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Hypersensitivity of Nonhomologous DNA End-joining Mutants to VP-16 and ICRF-193

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

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A number of clinically useful anticancer drugs, including etoposide (VP-16), target DNA topoisomerase (topo) II. These drugs, referred to as topo II poisons, stabilize cleavable complexes, thereby generating DNA double-strand breaks. Bis-2,6-dioxopiperazines such as ICRF-193 also inhibit topo II by inducing a distinct type of DNA damage, termed topo II clamps, which has been believed to be devoid of double-strand breaks. Despite the biological and clinical importance, the molecular mechanisms for the repair of topo II-mediated DNA damage remain largely unknown. Here, we perform genetic analyses using the chicken DT40 cell line to investigate how DNA lesions caused by topo II inhibitors are repaired. Notably, we show that *LIG4*$^{-/-}$ and *KU70*$^{-/-}$ cells, which are defective in nonhomologous DNA end-joining (NHEJ), are extremely sensitive to both VP-16 and ICRF-193. In contrast, *RAD54*$^{-/-}$ cells (defective in homologous recombination) are much less hypersensitive to VP-16 than the NHEJ mutants and, more importantly, are not hypersensitive to ICRF-193. Our results provide the first evidence that NHEJ is the predominant pathway for the repair of topo II-mediated DNA damage; that is, cleavable complexes and topo II clamps. The outstandingly increased cytotoxicity of topo II inhibitors in the absence of NHEJ suggests that simultaneous inhibition of topo II and NHEJ would provide a powerful protocol in cancer chemotherapy involving topo II inhibitors.

DNA double-strand breaks (DSBs)[1] 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 major pathways for repairing DSBs, homologous recombination (HR) and nonhomologous DNA end-joining (NHEJ) (2–5).

With the use of homologous DNA sequences, HR allows for accurate repair of DSBs. In eukaryotic cells, the HR reaction is performed by a wide variety of proteins including Rad51, Rad52, and Rad54 (2). *In vitro*, Rad51 protein assembles with single-stranded DNA to form the helical nucleoprotein filament that promotes DNA strand exchange, a basic step of HR (6–8). Rad54 protein is shown to interact with and stabilize the Rad51 nucleoprotein filament, stimulating its DNA pairing activity (9, 10). Interestingly, although Rad52 protein plays a pivotal role in DSB repair in *Saccharomyces cerevisiae*, the role of vertebrate and *Schizosaccharomyces pombe* Rad52 is much less significant (11–13).

In contrast to accurate repair by HR, NHEJ can lead to imprecise joining of DSB ends. It has been well established that NHEJ is responsible for V(D)J recombination in lymphocytes (3, 5). The NHEJ reaction relies on Ku (a heterodimer of Ku70 and Ku86), DNA-PKcs, Artemis, Xrcc4, and DNA ligase IV (the *LIG4* gene product) (3, 5, 14). Extensive biochemical studies propose a model for the mechanism of NHEJ (3, 5, 14, 15). First, Ku binds to the ends of a DSB and recruits the DNA-PKcs·Artemis complex. This complex would then trim the ends to make the ends ligatable. Additional nucleases and/or polymerases may also be involved in this process. Finally, the DNA ligase IV-Xrcc4 complex is recruited for ligation. 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 IV function (16, 17).

Consistent with the proposed functions of HR and NHEJ in DSB repair, cells deficient in HR or NHEJ proteins have been shown to be highly sensitive to DSB-generating DNA-damaging agents, such as ionizing radiation (16–23). In a chicken B-lymphocyte DT40 cell line, where HR activity is extraordinarily high as compared with other vertebrate cell lines (24), several knockout mutants deficient in HR and/or NHEJ have been constructed by gene targeting. *RAD54*$^{-/-}$ cells are shown to be hypersensitive to ionizing radiation (18). Similarly, *DNA-PKcs*$^{-/-/-}$ and *LIG4*$^{-/-}$ cells exhibit hypersensitivity to ionizing radiation (17, 23) (note that the chicken *DNA-PKcs* gene (*Prkdc*) lies on chromosome 2, which is trisomic in DT40 cells (23)). Importantly, the extent of radiosensitivity of the *RAD54*$^{-/-}$ cells is very similar to that of the *DNA-PKcs*$^{-/-/-}$ or *LIG4*$^{-/-}$ cells (17, 23), and *RAD54*$^{-/-}$*/DNA-PKcs*$^{-/-/-}$ double mutant cells are more radiosensitive than each single mutant (23). These observations indicate that in DT40 cells, HR, and NHEJ contribute equally to the repair of DSBs. *KU70*$^{-/-}$ DT40 cells also exhibit hypersensitivity to low doses of ionizing radiation, and *RAD54*$^{-/-}$*/KU70*$^{-/-}$ cells are more radiosensitive than each single mutant (18).

DNA topoisomerase (topo) II is a ubiquitous nuclear enzyme

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[1] The abbreviations used are: DSB, double-strand break; ES, embryonic stem; HR, homologous recombination; ICRF-193, meso-2,3-bis(2,6-dioxopiperazin-4-yl)butane; NHEJ, nonhomologous DNA end-joining; topo, DNA topoisomerase; *TOP2α*, the gene encoding topo IIα; VM-26, 4′-demethylepipodophyllotoxin thenylidene-β-D-glucoside (teniposide); VP-16, demethylepipodophyllotoxin ethylidene-β-D-glucoside (etoposide).

that alters the topological structure of DNA and chromosomes through a transient DSB and subsequent religation of the DSB (25). The enzyme has been implicated in many aspects of DNA metabolisms, including DNA replication, repair, transcription, and chromosome condensation/segregation (25). Vertebrate cells possess two genetically distinct topo II isoforms; topo IIα is essential for cell proliferation (26), whereas topo IIβ is non-essential (27). It has been established that topo IIα is more abundantly expressed in growing cells and its expression is cell cycle-regulated, peaking in G_2/M, whereas the expression level of topo IIβ is low and roughly constant during the cell cycle (28, 29).

Interestingly, some exogenous agents cause DSBs via inhibition of topo II. These agents include etoposide (VP-16), teniposide (VM-26), and 4′-(9-acridinylamino)methanesulfon-*m*-aniside, which are clinically useful in cancer chemotherapy (30, 31). A unique feature of these topo II inhibitors is the formation of "cleavable complex" (also called "cleavage complex"), in which a topo II-linked DNA strand-passing intermediate is stabilized, allowing the generation of a DSB (30, 31). Such topo II inhibitors are referred to as "topo II poisons" because they convert the essential enzyme into a cytotoxic DNA-damaging agent.

It is also known that some topo II inhibitors do not belong to topo II poisons and, thus, act as catalytic inhibitors without cleavable complex formation (31). Among these, bis-2,6-dioxopiperazines, such as meso-2,3-bis(2,6-dioxopiperazin-4-yl)butane (ICRF-193), have been most extensively analyzed thus far. Earlier biochemical studies have suggested that ICRF-193 inhibits topo II by trapping the enzyme in the form of a closed protein clamp (topo II clamp) (32, 33). However, more recent studies suggest that ICRF-193 is not a pure catalytic inhibitor; rather, it may act as a topo II poison (34–38). These studies raise the possibility that topo II clamp is a novel type of cytotoxic DNA damage.

Despite the importance of topo II-mediated DNA damage, the molecular mechanisms for the repair of topo II-mediated DNA damage are poorly understood. Topo II poison-induced cleavable complexes are known to trigger DNA damage responses, such as p53 stabilization, which can lead to apoptotic cell death or genomic instability (39, 40). Recently, topo II poison-induced cleavable complexes have been shown to be proteolytically degraded by the ubiquitin/26 S proteasome pathway, and a model is suggested in which the repair of cleavable complexes may involve transcription-dependent topo II proteolysis to reveal the protein-concealed DSBs (41). It is also suggested that the collision of DNA replication machinery with cleavable complexes leads to generation of overt DSBs (42). Such protein-free DSBs would then be repaired by the DSB repair pathway(s). Indeed, the *S. cerevisiae rad52* mutant is extremely sensitive to topo II poisons (43). In higher eukaryotes, several NHEJ mutants are shown to exhibit hypersensitivity to topo II poisons (17, 44, 45).

Much less is known about the mechanisms for the repair of ICRF-193-induced topo II clamps, assuming that these intermediates are actually cytotoxic DNA damage. Importantly, however, recent work has revealed that ICRF-193-induced topo II clamps also undergo 26 S proteasome-mediated degradation (46), raising an intriguing possibility that the repair machinery responsible for cleavable complexes are, at least in part, involved in the repair of topo II clamps as well. In this regard, it is interesting to note that Ku86-deficient Chinese hamster cells display hypersensitivity to ICRF-193 (47). The involvement of other NHEJ factors in the repair of ICRF-193-induced DNA damage is yet to be examined, however.

In this paper, we perform genetic analyses using the chicken DT40 cell line to investigate how topo II-mediated DNA damage caused by topo II inhibitors is repaired. With the use of *TOP2α*$^{+/-}$ mutants we demonstrate that ICRF-193 actually acts as a topo II poison in vertebrate cells. In addition, we confirm this notion by using DSB repair-deficient DT40 cell mutants; namely, *RAD54*$^{-/-}$ cells (an HR mutant), *LIG4*$^{-/-}$ and *KU70*$^{-/-}$ cells (NHEJ mutants), and *RAD54*$^{-/-}$/*KU70*$^{-/-}$ cells. Strikingly, the NHEJ mutants are extremely sensitive to VP-16 and ICRF-193 as compared with the wild-type or *RAD54*$^{-/-}$ cells. Our results indicate that topo II-mediated DNA damage, including both topo II poison-induced cleavable complexes and ICRF-193-induced topo II clamps, is predominantly repaired by the NHEJ pathway of DSB repair.

EXPERIMENTAL PROCEDURES

Vector Construction—A 4.6-kilobase genomic fragment containing exons 6–15 of the chicken *TOP2α* gene was isolated by PCR using DT40 genomic DNA as template. The primers used were pTop2a-E5 (5′-CAGTGTGTGGAACAATGGGAAAGGCATTCC-3′) and pTop2a-E16 (5′-GGGTGATGGCAGCATCATCTTCAGGACCAG-3′), designed based on the reported chicken *TOP2α* cDNA (DDBJ/EMBL/GenBank™ accession number AB007445). The genomic fragment was found to contain a unique *Sna*BI site, which was located in exon 9 of the *TOP2α* gene. To construct a targeting vector, we inserted a hygromycin resistance gene cassette into the *Sna*BI site.

Cell Culture and Transfection—DT40 cells were cultured in a 5% CO_2 incubator at 39 °C in ES medium (Nissui Seiyaku, Tokyo) supplemented with 10% fetal bovine serum and 1% chicken serum. For colony formation, cells were grown for 7–14 days in ES medium containing 0.15% agarose, 20% fetal bovine serum, and 2% chicken serum (soft agarose medium). DNA transfection was performed essentially as described (17, 48). Briefly, 4×10^6 cells were electroporated with 4 μg of DNA construct, and drug-resistant colonies were selected by incubation in soft agarose medium containing 1.5 mg/ml hygromycin B (Wako Pure Chemical, Osaka, Japan). Genomic DNA was isolated from the drug-resistant clones and subjected to Southern blot analysis as described previously (49).

Clonogenic Assays—VP-16 (Sigma) and ICRF-193 (Zenyaku Kogyo, Tokyo) were dissolved in Me_2SO and stored frozen in aliquots at −20 °C. Clonogenic assays were performed essentially as described (48). Briefly, cells were plated at 10^2–10^5 cells/dish into 60-mm bacterial dishes containing 5 ml of soft agarose medium with various concentrations of each drug. After incubation for 7–14 days, the number of resulting colonies was counted, and the percent survival was determined by comparing the number of surviving colonies to untreated controls.

RESULTS

Generation of TOP2α$^{+/-}$ *DT40 Cells*—We have previously shown that mouse embryonic stem (ES) cells heterozygous for the *TOP2α* gene exhibit an increased resistance to ICRF-193 as well as VP-16 (38). To further confirm these observations, we wished to establish *TOP2α*$^{+/-}$ DT40 cell lines by gene targeting. To perform this, we isolated a genomic clone containing the chicken *TOP2α* locus and constructed a targeting vector by inserting a hygromycin-resistance gene cassette into the *TOP2α* coding region (Fig. 1*A*). Electroporation of the targeting vector into wild-type cells gave rise to hygromycin-resistant clones. Heterozygous disruption of the *TOP2α* gene was examined by Southern blot analysis of *Bgl*II- or *Eco*RV-digested genomic DNA (Fig. 1, *B* and *C*), and two clonal lines, H5 and H7, exhibited band patterns indicative of the precise targeting event. The results indicate that these cell lines are heterozygous mutants for the *TOP2α* gene. It should be noted that the conservation of the *Sna*BI site located in exon 9 of the chicken and mouse *TOP2α* genes enabled us to introduce the same mutation as we did in the *TOP2α* gene of mouse ES cells (26, 38).

As expected, we found that the amount of topo IIα protein in *TOP2α*$^{+/-}$ cells (the H7 cell line) was reduced to ~50% that in wild-type cells (Fig. 1*D*). We then compared the growth rate of *TOP2α*$^{+/-}$ cells with that of wild-type cells and observed no

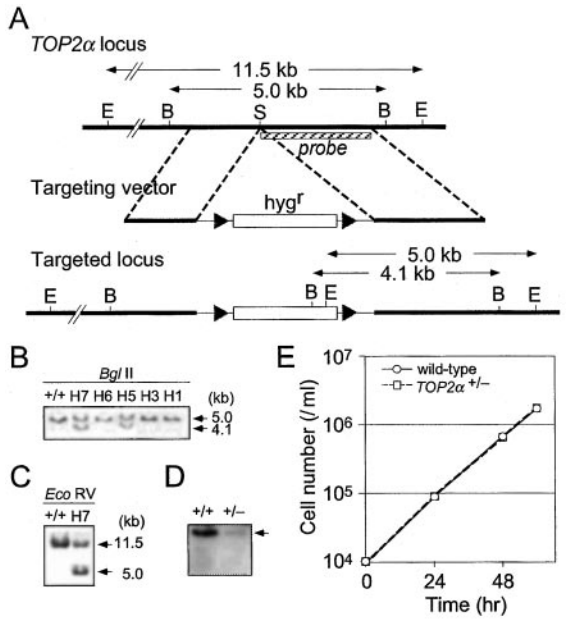

FIG. 1. **Generation of *TOP2*$\alpha^{+/-}$ clones.** *A*, schematic representation of heterozygous disruption of the chicken *TOP2*α gene. The *TOP2*α locus, targeting vector, and targeted locus are shown. The *triangles* flanking the hygromycin-resistance (hyg^r) gene designate *loxP* sequences. Note that the unique *Sna*BI site (denoted by *S*) is located in exon 9 of the *TOP2*α gene. *B*, *Bgl*II; *E*, *Eco*RV. *B*, Southern blot analysis of *Bgl*II-digested genomic DNA of wild-type cells (+/+) and five hygromycin-resistant clonal cell lines (H1, H3, H5, H6, H7). Note that two cell lines H5 and H7 exhibit band patterns indicative of the precise targeting event. The probe used is shown in *A*. *C*, Southern blot analysis of *Eco*RV-digested genomic DNA of wild-type cells (+/+) and the cell line H7. The probe used is shown in *A*. *D*, Western blot analysis for topo IIα in wild-type (+/+) and heterozygous H7 (+/–) cells. *E*, growth curves of wild-type and *TOP2*$\alpha^{+/-}$ cells. Data are the mean of three independent experiments. *kb*, kilobases.

difference between the two (Fig. 1*E*), indicating that a one-half reduction in the topo IIα expression level does not affect DT40 cell growth.

TOP2$\alpha^{+/-}$ *Cells Exhibit an Increased Resistance to VP-16 and ICRF-193*—We examined the sensitivity of *TOP2*$\alpha^{+/-}$ cells to the topo II poison VP-16. As shown in Fig. 2*A*, the *TOP2*$\alpha^{+/-}$ cells were more resistant to VP-16 than wild-type cells. We next examined the sensitivity to ICRF-193. Consistently, the mutant cells did exhibit an increased resistance to ICRF-193 as compared with wild-type cells (Fig. 2*B*). These results are quite similar to that obtained with mouse ES cells (38). Together, our data demonstrate that ICRF-193, like VP-16, does act as a topo II poison in vertebrate cells. We note that *TOP2*$\alpha^{+/-}$ cells are not hypersensitive to an alkylating agent methyl methanesulfonate (Fig. 2*C*) or UV light (data not shown).

NHEJ Mutants Are Hypersensitive to VP-16—In the DT40 cell line, several mutants defective in DNA repair/recombination have been created thus far. *KU70*$^{-/-}$ and *LIG4*$^{-/-}$ cells are NHEJ mutants that exhibit hypersensitivity to DSB-generating DNA-damaging agents such as ionizing radiation (17, 18). *RAD54*$^{-/-}$ cells are defective in HR and also display increased radiosensitivity, and *RAD54*$^{-/-}$/*KU70*$^{-/-}$ cells, defective in both HR and NHEJ, are much more radiosensitive than each single mutant (18). Interestingly, the extent of radiosensitivity of *RAD54*$^{-/-}$ cells is very similar to that of *LIG4*$^{-/-}$ cells (and also *DNA-PKcs*$^{-/-/-}$ cells) (17, 18, 23), indicating that the two repair pathways, HR and NHEJ, contribute equally to the repair of DSBs in the DT40 cell line.

We previously showed that *LIG4*$^{-/-}$ cells were more VP-16-sensitive than wild-type cells (17). This result was simply interpreted as a DSB repair defect of the *LIG4*$^{-/-}$ cells, based on

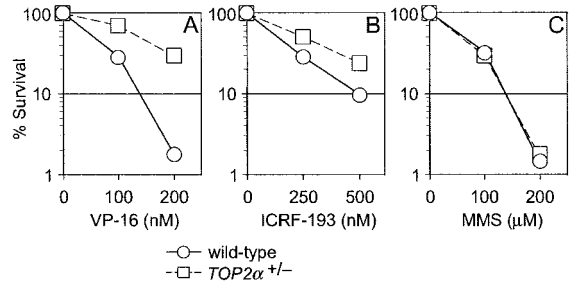

FIG. 2. ***TOP2***$\alpha^{+/-}$ **cells exhibit an increased resistance to VP-16 and ICRF-193.** *A*, sensitivity of cells to VP-16. *B*, sensitivity of cells to ICRF-193. *C*, sensitivity of cells to MMS. Data are the mean of four independent experiments. *MMS*, methyl methanesulfonate.

the fact that VP-16 is a topo II poison that generates DSBs. However, other possibilities have not been fully excluded. For example, there might be an additional repair pathway(s) that relies on DNA ligase IV and contributes to the repair of VP-16-induced DNA damage. Alternatively, because VP-16 induces, at least primarily, protein-concealed DSBs, the repair of such lesions might require DNA ligase IV but not the NHEJ pathway. We therefore used other DT40 cell mutants to examine the sensitivity to VP-16.

By clonogenic assays, we found that *KU70*$^{-/-}$ cells, like *LIG4*$^{-/-}$ cells, were hypersensitive to VP-16 (Fig. 3*A*). Likewise, the *KU70*$^{-/-}$/*LIG4*$^{-/-}$ cells (17) were hypersensitive to VP-16, and the extent of this hypersensitivity was very similar to that of *KU70*$^{-/-}$ or *LIG4*$^{-/-}$ cells (data not shown). These data clearly indicate that the NHEJ pathway is required for the repair of VP-16-induced DNA damage. We also found that *RAD54*$^{-/-}$ cells were more VP-16-sensitive than wild-type cells (Fig. 3*A*). Significantly, however, the hypersensitivity was considerably mild as compared with *KU70*$^{-/-}$ or *LIG4*$^{-/-}$ cells. For instance, at 25 nM, the NHEJ mutants displayed ~4 orders of magnitude higher sensitivity than wild-type cells, whereas *RAD54*$^{-/-}$ cells retained ~60% survival. These observations suggest that HR plays only a minor role in the repair of VP-16-induced DNA damage. This contrasts with the case in *S. cerevisiae*, where HR plays a pivotal role in the repair of topo II poison-induced DNA damage (43).

The predominance of NHEJ (over HR) in the repair of VP-16-induced DNA damage was further confirmed by the use of *RAD54*$^{-/-}$/*KU70*$^{-/-}$ cells. Strikingly, the *RAD54*$^{-/-}$/*KU70*$^{-/-}$ cells were not more VP-16-sensitive than *KU70*$^{-/-}$ cells (Fig. 3*A*), as is in marked contrast to the results obtained with ionizing radiation (see above). Clearly, these results support the notion that VP-16-induced DNA damage is preferentially repaired by NHEJ, whereas HR plays a minor role in the repair. Rather, we note that the double mutant is less sensitive to VP-16 than *KU70*$^{-/-}$ cells. It is intriguing that *KU70*$^{-/-}$ cells are more resistant to VP-16 in the absence of the HR protein Rad54.

NHEJ Mutants Are Hypersensitive to ICRF-193, but RAD54$^{-/-}$ *Cells Are Not*—To further unveil the mechanisms for the repair of topo II-mediated DNA damage, we then examined the sensitivity to the topo II inhibitor ICRF-193 of the various DT40 cell mutants. As shown in Fig. 3*B*, *KU70*$^{-/-}$ cells displayed a marked sensitivity to ICRF-193. More importantly, we found that *LIG4*$^{-/-}$ cells were also hypersensitive to ICRF-193 (Fig. 3*B*), indicating that both Ku and DNA ligase IV participate in the repair of ICRF-193-induced DNA damage. From these results, we conclude that the NHEJ pathway is essential for the repair of ICRF-193-induced DNA damage as well as topo II poison-induced DNA damage.

Interestingly, the sensitivity of *RAD54*$^{-/-}$ cells to ICRF-193 was comparable with that of wild-type cells (Fig. 3*B*). Rather,

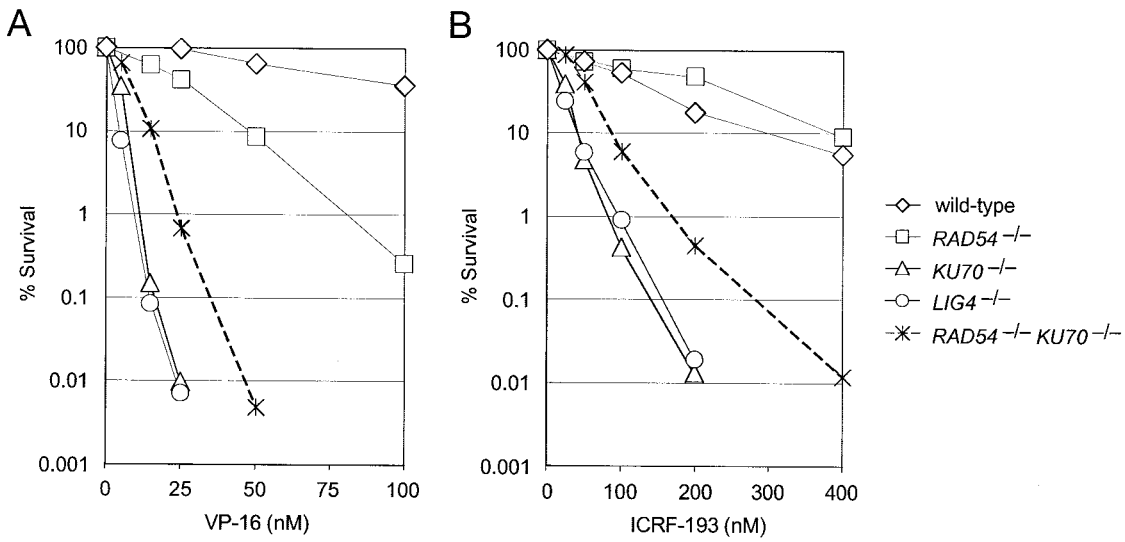

FIG. 3. **NHEJ mutants are hypersensitive to both VP-16 and ICRF-193.** *A*, sensitivity to VP-16 of wild-type, *RAD54*$^{-/-}$, *KU70*$^{-/-}$, *LIG4*$^{-/-}$, and *RAD54*$^{-/-}$/*KU70*$^{-/-}$ cells. Data are the mean of at least three independent experiments. *B*, sensitivity to ICRF-193 of wild-type, *RAD54*$^{-/-}$, *KU70*$^{-/-}$, *LIG4*$^{-/-}$, and *RAD54*$^{-/-}$/*KU70*$^{-/-}$ cells. Data are the mean of at least two independent experiments.

at higher concentrations, *RAD54*$^{-/-}$ cells were slightly more resistant. Also, similar to the case of VP-16, *RAD54*$^{-/-}$/*KU70*$^{-/-}$ cells were less sensitive to ICRF-193 than *KU70*$^{-/-}$ cells were (Fig. 3*B*). Clearly, these results eliminate the importance of HR in the repair of ICRF-193-induced DNA damage.

DISCUSSION

In this paper, we have performed genetic analyses to investigate how topo II-mediated DNA damage is repaired in vertebrate cells. Our data provide the first evidence that NHEJ is the predominant pathway for the repair of any topo II-mediated DNA damage. Based on the results described here, we propose a hypothetical model as to the formation and repair of DNA damage induced by topo II inhibitors (Fig. 4).

We have shown that the topo II inhibitor ICRF-193, like VP-16, does act as a topo II poison *in vivo*. Furthermore, we have revealed that NHEJ mutants are hypersensitive to ICRF-193 as well as VP-16. Together, these data strongly suggest that the two types of DNA damage, *i.e.* the VP-16- and ICRF-193-induced DNA damage, behave similarly in the cell, being detected and/or repaired in a similar manner. This notion is further supported by recent findings that both topo II poison-induced cleavable complexes and ICRF-193-induced topo II clamps undergo 26 S proteasome-mediated degradation (41, 46). Furthermore, both cleavable complexes and topo II clamps are shown to up-regulate p53 and induce apoptosis (39, 46). It may be that as long as topo II is locked on DNA, cleavable complexes and topo II clamps are intrinsically similar as DNA damage for the cell. Although such drug-locked topo II is reversible DNA damage, subsequent collision of the damage with cellular processes such as DNA replication or transcription machinery would lead to generation of irreversible DNA damage that absolutely requires NHEJ for its repair (Fig. 4). Intriguingly, the HR pathway may also participate in the generation of such irreversible DNA damage (Fig. 4), as *KU70*$^{-/-}$ cells were more resistant to either topo II inhibitor in the absence of Rad54 (see below).

Because NHEJ is the major pathway for repairing DSBs in vertebrate cells, we assume that the cytotoxic lesions resulting from topo II inhibition are most likely DSBs. Why, then, is NHEJ predominantly required for the repair? Considering the equal contribution of HR and NHEJ in repair of radiation-induced DSBs in DT40 cells (17, 23), the predominance of NHEJ in the repair of topo II-mediated DNA damage would be

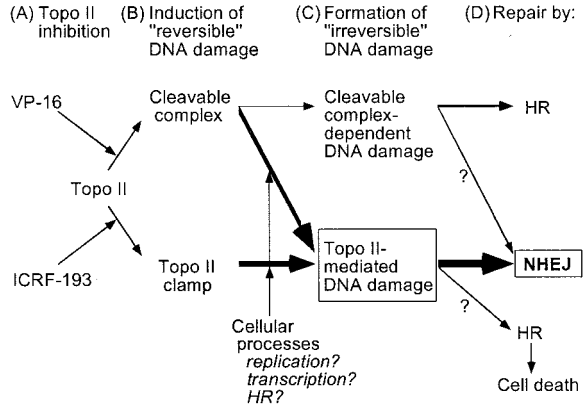

FIG. 4. **Hypothetical model for topo II-mediated DNA damage and its repair.** VP-16 and ICRF-193 inhibit topo II via different mechanisms (*A*). VP-16 stabilizes cleavable complexes, whereas ICRF-193 induces topo II clamps. Importantly, both of these intermediates include drug-locked topo II and are intrinsically reversible (*B*). Collision of the drug-locked topo II with cellular processes, such as DNA replication or transcription machinery, would lead to formation of irreversible DNA damage (topo II-mediated DNA damage), which is common to cleavable complex and topo II clamp (*C*). Intriguingly, HR may also be involved in the formation of such irreversible DNA damage. The repair of irreversible topo II-mediated DNA damage relies absolutely on NHEJ (*D*). Conversely, another DSB repair pathway HR, if involved in the relevant repair, might be deleterious for the cell, leading to cell death (*D*). Another type of irreversible DNA damage may also be generated by VP-16, a strong topo II poison (cleavable complex-dependent DNA damage), particularly at high doses (*C*). This type of DNA damage would be repaired by HR (and possibly by NHEJ) (*D*). See "Discussion" for details.

enigmatic. Possibly, as yet undefined molecular interactions between topo II and an NHEJ factor(s) might facilitate the preferential usage of NHEJ. It is also possible that an unknown mechanism(s) might render the HR machinery incapable of accessing to, or initiating repair of, topo II-mediated DNA damage. Alternatively, an HR-mediated repair, if any, might be unfavorable for cell survival. Supportive of this idea may be our observations with the *RAD54*$^{-/-}$/*KU70*$^{-/-}$ mutant, which is less sensitive than the *KU70*$^{-/-}$ mutant to either topo II inhibitor. Furthermore, toward higher doses of ICRF-193, the *RAD54*$^{-/-}$ mutant exhibits slightly increased resistance compared with wild-type cells. Together, these observations raise an intriguing possibility that HR may be a cytotoxic repair

pathway for topo II-mediated DNA damage (Fig. 4). Importantly, however, the increased resistance in the absence of Rad54 could be interpreted differently as mentioned above, although these possibilities are not mutually exclusive.

Recently, Helleday and co-workers (50) have investigated the molecular mechanism by which recombination resolves DSBs at DNA replication forks and proposed that HR plays a more prominent role in the repair of such DSBs than does NHEJ. They have also shown by using thymidine that HR alone is required for the repair of slowed replication forks in the absence of detectable DSBs (51). Thus, the prominent role of HR in the repair of replication fork-associated DNA damage is in sharp contrast to the case of topo II-mediated DNA damage.

Earlier work by Muñoz *et al.* (47) suggested a novel role for Ku at G_2/M to explain their observation that Ku86-deficient cells were hypersensitive to ICRF-193. This was, however, based on the assumption that the drug is a pure catalytic inhibitor of topo II. Our current study has revealed the involvement of DNA ligase IV in the (Ku-dependent) repair of ICRF-193-induced DNA damage. This clearly indicates that the damage involves DNA strand breaks, the repair of which absolutely requires the ligase function. Unfortunately, however, we are currently unable to rule out the possibility that DNA ligase IV is (at least in part) required for the repair of single-strand breaks but not DSBs. Further studies will be needed to clarify this point.

Our results with the *RAD54*$^{-/-}$ mutant indicate that although HR plays a (minor) role in the repair of VP-16-induced DNA damage, it is inert in the repair of ICRF-193-induced DNA damage. As discussed above, it is highly unlikely that the difference reflects the longstanding notion that VP-16 causes DSBs while ICRF-193 does not. Most importantly, Snapka and co-workers (52) have recently shown that ICRF-193 does cause DSBs both *in vitro* and *in vivo*. Thus, the differential requirement for HR may be due to differences in strength and/or reversability of topo II poisoning. It is conceivable that topo II poisoning by ICRF-193 is weaker than that by classical topo II poisons that stabilize cleavable complexes. We speculate that strong topo II poisons, especially at high doses, may generate another type of irreversible DNA damage, which cannot be produced by topo II poisoning by ICRF-193 and that this type of damage would require HR (and possibly NHEJ) for its repair (Fig. 4). In contrast, the repair of topo II-mediated DNA damage, which is common to cleavable complexes and topo II clamps, would rely absolutely on NHEJ (Fig. 4). It is also possible that the isoform selectivity of ICRF-193 may contribute to the difference in the involvement of HR. ICRF-193 has recently been shown to target topo IIβ to a greater extent than it targets topo IIα (46, 52). Possibly, the recovery from topo IIβ inhibition may have a stronger preference for NHEJ than topo IIα inhibition. It should be noted, however, that because of the predominance of topo IIα in actively dividing cells, topo IIα poisoning contributes significantly to ICRF-193 cytotoxicity, as evidenced by our present study showing that *TOP2α*$^{+/-}$ cells are less sensitive than wild-type cells to ICRF-193.

Our current findings may shed light on the molecular mechanism for random integration. Random integration is a nonhomologous recombination event that occurs between chromosomal DNA and foreign DNA(s). This phenomenon has been practically applied to generate stable (drug-selectable) transfectants but has been a barrier to efficient gene targeting owing to its overwhelmingly high frequency. Although the precise mechanism of random integration has not been clarified yet, recent studies strongly suggest the involvement of NHEJ. In a murine cell mutant deficient in DNA ligase IV, the random integration frequency is reduced to ~5% (21). Also, the DT40 NHEJ mutants used in this study have a significantly reduced frequency of random integration.[2] Intriguingly, topo II inhibitors have been shown to significantly enhance the random integration frequency in animal cells (49, 53). In contrast, a topo I inhibitor camptothecin does not affect the random integration frequency (49), indicating that the enhancement is specific to topo II inhibition. The mechanism for the enhancement by topo II inhibitors has been largely unclear, especially because VP-16 and ICRF-193 stimulate random integration to a similar extent (49, 53). Significantly, however, given that NHEJ is responsible for random integration, the effect of topo II inhibitors on random integration is very well explained by our conclusion that any DNA damage induced by topo II inhibitors is predominantly repaired by NHEJ. It is, thus, tempting to speculate that topo II poisoning that occurs endogenously can lead to activation of NHEJ, thereby stimulating the random integration. This might be supported by several reports showing that physiological stresses such as acidic pH as well as abasic sites, including base excision repair intermediates, act as topo II poisons (54–57). Thus, the reduction in the level of topo II expression may result in reduced endogenous topo II poisoning, leading to a reduced frequency of random integration. Because the silencing of random integration is one of the desirable strategies for efficient gene targeting, it will be of importance to elucidate the role of topo II in random integration.

Finally, the prominent role of NHEJ in repairing topo II-mediated DNA damage would have significant implications for cancer chemotherapy. Because topo II inhibitors exert much stronger cytotoxicity in the absence of NHEJ (even at very low concentrations), these agents (including not only classical topo II poisons but also weaker poisons such as ICRF-193) should become more powerful anticancer drugs when used in combination with NHEJ inhibitors. For the aim of NHEJ inhibition, gene knockdown of the NHEJ factor(s) by RNA interference may also be feasible. Thus, it is conceivable that the combined use of topo II inhibitors and NHEJ inhibition will be of immense clinical importance.

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Hypersensitivity of Nonhomologous DNA End-joining Mutants to VP-16 and ICRF-193: IMPLICATIONS FOR THE REPAIR OF TOPOISOMERASE II-MEDIATED DNA DAMAGE

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