Severe Combined Immunodeficient Cells Expressing Mutant hRAD54 Exhibit a Marked DNA Double-Strand Break Repair and Error-prone Chromosome Repair Defect

Janice M. Pluth, Laura M. Fried and Cordula U. Kirchgesnner

Department of Radiation Oncology, Stanford University School of Medicine, Stanford, California 94305-5152

ABSTRACT

DNA double-strand breaks (DSBs) can be induced by a number of endogenous and exogenous agents and are lethal events if left unrepaired. DNA DSBs can be repaired by homologous recombination (HR) and nonhomologous end joining (NHEJ). In mammals and higher eukaryotes, NHEJ is thought to be the primary pathway for repair, but the role for each pathway in DNA DSB repair has not been fully elucidated. To define the relative contributions of HR and NHEJ in mammalian DNA DSB repair, cells defective in both pathways were produced. Double-mutant cells were created by expressing a dominant mutant hRAD54 protein in a DNA-dependent protein kinase (DNA-PK)-deficient severe combined immunodeficient cell line. Double-mutant cells demonstrate an increase in ionizing radiation sensitivity and a decrease in DNA DSB repair as compared with either single mutant, whereas single-mutant hRAD54 cells exhibit a wild-type phenotype. Unexpectedly, DNA-PK-null cells were more resistant to mitomycin-C damage than were wild-type cells. Chromosome aberration analysis reveals numerous incomplete chromatid exchange aberrations in the majority of the double-mutant cells after ionizing radiation exposure. Our findings confirm a role for HR in DSB repair in higher eukaryotes, yet indicate that its role is not evident unless the primary repair pathway, NHEJ, is nonfunctional. Mitomycin-C resistance in DNA-PK-null cells compared with wild-type cells suggests that the HR pathway may be more efficient in cross-link repair in the absence of NHEJ. Lastly, the incorrectly repaired chromatid damage observed in double-mutant cells may result from failed recombination or another error-prone repair process that is apparent in the absence of the two primary repair pathways.

INTRODUCTION

DNA DSB repair is critical for the maintenance of genetic integrity. DSBs may occur spontaneously or be induced by IR or radiomimetic drugs. Inaccurate repair of DSBs may result in genetic instability and lead ultimately to carcinogenesis through the activation of oncogenes, inactivation of tumor suppressor genes, or loss of heterozygosity. There are two major DSB repair pathways, NHEJ and HR, and the relative contribution of each of these repair pathways in mammalian DSB repair is still being elucidated. HR, as studied in yeast systems, uses a large region of homologous sequence, such as a sister chromatid or a homologous chromosome, as a template to restore a double-stranded break in DNA with high fidelity (1, 2). Saccharomyces cerevisiae RAD52 epistasis group proteins have been shown to be involved in HR with mutants displaying sensitivity to IR and chemicals known to induce DSBs (3–6). The most sensitive yeast phenotypes are the RAD51, RAD52, and RAD54 mutants, and mammalian homologues of these genes have all been identified and cloned (5, 7–10). The role of these RAD52 epistasis group proteins in mammalian cells has been difficult to study, because knockout mice were either embryonically lethal (RAD51−/−) or displayed no apparent defect (RAD52−/−; Refs. 11–13). Essers et al. (14) reported that ES cells from RAD54−/− mice show reduced HR and increased IR sensitivity. Increased IR sensitivity was also observed in RAD54−/− chicken DT40 cells and RAD54−/− Drosophila (15, 16). Recently Essers et al., (17) further modifiﬁed our understanding of the role of HR in repair of IR damage, by revealing that RAD54-deﬁcient mice exhibit IR sensitivity only at an embryonic stage and not as adults.

The NHEJ pathway of DNA DSB repair does not rely on large regions of DNA sequence homology and often results in deletions and other genomic alterations; thus, it is a low-fidelity type of repair (18, 19). The three subunits of DNA-PK (Ku p70, Ku p86, and DNA-PKcs), the XRCC4 gene product, and DNA ligase IV are all involved in NHEJ, and cells defective in any of these proteins are radiosensitive (20–24). In yeast, HR is the primary pathway for DNA DSB repair; however, in mammals, NHEJ appears to be the major pathway (25). Although HR is the dominant mode of DNA DSB repair in S. cerevisiae, NHEJ has been detected in yeast when HR is defective (26, 27).

Mouse SCID cells defective in DNA-PKcs are ~2- to 3-fold more sensitive to IR than its wild-type, parental strain, C.B-17 (28–31). This IR sensitivity is attributable to a defect in the SCID cells’ ability to perform NHEJ (29, 32–35). The SCID cells’ defect in NHEJ results from a point mutation in the gene encoding for the catalytic subunit of DNA-PK (21, 36–38). Although a truncated protein is produced in SCID cells, it likely has little effect because the phenotype of DNA-PKcs-null mouse cells is essentially identical to that of SCID cells (39–41).

Because NHEJ-defective SCID cells still manage to repair about one-half of DNA DSBs after IR (35), we investigated whether the level of DNA DSB repair observed in SCID cells is attributable, at least in part, to the presence of the HR repair pathway. Others have also attempted to uncover the relative roles of HR and NHEJ in IR sensitivity and DSB repair. Recent work in mammalian cells using a tandemly repeated substrate integrated on a chromosome inferred the use of HR in up to 40% of all of the joining events, of which 30% were a deletion-type HR, and only 10% were gene conversion (42). Given that HR uses homologous DNA as a template, there is probably a cell cycle preference to which pathway is used at any given time. HR is likely preferred during late S and G2 phases, whereas in other phases of the cell cycle, NHEJ is favored. This cell cycle-related preference for each of the pathways was recently documented in chicken DT40 cells (15), with the authors concluding that both path-
ways contribute significantly to maintaining chromosomal DNA during the cell cycle. In addition, work using Drosophila KU70−/−/RAD54−/− double-mutants has shown that there is a synergistic effect of the two DSB repair pathways in repair of IR but not MMS damage (16). Lastly, Essers et al. (17) have revealed that both pathways play a role in IR damage repair at different times during development using SCID/RAD54−/− knockout mice, with HR prominent during early development and NHEJ being the primary pathway in the adult animal.

In the present study, we show the effects of a double defect in HR and NHEJ on DNA DSB repair and chromosome repair in differentiated mammalian cells. We reveal that the role of HR in DNA DSB repair is hidden unless NHEJ is disabled. In addition, the types of chromosome aberrations observed after IR in SCID cells expressing the mutant hRAD54 reveal error-prone repair, showing a predominance of unresolved, incorrectly joined, chromatid exchange aberrations.

MATERIALS AND METHODS

Cell Culture. Transformed fibroblasts from ST. SCID, obtained in our laboratory (29) and the genetically matched parent, C.B-17, were grown in DMEM containing 10% FCS. S/MHB-2, S/mut-4, S/mut-6, C/MHB-2, C/mut-1, and C/mut-3 were grown in the same medium supplemented with 500 µg/ml Genetin (Life Technologies, Inc.).

Subcloning. The point mutant hHR54 cDNA was copied using PCR in such a way as to create KpnI (5′) and XbaI (3′) restriction sites in the cDNA sequence. The primers designed were hR54F-KpnI (5′-AAGGTACCAT-GAGG AGGACGTGGGC TCCACAGCAG-3′) and hHR54R-XbaI (5′-TCCATTGACCCGCAGG GCCCCCCGCTTTCCATGGA-3′). The cDNA was subcloned into the pCIIN3.1-Myc/His B expression vector (Invitrogen), and expanded in Top10™ cells (Invitrogen). Maxipreps were performed using the QIAGEN protocol.

Mammalian Cell Transfections. Prior to transfection, Myc-His B and hHR54-GRT/Myc-His B vectors were linearized with SSPI endonuclease. The cDNA was subcloned into the pcDNA3.1-Myc/His B expression vector (Invitrogen), and expanded in Top10™ cells (Invitrogen). Maxipreps were performed using the QIAGEN protocol.

DSB Repair Assay. DSB repair analysis was performed as a variation of experiments described elsewhere (43). Fibroblasts were seeded at 4 × 10³ cells in T-175 flasks. One day later, the medium was changed to contain 0.02 µCi/ml BrdU at a final concentration of 0.02 µCi/ml. Twenty-four h later, the medium was replaced with nonradioactive medium for ~24 h. Monolayers of cells were trypsinized and resuspended in PBS at 1 × 10⁶ cells/ml in 1.5% low-melt agarose (at 40°C). The cell/agarose mixture was pipetted into 2-mm tubing and was allowed to cool on ice. Solidified cells in agarose were removed from the tubing, cut into 5-mm-long agarose plugs, and placed in cold PBS for irradiation. Plugs were irradiated in PBS at 40 Gy using a 137Cs-irradiator (dose rate of ~3.4 Gy/min) on ice. Asymmetric field inversion gel electrophoresis was performed as described previously (44). Each time point was run three times per experiment. The amount of damage in the unirradiated control was subtracted from the different time points after IR in a given cell line, and each cell line was normalized to the IR-0 time point.

Cell Cycle Analysis. Cell cycle analysis was performed as per manufacturer’s instructions using fluorescence-activated cell sorting (FACS)-Scan (Becton Dickinson). Twenty thousand cells were analyzed per sample.

Cell Culture and Radiation Exposure for Chromosome Aberration Analysis. SCID cells transfected with empty vector (S/MHB-2) and two SCID cell lines transfected with mutant hRAD54 (S/mut-6, S/mut-3) were suspended at a concentration less than 1 × 10⁶/ml and irradiated for 3.92 min (1.53 Gy/min) for a dose equivalent to 6 Gy. After irradiation, cells were released into fresh medium and allowed to recover 24 h prior to harvest. Colcemid was added 4 h prior to harvest at a concentration of 0.1 µg/ml 24 h after irradiation cells were harvested and dropped using standard conditions (0.075 M KCl for 30 min at 37°C, followed by three fixations in methanol/acidic acid 3:1, v/v).

Chromosome Painting. One µl of mouse digoxigenin- and FITC-labeled composite DNA probe specific for mouse chromosomes 2 and 8 (courtesy of Dr. James Tucker, Lawrence Livermore National Laboratory, Livermore, CA) and a 10-kb composite DNA probe specific for mouse chromosomes 2 and 8 (courtesy of Dr. James Tucker, Lawrence Livermore National Laboratory, Livermore, CA) was added to the hybridization mix (50% formamide, 2× SSC, 10% dextran sulfate, and 3.5 µl of mouse Cot-1), for a total volume of 15 µl. Hybridization and washes were performed as described by Spruijt et al. (45). Slides were counterstained with propidium iodide containing Vectorshield (Vector Laboratories, Burlingame, CA) before viewing.

RESULTS

To impair HR, a human RAD54 cDNA containing a mutation changing the lysine residue at position 189 to an arginine in the putative GKT Walker-type nucleotide-binding domain (hHR54-GRT) was transfected into wild-type C.B-17 and DNA-PK-deficient SCID cells. Previous work has shown that this mutant human RAD54 cDNA
is unable to rescue the MMS-sensitive RAD54Δ phenotype in S. cerevisiae cells (10), and overexpression of the equivalent mutant protein in S. cerevisiae leads to a dominant-negative phenotype in yeast (46). In addition, RAD54 belongs to the SNF2/SWI2 family of proteins, and mutation of this ATPase site in these proteins disrupts their function (47). The approximate location of the mutation in the human cDNA (Fig. 1B, * ) and the sites for the primers within the cDNA and pcDNA3.1/Myc-His B vector (Invitrogen) that produce a 1.4-kb fragment are shown in the diagram (Fig. 1B). To confirm expression of the mutant hRAD54 cDNA and verify stability of the transfecants from the vector, RT-PCR was performed throughout the course of experimentation. Fig. 1A demonstrates amplification of the predicted 1.4-kb fragment (Lanes 3 and 5) in C.B-17 and SCID cell lines stably transfected with the mutant cDNA. In comparison, cDNA from cell lines transfected with vector only, C/MHB-2 and S/MHB-2, do not amplify the 1.4-kb fragment (Lanes 2 and 4). Primers to the proapoptotic cDNA, Bax, were used to confirm that the RT-PCR worked and the resulting PCR product was 163 bp.

Sensitivity to MMC was determined in the cell lines to measure impairment of the HR pathway as interstrand cross-links appear to be repaired by HR (48), and cell lines mutant for proteins in the RAD52 epistasis group in yeast demonstrate hypersensitivity to MMC (49). An ~2-fold increase in sensitivity to MMC was observed in two separate cell lines of C.B-17 stably transfected with mutant hRAD54 (C/mut-1 and C/mut-3) compared with C.B-17 cells transfected with vector alone (C/MHB-2; Fig. 2A). Likewise, two SCID cell lines expressing mutant hRAD54 (S/mut-4 and S/mut-6) demonstrated an increased sensitivity to MMC when compared with SCID cells transfected with vector alone (S/MHB-2; Fig. 2A). As a control, RAD54+/− MEFs (No. 2) were assayed for MMC sensitivity (Fig. 2C) and showed a similar level of hypersensitivity to MMC when compared with RAD54+/− MEF cells transfected with wild-type RAD54 (No. 36). Although the various cell lines show differences in their baseline MMC sensitivity, in all cases when RAD54 function is affected, either by mutation or by deletion, an increase in MMC sensitivity is observed.

SCEs have been shown to be mediated by HR; thus, lower levels of SCEs have been observed in cells defective in RAD54 (50). Therefore, to further confirm a defect in the HR pathway in our transfecants, SCE frequencies were determined for wild-type C.B-17 cells transfected with empty vector (C/MHB-2) and C.B-17 cells transfected with mutant hRAD54 (C/mut1). Wild-type C/MHB-2 cells showed roughly twice the level of SCEs compared with the cells expressing the mutant hRAD54 cDNA (24 ± 6 versus 11 ± 4, respectively), confirming the dominant-negative effect of the mutant hRAD54 cDNA on HR function. Thus, the increase in MMC sensitivity and reduced SCE frequencies in cell lines overexpressing the point mutant hRAD54 cDNA strongly indicate that the mutant protein induces a dominant-negative effect on the ability of these cells to perform HR.

Given that HR is impaired in the wild-type C.B-17 and SCID cell lines overexpressing mutant hRAD54, we wanted to determine whether this defect in HR contributes to the IR sensitivity of SCID cells. Wild-type C.B-17 transfecants expressing mutant hRAD54 (C/mut-1 and C/mut-3) did not demonstrate an increase in sensitivity to IR compared with wild-type cells transfected with vector alone (C/MHB-2; Fig. 3A). Likewise, RAD54−/− MEFS were not more sensitive to IR than are RAD54+/− MEFs stably transfected with wild-type hHR54 cDNA (Fig. 3B), although they exhibited hypersensitivity to MMC (Fig. 2C). The MEF cell lines show slightly higher levels of baseline radiosensitivity as compared with the transfected wild-type C.B-17 cells, but in both lines, a defect in RAD54 does not increase radiosensitivity as compared with each line’s respective control. In contrast, SCID transfecants overexpressing mutant hRAD54 (S/mut-4 and S/mut-6) demonstrate an increase in IR sensitivity compared with SCID cells transfected with vector alone (S/MHB-2; Fig. 3A).

To further characterize the IR response, an assay for DNA DSB repair was performed on wild-type C.B-17 cells transfected with vector alone (C/MHB-2), C.B-17 cells transfected with mutant hRAD54 (C/mut-1), SCID cells transfected with empty vector (S/MHB-2), and SCID cells transfected with mutant hRAD54 (S/mut-6). Wild-type C/MHB-2 cells repaired ~85% of DNA DSBs by 6 h after 40 Gy of IR, whereas DNA-PK-deficient S/MHB-2 cells repaired only 57% of DSBs by 6 h postirradiation (Fig. 4). In comparison, SCID/RAD54-defective S/mut-6 cells were capable of repairing only 38% by 6 h after 40 Gy of IR. Wild-type cells transfected with mutant hRAD54 (C/mut-1) performed DSB repair similarly to wild-type cells transfected with vector alone (C/MHB-2, Fig. 4). Thus, defective DSB repair was exhibited with mutant hRAD54 expression only in a SCID background, not in a wild-type C.B-17 background, implying that a pathway involving RAD54 becomes more critical when NHEJ is impaired in mammalian cells.

A surprising observation that warranted further investigation was the finding that wild-type cells transfected with empty vector (C/MHB-2) were more sensitive to MMC than were SCID cells transfected with empty vector (S/MHB-2; Fig. 2, A and B). To determine whether the MMC resistance was attributable to the mutant DNA-PKcs in SCID cells, we analyzed MMC sensitivity in SCID cells complemented with a human chromosome 8 fragment containing the...
DNA-PKcs gene (SCID/100E) and in SCID cells containing part of human chromosome 8 without the DNA-PKcs gene (SCID/50D; Ref. 31). Fig. 5 demonstrates that the DNA-PK-complemented SCID cell line, 100E, displays a MMC sensitivity similar to that of the wild-type C.B-17 cell line. In comparison, DNA-PK-deficient 50D cells show an increased resistance to MMC similar to that of SCID cells. These results suggest that the HR pathway of cross-link repair is more efficient in mammalian cells when the NHEJ pathway is defective. A similar resistance to MMS was observed by Takata et al. (15) in Ku70−/− cells.

To determine the effect of impaired HR and NHEJ on chromosome repair, we looked at chromosome aberrations in both the SCID cells with empty vector and the double-mutant cells transfected with mutant hRAD54. Fifty metaphase spreads were scored for both irradiated and unirradiated samples of SCID cells transfected with empty vector (S/MHB-2) and two SCID lines transfected with the mutant hRAD54 (S/mut-3 and 6 designated #3 and #6, respectively, in Table 1). All of the cell lines had approximately equal modal numbers of chromosomes per metaphase (S/MHB-2, 48 ± 5.4; S/mut-6, 49 ± 10.0; S/mut-3, 47 ± 10.2). Spontaneous aberration frequencies varied between cell lines ranging from 0.22 aberrations per cell in S/mut-3 to 2.22 aberrations per cell in S/mut-6. After irradiation, double-mutant cells (S/mut-3 and S/mut-6) showed an ~3-fold higher aberration frequency compared with that of the cell line with vector alone (S/MHB-2). Inductions observed within each line after irradiation differed because all of the cell lines had distinct baseline aberration frequencies. After IR, S/MHB-2 showed a <2-fold induction (1.8) in aberrations, whereas S/mut-6 showed nearly a 3-fold induction (2.9), and S/mut-3 showed a greater than a 33-fold induction (33.1). Especially striking were the large number of unresolved chromatid exchange aberrations observed in the double-mutant cells (Fig. 6). In the

Fig. 4. DNA DSB repair assay. Asymmetric field inversion gel electrophoresis of cells IR in agarose plugs was used to determine the percentage of DNA DSB rejoined as a function of repair time for wild-type C.B-17 cells transfected with empty vector (C/MHB-2), C.B-17 transfected with mutant hRAD54 (C/mut-1), SCID cells transfected with empty vector (S/MHB-2), and SCID cells transfected with mutant hRAD54 (S/mut-6) after IR with 40 Gy. Values were determined from two independent experiments with three observations at each time point. Error bars, SE.

Fig. 5. MMC resistance in SCID cells attributable to lack of DNA-PK. DNA-PK-complemented SCID cells (100E) exhibit a wild-type degree of MMC sensitivity. SCID cells transfected with chromosome fragment not containing DNA-PK (S/MHB-2) and SCID cells transfected with mutant hRAD54 (S/mut-3 and 6 designated #3 and #6, respectively, in Table 1). All of the cell lines had approximately equal modal numbers of chromosomes per metaphase (S/MHB-2, 48 ± 5.4; S/mut-6, 49 ± 10.0; S/mut-3, 47 ± 10.2). Spontaneous aberration frequencies varied between cell lines ranging from 0.22 aberrations per cell in S/mut-3 to 2.22 aberrations per cell in S/mut-6. After irradiation, double-mutant cells (S/mut-3 and S/mut-6) showed an ~3-fold higher aberration frequency compared with that of the cell line with vector alone (S/MHB-2). Inductions observed within each line after irradiation differed because all of the cell lines had distinct baseline aberration frequencies. After IR, S/MHB-2 showed a <2-fold induction (1.8) in aberrations, whereas S/mut-6 showed nearly a 3-fold induction (2.9), and S/mut-3 showed a greater than a 33-fold induction (33.1). Especially striking were the large number of unresolved chromatin exchange aberrations observed in the double-mutant cells (Fig. 6). In the
majority of double-mutant cells (58% of S/mut-3 cells and 86% of S/mut-6 cells) incomplete chromatid exchange aberrations were observed that joined ~2–26 chromosomes together. These types of aberrations were observed only rarely in wild-type C.B-17 cells transfected with mutant hRAD54 (C/mut -1; <1% of cells) or in SCID cells transfected with empty vector (S/MHB-2; 2% of cells), and when present in these cell lines, the exchanges always involved four or less chromosomes (data not shown).

**DISCUSSION**

Using a dominant-negative system, we examined the roles of HR and NHEJ in mammalian DNA DSB repair. When mutant hRAD54 was expressed in wild-type C.B-17 cells, a hypersensitivity to MMC and a decrease in SCE frequency was observed; however, no change in IR sensitivity as compared with controls was noted. The increased MMC sensitivity and decreased SCE frequency indicate that we have negatively altered HR function in these cells. Although we cannot rule out that there is another function (other than HR) for RAD54, or that our dominant-negative hRAD54 has affected something other than HR (which happens to also affect DNA DSB repair), our dominant-negative hRAD54 appears to be specific to RAD54 function because our cells behave similar to the SCID/RAD54−/− whole animals described by Essers et al. (17). In addition, the results that we observe in our dominant-negative system are also consistent with the findings in RAD54−/− MEFs, which fail to show increased sensitivity to IR but show increased MMC sensitivity compared with that of control RAD54−/− MEFs expressing wild-type hHR54 cDNA. Thus, we conclude that HR has been impaired; however, impairment of HR does not affect IR sensitivity. Therefore, in NHEJ functional, differentiated mammalian cells, HR plays no role, or plays a very minor role, in the repair of IR damage and in DSB repair. In all of the previously studied higher eukaryotic systems (ES cells, DT40 chicken cells, and *Drosophila*) a defect in RAD54 increased IR sensitivity compared with wild-type cells (14). However, a report by Essers et al., (17) reveals that RAD54−/− embryonic and adult differentiated cells respond differently to IR. Mouse embryonic RAD54−/− cells exhibit IR sensitivity, whereas adult RAD54−/− mice fail to show any sensitivity. Thus, DT40 chicken cells and *Drosophila* appear more similar to mouse embryonic cells in terms of the relative importance of HR in repair of IR damage, which is clearly different from what we observe in differentiated eukaryotic cells. This finding is perhaps expected given that both ES and DT40 cells exhibit higher levels of HR and different cell cycle characteristics (higher population in S-G2 phase and a lower population in G1 in asynchronously growing cultures) compared with our differentiated fibroblast cells.

A reciprocal pattern is observed in *S. cerevisiae*, which uses the HR pathway predominately (26, 27, 51, 52). A complete deletion mutant of *HDF1*, the *S. cerevisiae* homologue of the gene-encoding mammalian Ku70, did not show any sensitivity to IR. However, in a RAD52 mutant background in which HR is impaired, additional sensitization in response to IR was observed, leading to the conclusion that HDF1 plays a role in a non-HR repair process that can only be observed when HR is disabled. Takata et al. (15) created RAD54−/−, *KU70−/−*, and RAD54−/−/IKU70−/− cells from the chicken B-cell line DT40. In these cell lines, the γ-radiation sensitivity of the double-mutant cells was profoundly greater than that of either single mutant, indicating two complimentary repair pathways. These researchers also demonstrated that RAD54−/− chicken cells were more sensitive to IR than are *KU70−/−* chicken cells, indicating HR as the primary DSB repair pathway and NHEJ as a secondary pathway, similar to what was observed in yeast. In fact, *KU70−/−* chicken cells display an increased resistance to IR compared with wild-type chicken cells at higher doses. These researchers interpret these results as being attributable to stimulation of HR in cells defective in NHEJ. In

![Fig. 6. Fluorescence in situ hybridization analysis of chromosome aberrations in SCID cells transfected with mutant hRAD54 after exposure to 6 Gy of IR.](image-url)}
contrast to what was observed in yeast and chicken cells, we find that the NHEJ pathway predominates over HR in the repair of IR-induced damage and in DSB repair in mammalian differentiated cells.

Although no increase in radiation sensitivity was observed in a wild-type, C.B-17 background, expression of dominant-negative hRAD54 in a SCID background resulted in an increase in radiation sensitivity. The implications of this are 2-fold. First, SCID cells, which are defective in DNA-PKcs, a protein involved in NHEJ, are more sensitive to IR than wild-type C.B-17 cells. Thus, to observe an augmentation of this radiosensitivity strongly implies that DNA-PKcs and RAD54 are part of two separate pathways of DNA DSB repair. These results are confirmed in the DSB break repair assay. Second, mutant hRAD54 only affects IR sensitivity in a SCID mutant background, not in a wild-type C.B-17 background, indicating that HR becomes more crucial when NHEJ is impaired in differentiated mammalian cells. Therefore, in mammalian fibroblasts, the importance of HR is uncovered only in NHEJ-deficient cells. In addition, an increase in resistance to MMC is observed in NHEJ-deficient cells (Fig. 2, A and B), with SCID cells containing empty vector (S/MHB-2) exhibiting more resistance to MMC than wild-type C.B-17 cells containing empty vector (C/MHB-2). Correspondingly, SCID cells complemented for DNA-PK (100E) show a sensitivity to MMC (Fig. 5) similar to wild-type C.B-17 cells, whereas DNA-PK-deficient cells (50D) show an increased resistance to MMC similar to that of SCID.

Higher chromosome aberration frequency inductions were observed in the double-mutant cells after IR when compared with the SCID cells containing the vector alone. Particularly striking was the large number of unresolved complex chromatid-exchange-type aberrations observed in the double-mutant cells (Fig. 6). These incomplete exchange aberrations, resulted in multiple chromatid attachments among several chromosomes (up to 26 chromosomes in one case). Chromatid aberrations are often indicative of damage occurring in the preceding S-G2 phase of the cell cycle. Interestingly, no comment was made of these types of incomplete chromatid aberrations in the chromosome analysis of chicken DT40 cells deficient in Ku70 and RAD54, although the authors (15) did note an overall increase in chromatid aberrations. The lack of this finding in chicken DT40 cells could be attributable to the differing level of importance of the HR pathway in the two cell types (as was previously mentioned) or because Ku was deficient in these cells rather than DNA-PK as in our cells. The error-prone repair observed in our double-mutant cells could represent incomplete repair attributable to the presence of an aberrant hRAD54 protein, or it could be the result of a low-fidelity repair process (perhaps an additional pathway initiated in the absence of both the NHEJ and HR). In both yeast and T4 bacteriophage, other repair pathways besides the two predominant pathways have yet to be elucidated.

Numerous unresolved complex chromatid-exchange aberrations have also been observed in ataxia telangiectasia cells exposed to camptothecin, a topoisomerase I poison specific to S phase cells (55). These researchers concluded that in the absence of a functional ATM protein DSBs formed were processed abnormally, resulting in many heterologous chromatid exchanges that were observed only infrequently in camptothecin-treated wild-type cells. The fact that we also observed high levels of incorrectly repaired S-phase damage does not imply that our double-mutant cells have a defect only in S-phase repair. Observing primarily error-prone S-phase damage could reflect the fact that we harvested cells 24 h after IR, because of the slow recovery of these cells, the majority of the cells that made it to metaphase by harvest were ones in S-G2 phase at the time of IR. Further analysis harvesting cells at longer time periods after IR would be necessary to determine whether error-prone repair is also observed in other phases of the cell cycle in these double-mutant cells.

Unresolved chromatid exchanges are also frequently observed in FA patients’ cells when treated with a DNA cross-linker, diepoxybutane (56). FA is an inherited disorder displaying chromosome instability and extreme cancer proneness (57). Cells from FA patients show extreme sensitivity to cross-linking agents, and the underlying defect has been hypothesized to be in a DNA-repair pathway. Although no obvious DNA-repair defect has been firmly established in FA, recent reports indicate possible defects in NHEJ and in the fidelity of repair (58–60). The similarity between aberrations in FA cells and those we describe for our double-mutant cells may suggest that an in-depth investigation of the NHEJ and HR pathways in FA cells might prove illuminating. Perhaps various FA complementation group proteins provide an interface for the NHEJ and HR pathways and aid in coordinating DNA repair after DNA damage.

In summary, by constructing a SCID/RAD54 double-mutant cell line, we have created a unique genetic system to study the involvement of two pathways, NHEJ and HR, in DNA DSB repair in response to IR. Our finding that HR plays a minor role in DNA DSB repair unless NHEJ is defective confirms the notion that NHEJ is the primary DNA DSB repair pathway in mammalian fibroblast cells. This result is in contrast to previously published reports in mouse ES cells, chicken DT40 cells, and Drosophila, which all show a role for HR even in NHEJ functional cells. In addition, our studies reveal HR to be more efficient in repairing MMC damage in the absence of NHEJ. And lastly, data from chromosome aberration studies after IR reveal that a defect in both HR and NHEJ, results in severe genetic instability and error-prone repair.

ACKNOWLEDGMENTS

We thank Roland Kanaar and Jeroen Essers for RAD54+/− MEFs (no. 2), RAD54−/− MEFs expressing wild-type hRAD54 cDNA (no. 36), and hHR54 cDNA, as well as for helpful discussions. We also thank James Tucker for the mouse 2:8 probe for fluorescence in situ hybridization analysis and Jim Evans, Vikky Yamazaki, and Susannah Green for their assistance.

REFERENCES


Severe Combined Immunodeficient Cells Expressing Mutant hRAD54 Exhibit a Marked DNA Double-Strand Break Repair and Error-prone Chromosome Repair Defect

Janice M. Pluth, Laura M. Fried and Cordula U. Kirchgessner


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/61/6/2649

Cited articles
This article cites 57 articles, 24 of which you can access for free at:
http://cancerres.aacrjournals.org/content/61/6/2649.full.html#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/61/6/2649.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.