Loss of Nonhomologous End Joining Confers Camptothecin Resistance in DT40 Cells

IMPLICATIONS FOR THE REPAIR OF TOPOISOMERASE I-MEDIATED DNA DAMAGE*

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DNA topoisomerase I (Top1) generates transient DNA single-strand breaks via the formation of cleavage complexes in which the enzyme is linked to the 3'-phosphate of the cleavage strand. The anticancer drug camptothecin (CPT) poisons Top1 by trapping cleavage complexes, thereby inducing Top1-linked single-strand breaks. Such DNA lesions are converted into DNA double-strand breaks (DSBs) upon collision with replication forks, implying that DSB repair pathways could be involved in the processing/repair of Top1-mediated DNA damage. Here, we report that Top1-mediated DNA damage is repaired primarily by homologous recombination, a major pathway of DSB repair. Unexpectedly, however, we found that nonhomologous end joining (NHEJ), another DSB repair pathway, has no positive role in the relevant repair; notably, DT40 cell mutants lacking either of the NHEJ factors (namely, Ku70, DNA-dependent protein kinase catalytic subunit, and DNA ligase IV) were resistant to killing by CPT. In addition, we showed that the absence of NHEJ alleviates the requirement of homologous recombination in the repair of CPT-induced DNA damage. Our results indicate that NHEJ can be a cytotoxic pathway in the presence of CPT, shedding new light on the molecular mechanisms for the formation and repair of Top1-mediated DNA damage in vertebrates. Thus, our data have significant implications for cancer chemotherapy involving Top1 inhibitors.

DNA double-strand breaks (DSBs) can be caused by a variety of exogenous and endogenous agents, posing a major threat to genome integrity. If left unrepaired, DSBs may cause cell death (1, 2). Vertebrate cells have evolved two distinct pathways for repairing DSBs, homologous recombination (HR) and nonhomologous end joining (NHEJ) (2–5). The HR reaction requires a wide variety of proteins including Rad51, Rad52, and Rad54 (2), whereas NHEJ relies on Ku (a heterodimer of Ku70 and Ku86), DNA-PKcs, Artemis, Xrcc4, and DNA ligase IV (the LIG4 gene product) (3, 5, 6). The requirement for DNA ligase IV in this pathway is exclusive as other DNA ligases (I and III) are unable to substitute for the ligase function (7, 8). In contrast to HR that allows for accurate repair of DSBs, NHEJ is typically an imprecise, error-prone pathway.

In accordance with the essential roles of HR and NHEJ in DSB repair, cells deficient in HR or NHEJ have been shown to be highly sensitive to ionizing radiation (7–14). In the chicken B-lymphocyte DT40 cell line (15), the extent of radiosensitivity of RAD54−/− cells is similar to that of DNA-PKcs−/− or LIG4−/− cells (8, 14), and RAD54−/−/DNA-PKcs−/− double mutant cells are much more radiosensitive than each single mutant (14). These results clearly indicate that HR and NHEJ contribute equally, and independently, to the repair of radiation-induced DSBs.

Perturbation of fundamental cellular processes such as DNA replication or the action of DNA topoisomerases often causes DSBs. DNA topoisomerases are ubiquitous nuclear enzymes that participate in many aspects of DNA metabolisms, including DNA replication, transcription, and chromosome condensation/segregation (16). DNA topoisomerase II (Top2) alters the topology of DNA through a transient DSB and subsequent religation of the DSB (16). Inhibition of the enzyme by Top2 inhibitors, such as VP-16 or ICRF-193, has been shown to generate DSBs (17–20). We have recently shown that Top2-mediated DNA damage is predominantly repaired by the NHEJ pathway as evidenced by the observations that NHEJ mutants (LIG4−/− and Ku70−/−) cells are extremely sensitive to Top2 inhibitors compared with wild-type or RAD54−/− cells (21). Interestingly, the absence of Rad54 alleviated the hypersensitivity of Ku70−/− cells, suggesting that the cytotoxicity of Top2 inhibitors is enhanced by HR (21). On the other hand, Hellday and co-workers (22, 23) have reported that HR plays a more prominent role in the repair of replication fork-associated DNA damage than does NHEJ. The importance of repair by HR in response to replication inhibition has also been suggested by Lopes and coworkers (24). Thus, it is evident that HR and NHEJ operate very differently depending on the nature of DNA damage.

In multicellular organisms, DNA topoisomerases I (Top1) is much more abundant than Top2 and is indispensable for embryonic development (25, 26). In contrast to Top2 that relies on a transient DSB, Top1 generates a transient DNA single-strand break (SSB) with a free 5'-hydroxyl and a Top1-linked 3'-phosphate (16, 17). Such an intermediate, referred to as Top1 cleavage (or cleavable) complex, is normally short lived as the transient SSB is subsequently religated. Top1 has been
shown to be the target of a number of anticancer agents, including camptothecin (CPT) (17). These agents, referred to as Top1 poisons, selectively trap Top1 cleavage complexes by inhibiting the religation step, thereby inducing Top1-linked SSBs (17, 27). Intriguingly similar Top1 poisoning can be caused by a variety of endogenous and exogenous DNA lesions, including base modifications, mismatches, and abasic sites (27). This implies that the processing/repair of spontaneous (drug-independent) Top1 cleavage complexes is important for cell growth given the abundance of Top1 proteins in the cell. Recent work by Vance and Wilson (28) supports this notion.

The repair of Top1-mediated DNA damage is probably an important determinant for the cellular responses to Top1 poisons and for the selectivity of these agents for cancer cells. Despite the biological and clinical importance, the molecular mechanisms for the repair of Top1-mediated DNA damage remain largely unknown. This is partly because the repair may involve multiple pathways possibly in combination (27). Increasing evidence suggests that Top1 cleavage complexes are converted into irreversible DNA lesions in a replication-dependent or -independent fashion (27, 29–33). The replication-dependent cytotoxic DNA lesions, which arise from collisions between replication forks and Top1 cleavage complexes, involve DSBs and Top1 covalent complexes (30–34) (see Fig. 6). The generation of DSBs during the processing of Top1 cleavage complexes implies that DSB repair may play a role in the relevant repair. Indeed, in the yeast Saccharomyces cerevisiae, HR plays a pivotal role in Top1 damage repair: cells deficient in HR, such as rad52 mutants, are extremely sensitive to CPT (28, 35). CPT sensitivity of NHEJ-deficient yeast cells is essentially the same as that of wild-type cells (28). In vertebrate cells, several studies have shown that cells deficient in either HR or NHEJ are more CPT-sensitive than their respective controls (parental cell lines or corrected cells), and HR is likely more important than NHEJ (22, 36–38). However, because of the nonisogenic systems used in these studies, the respective contribution of HR and NHEJ in Top1 damage repair remains obscure.

In this study, we performed genetic analyses using the DT40 cell line to investigate the roles of HR and NHEJ in the repair of Top1-mediated DNA damage. We showed that HR is indeed important for Top1 damage repair. Strikingly we found that all NHEJ mutants (KU70/−, DNA-PKcs/−/−, and LIG4/−/−) are resistant to killing by CPT compared with wild-type cells. Furthermore the absence of NHEJ alleviated the requirement of HR in the repair of CPT-induced DNA damage. Our results indicate that NHEJ can be cytotoxic in the presence of Top1 poisons and that HR and NHEJ have opposing roles in the repair of Top1-mediated DNA damage. We suggest a model for the negative role of NHEJ in the repair of Top1-mediated DNA damage.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Clonogenic Assays—**DT40 mutant cell lines used in this study (LIG4/−/− cells, KU70/−/− cells, DNA-PKcs/−/− cells, RAD54/−/− cells, and RAD54/−/−KU70/−/− cells) have been created by gene targeting and extensively characterized in earlier studies (8, 9, 14, 39). Note that the chicken DNA-PKcs gene (Prkd) lies on chromosome 2, which is trisomic in DT40 cells (14). All the DT40 cell lines were cultured in a 5% CO2 incubator at 39 °C in ES medium (Nissui Seiyaku, Tokyo) supplemented with 10% fetal bovine serum and 1% chicken serum.

Clonogenic assays were performed as described previously (8). Briefly, cells were plated at 10–106 cells/dish into 60-mm bacterial dishes containing 4 ml of soft agarose medium (ES medium containing 0.12% agarose, 20% fetal bovine serum, and 2% chicken serum) with various drug concentrations. The cells were grown for 7–11 days, and the resulting visible colonies were counted. The percentage of survival was determined by comparing the number of surviving colonies to untreated controls. For each assay, at least three independent experiments were performed. Camptothecin, purchased from Sigma, was dissolved in Me2SO and stored frozen in aliquots at −20 °C.

**Relaxation Assays—**Nuclear extracts were prepared from DT40 cells as described previously (40). Relaxation assays were performed in the absence of ATP by using supercoiled plasmid DNA as a substrate. Briefly, 0.1–1 μg of nuclear extracts were added to 20 μl of reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, 15 μg/ml bovine serum albumin, and 1–2 μg of pBurescript II SK(−). After incubation at 37 °C, the reaction was stopped by adding SDS to 0.5%. The samples were then electrophoresed in a 0.9% agarose gel, and the DNA was stained with ethidium bromide, visualized by illumination with UV light, and photographed.

**In Vivo Complex of Topoisomerase Bioassays—**Top1 cleavage complexes formed in vivo were measured by an antibody-based in vivo complex of topoisomerase bioassay as described previously (41, 42). Briefly, 2 × 106 cells were treated with CPT for 1 h at 39 °C in serum-free medium. The cells were directly lysed with 1% Sarkosyl, and the viscous lysate was overlaid onto a preformed CsCl step gradient. After centrifugation at 20 °C in a Beckman SW55 rotor at 31,000 rpm for 20–24 h, gradients were fractionated from the bottom. DNA peaks were located using Anoo determinations on each fraction. Each fraction (50 μl) was applied to a nitrocellulose membrane (Hybond-C extra, Amersham Biosciences) using a slot blot device. The membrane was incubated with anti-human Top1 antibody (Scl-70) obtained from Topogen, Inc. (Columbus, OH) followed by incubation with horseradish peroxidase-conjugated Protein A. (Note that Scl-70 is cross-reactive with chicken Top1 (43).) Signals were detected using the ECL system (Amersham Biosciences) according to the manufacturer’s instruction.

**RESULTS**

**NHEJ-deficient DT40 Cells Are Resistant to Killing by CPT—**By clonogenic assays, we examined the sensitivity of various DT40 cell lines to the Top1 poison CPT. As shown in Fig. 1A, wild-type cells were not so sensitive to CPT at low doses (~30 nM). At higher doses (~90 nM), however, they were...
Top1 Activity Is Not Altered in NHEJ Mutants—A large body of evidence indicates that a reduction in Top1 activity confers resistance to Top1 poisons (26, 44, 45). We therefore prepared nuclear extracts from the wild-type and mutant cell lines and examined the Top1 activity by ATP-independent relaxation assays. As shown in Fig. 4A, relaxation activity of Top1 from NHEJ mutants was essentially the same as that from wild-type cells. This was further confirmed by a time course experiment with LIG4−/− cells and those transfected with the LIG4 transgene (Fig. 4B). We also compared the level of Top1 expression by immunoblot analysis and found no significant difference between wild-type and NHEJ-deficient cells (data not shown). These results suggest that neither the amount of Top1 nor its activity is altered in the NHEJ mutants. To further verify this, we next used an antibody-based in vivo complex of topoisomerase bioassay, which allows detection of covalent Top1-DNA complexes in cells (41, 42). As shown in Fig. 4C, a significant amount of Top1-DNA complexes was indeed trapped in CPT-treated LIG4−/− cells, which was comparable to that of CPT-treated wild-type cells. Very similar results were obtained with KU70−/− and DNA-PKcs−/− cells (data not shown). In the absence of CPT, such Top1-DNA complexes were barely detectable in either cell line (data not shown). (We note that under our experimental conditions, a slightly (but reproducibly) larger amount of Top1-DNA complexes was detected in the NHEJ mutants, although the reason for this increase is currently unclear.) Most importantly, the pattern of CPT-treated LIG4−/− cells was indistinguishable from those corrected with the LIG4 transgene (Fig. 4C), indicating that the absence of DNA ligase IV does not affect the formation of covalent Top1-DNA complexes in vivo. Taken together, the results clearly show that the NHEJ mutants possess normal Top1 activity. Thus, the CPT resistance of NHEJ mutants is not accounted for by a decrease in cellular Top1 activity.

HR Is Involved in the Repair of Top1-mediated DNA Damage—To investigate the role of HR in the repair of Top1 poison-induced DNA damage, we examined the sensitivity to CPT of...
Fig. 3. NHEJ mutants are not resistant to DNA replication inhibition. A, growth curves of wild-type, KU70+/−, DNA-PKcs−/−, and LIG4−/− cells in the presence of 0.2 mM hydroxyurea. Symbols are as in Fig. 1A. B, relative cell growth of each cell line after a 45-h incubation in the presence of 0, 0.1, or 0.2 mM hydroxyurea. C, growth curves of wild-type, KU70−/−, and LIG4−/− cells in the presence of 1.0 mM thymidine. Symbols are as in Fig. 1A. D, relative cell growth of each cell line after a 48-h incubation in the presence of 0.2 mM hydroxyurea. Data are representative of three independent experiments with similar results.

Fig. 4. NHEJ mutants possess normal Top1 activity. A, nuclear extracts prepared from wild-type (lanes 1, 5, and 9), DNA-PKcs−/− (lanes 2, 6, and 10), KU70−/− (lanes 3, 7, and 11), and LIG4−/− cells (lanes 4, 8, and 12) were assayed for Top1 activity as described under "Experimental Procedures." The amount of nuclear extracts used was: lanes 1–4, 1.0 μg; lanes 5–8, 0.2 μg; lanes 9–12, 0.1 μg. The reaction was performed at 37 °C for 1 h. The positions of supercoiled and relaxed plasmid DNA are indicated. B, nuclear extracts (250 ng) prepared from wild-type (lanes 1, 4, 7, 10, and 13), LIG4−/− cells (lanes 2, 5, 8, 11, and 14), and LIG4−/− cells corrected with a LIG4 transgene (lanes 3, 6, 9, 12, and 15) were assayed for Top1 activity. The reaction was performed at 37 °C for the times indicated (lanes 1–3, 5 min; lanes 4–6, 10 min; lanes 7–9, 15 min; lanes 10–12, 30 min; lanes 13–15, 45 min). C, the in vivo complex of topoisomerase assay was performed as described under "Experimental Procedures." Wild-type (lane 1), LIG4−/− (lane 2), and the transgene-corrected LIG4−/− cells (lane 3) were treated with 100 μM CPT for 1 h. Cells were lysed with Sarkosyl and subjected to CsCl gradient centrifugation. Covalent Top1-DNA complexes sedimented near the bottom of the gradient (see fractions marked "Top1-DNA complexes"), whereas free Top1 proteins sedimented near the top of the gradient (see fractions marked "Free Top1").

RAD54−/− cells (9, 39). We found that the RAD54−/− cells were more sensitive than wild-type cells (Fig. 5). It is worth mentioning that even at low doses of CPT (e.g. 30 nM), the size of RAD54−/− colonies was considerably small (data not shown). These results thus indicate that HR plays an important role in the repair of Top1-mediated DNA damage. Finally, we examined the sensitivity of a RAD54−/−/KU70−/− double mutant, which is defective in both HR and NHEJ and extremely hypersensitive to ionizing radiation (9). Consistent with a role of HR in Top1 damage repair, RAD54−/−/KU70−/− cells were more CPT-sensitive than KU70−/− cells (Fig. 5). Intriguingly, however, the double mutant was more resistant to the drug than RAD54−/− cells and, less pronouncedly, than wild-type cells. This suggests that NHEJ deficiency does confer CPT resistance even in cells defective for HR. These findings were further confirmed by comparing growth curves of the mutant cell lines; in the presence of CPT (60 or 100 nM), RAD54−/−/KU70−/− cells proliferated more slowly than NHEJ-deficient cells, but the growth was indeed better than that of wild-type cells (data not shown).

DISCUSSION

In vertebrate cells, the molecular mechanisms for the repair of Top1-mediated DNA damage remain largely unknown. In this study, we investigated the respective roles of HR and NHEJ in the repair of Top1-mediated DNA damage induced by
the anticancer drug CPT. Our data provide evidence that HR, but not NHEJ, is important for Top1 damage repair in vertebrate cells. Most importantly, we found that DT40 cells deficient in NHEJ are resistant to killing by CPT. The CPT tolerance was observed even in the absence of a functional repair pathway (HR), indicating that the loss of NHEJ alleviates the HR requirement in the repair of Top1-mediated DNA damage. It should be noted that the observed CPT resistance of NHEJ mutants is not attributable to changes in cellular Top1 activity or to resistance to replication inhibition.

The importance of HR in the repair of Top1-mediated DNA damage has been suggested (22, 36, 37, 46–48). Clearly, our findings with RAD54<sup>−/−</sup> cells support this notion. More importantly, however, our current results with RAD54<sup>−/−</sup>/KU70<sup>−/−</sup> cells indicate that NHEJ deficiency does confer CPT resistance in cells defective for HR. Of particular interest is that the RAD54<sup>−/−</sup>/KU70<sup>−/−</sup> double mutant was more resistant to CPT than wild-type cells (Fig. 5). This suggests that although HR plays an important role in Top1 damage repair, the role is negligible in cells lacking functional NHEJ. These results imply that HR may not be the sole pathway that repairs Top1-mediated DNA damage, compatible with recent evidence that multiple pathways are involved in the repair of Top1-mediated DNA damage (27). The idea that efficient alternative pathways (other than HR) may work to repair Top1-mediated DNA damage contrasts with the situation in yeast where HR plays essential roles in Top1 damage repair (28); specifically, several redundant pathways are involved in the initial processing of Top1 damage, but all the pathways eventually rely on HR to complete repair. Yet, even the partial contribution from HR in vertebrate Top1 damage repair would be intriguing in light of our recent finding that vertebrate Top2-mediated DNA damage (induced by Top2 inhibitors) is predominantly repaired by NHEJ, not HR (21). In yeast, HR is responsible for the repair of Top2-mediated DNA damage, whereas NHEJ has only a minor role (49). Thus, the roles of DSB repair pathways in topoisomerase-mediated DNA damage are quite different between yeast and vertebrates. Taken together, our studies revealed that vertebrate HR and NHEJ have opposing roles in the repair of Top1- as well as Top2-mediated DNA damage. Namely, HR is involved in Top1 damage repair in which NHEJ can play a negative role; conversely, NHEJ is an essential requirement for Top2 damage repair in which HR may be cytotoxic.

An increased resistance caused by NHEJ deficiency has been observed toward high doses (>6 grays) of ionizing radiation in DT40 cells; this was, however, specific to KU70<sup>−/−</sup> cells (8, 9, 14). In the present study, an increased resistance to CPT was observed in LIG4<sup>−/−</sup> and DNA-PKcs<sup>−/−</sup> cells as well as KU70<sup>−/−</sup> cells. Moreover, ectopic expression of a LIG4 transgene in LIG4<sup>−/−</sup> cells sensitized the cells to CPT. These results clearly indicate that NHEJ deficiency itself confers CPT resistance.

Why then does the loss of NHEJ confer CPT resistance? In other words, how can NHEJ be cytotoxic in the presence of Top1 poisons? Several explanations might address these questions. However, because NHEJ is the major DSB repair pathway and DNA ligase IV function is critical for this pathway, it would be reasonable to speculate that NHEJ unfavorably operates to join the ends of DSBs that, in order for cells to survive, must be repaired by other pathways. Joining of such DSBs by NHEJ may result in a deleterious dead end structure of some sort, eventually leading to cell death. Supportive of this idea is increasing evidence that CPT induces DSBs in vivo (22, 27, 29–31, 34, 38, 50). In particular, collisions of replication forks with Top1 cleavage complexes have been suggested to generate free DSBs along with irreversible Top1 covalent complexes that may also be potentially causative of DSBs (Fig. 6). Thus, the generation of DSBs during the processing of Top1 cleavage complexes would provide opportunities to use NHEJ. It is possible that similar DSBs that do not favor NHEJ-mediated repair may be generated in a replication-independent manner. For example, SSBs occurring in close proximity of Top1 cleavage complexes can directly cause DSBs (27).

To our knowledge, this is the first demonstration that defects in NHEJ confer resistance to Top1 poisons. It will thus be interesting to examine whether certain cancer cells with acquired resistance to Top1 poisons have some defect in NHEJ possibly in combination with other genetic and/or epigenetic changes. It has been shown, however, that NHEJ has no impact on CPT sensitivity/resistance in yeast (28). Additionally, earlier work has suggested that Chinese hamster ovary cell lines deficient in Ku or DNA-PKcs are slightly more CPT-sensitive than their respective controls (36, 37), although these mutants may have accompanied additional genetic (or epige-
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...networks (7) and found that the targeted deletion of LIG4 had no impact on CPT sensitivity. This finding strongly suggests that NHEJ does not play a significant role in Top1 damage repair, consistent with our current observations with DT40 cells. Hence, it is highly likely that NHEJ in general plays little or no positive role in Top1 damage repair. The result with the human cell line, however, does not support the negative role of NHEJ discussed here. The reason for apparent differences in cell lines with respect to NHEJ and CPT is currently unclear. It may be that under certain conditions NHEJ becomes cytotoxic in the presence of Top1 poisons. Given that NHEJ processing of Top1 damage results in a lethal dead end structure (see above), it is possible that such lethal damage is not significantly repairable by any other process in DT40 cells, while a pathway(s) might exist in some cells (at least in Nalm-6) to cope with the lethal damage. Alternatively, some cells might suffer less (or no) damage induced by NHEJ and CPT, for example, by preventing the NHEJ machinery from accessing Top1 damage on the genome. Clearly, further studies are needed to fully understand the negative role of NHEJ in Top1 damage repair. Finally, our findings presented here shed new light on the molecular mechanisms for the formation and repair of Top1-mediated cytotoxic DNA damage in vertebrates and have significant implications for cancer chemotherapy involving Top1 inhibitors.

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