid culture, and the sensitivity was assessed at 48 hours (*top right*; see Materials and Methods).

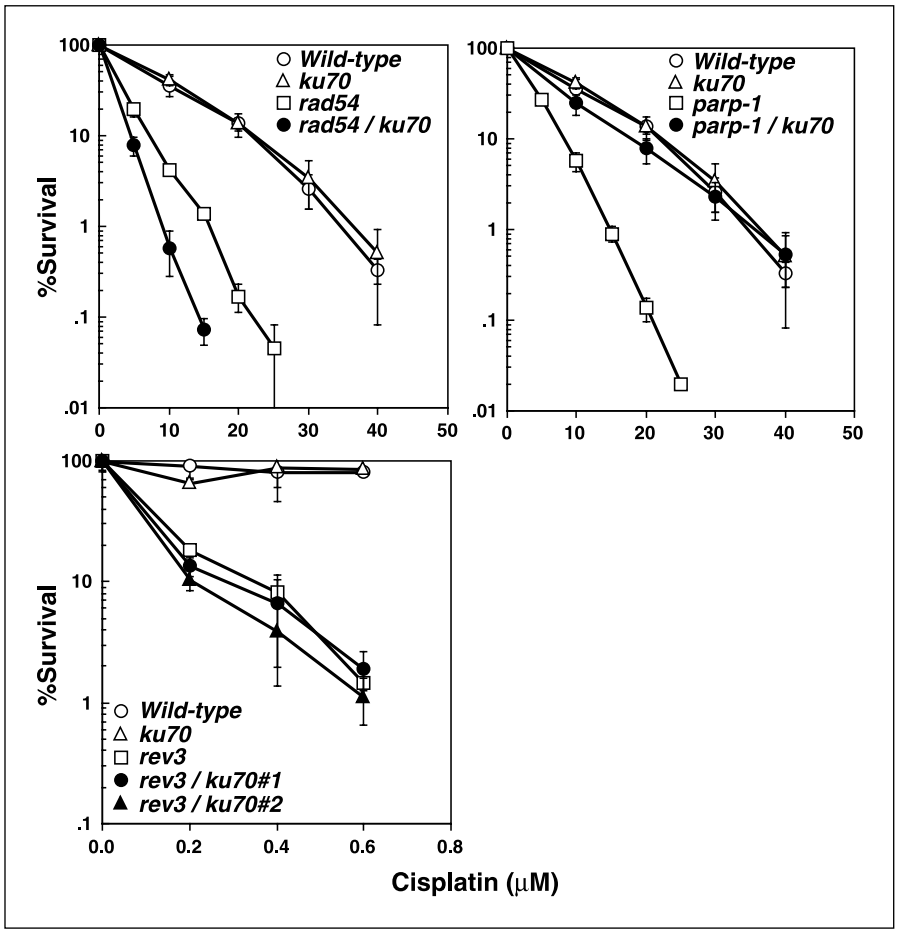

Figure 4. Effect of *KU70* deletion on the tolerance to killing by cisplatin. Sensitivity of *ku70, rad54*, and *rad54/ku70* double mutant (*top left*), *ku70, parp-1*, and *parp-1/ku70* double mutant (*top right*), and *ku70, rev3*, and *rev3/ku70* mutants (*bottom left*) to cisplatin. Sensitivity was measured by colony survival assay after 1-hour exposure to cisplatin.

In contrast, a defect in Fanc-c or Rev3 dramatically decreases the level of cisplatin-induced SCE compared with wild-type cells (31). Moreover, mammalian FA as well as *fanc-c* DT40 cells show virtually no sensitivity to MMS or UV (Fig. 2*D*; refs. 21, 40), and *fanc-c* had only moderate sensitivity to transplatin. Thus, the Fanc pathway may have a minor role, if any, in Polζ-dependent TLS past MMS or UV lesions. Taking the role for both the Fanc pathway and Rev3 in homologous recombination into account, it is tempting to speculate that this unknown mechanism for ICL repair might involve a specialized form of homologous recombination. The role of Rev3 in this ICL-induced homologous recombination could involve the start of strand elongation from the damaged structure. In summary, Polζ may have dual function in cellular tolerance to cross-linking agents, one in TLS past monoadducts and intrastrand cross-links and the other in ICL repair possibly involving FA signal transduction pathway as well as homologous recombination. This dual action might explain the extremely high sensitivity of the *rev* mutants to cross-linking agents. Taking the low fidelity of TLS polymerases into account, a significant contribution of Rev3 to bypassing a large number of cisplatin lesions implies that the use of cisplatin in chemotherapy could result in mutations through error-prone DNA synthesis.

Application to clinical research. DT40 is a unique cell line that offers a panel of isogenic mutants derived from a stable parental line. DT40 cells have the following characteristics that might affect the cellular responses to genotoxic stresses. First, DT40 seems, for unknown reasons, to possess significantly higher homologous recombination efficiency than any mammalian cell line. This special feature might substitute for a defect of known homologous recombination gene, as a defect in Xrcc2 has a more profound effect on rodent cells than on DT40 (25, 41). Second, DT40 divide thrice daily and spend only a short time in the G_1 phase of the cell cycle. Thus, an asynchronous population of DT40 cells consists only of a small G_1 fraction ($\sim$15%) and a much larger S-phase fraction (70%). Third, like many cancer cells, DT40 lacks the functional p53 and as a result has no G_1-S damage checkpoint (42). Thus, DNA damage at any phase of the cell cycle may have direct effect on DNA replication. These DT40-specific characteristics suggest that a defect in the DNA repair that is associated with DNA replication may display a more prominent phenotype in DT40 cells than in other cell lines that have longer G_1 phase and/or normal G_1-S checkpoint. Bearing these DT40-specific characteristics in mind, DT40 is a valuable tool and has been used extensively to explore relevant cellular pathways responsible for cancer therapy. In general, most phenotypes, especially those in homologous recombination mutants, observed in DT40 correlate well with those in mammalian model systems. Moreover, genes that are essential for embryonic development, such as Rev3, are more readily analyzed in cellular model systems, such as DT40. Thus, the findings in this study could prove very useful for improvements in the chemotherapeutic application of cisplatin and related compounds. A novel strategy, for example, could be a screen to identify chemical compounds that inhibit the TLS pathway. According to our findings, inhibitors of Rev3 or Rad18 could be highly efficient in sensitizing tumor cells to killing by cisplatin. Moreover, inhibition of Rev3 may reduce unwanted mutations induced by its error-prone activity. Similarly,

selective inhibitors of Parp, which are already available, in combination with cross-linking agents could be useful to further sensitize cancer cells to chemotherapy. The data presented in this study thus provide a rational approach to measure the importance of individual of repair pathways as targets in chemotherapy.

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