[CANCER RESEARCH 64, 7139–7143, October 1, 2004]

ATR Affecting Cell Radiosensitivity Is Dependent on Homologous Recombination Repair but Independent of Nonhomologous End Joining

Hongyan Wang,[1] Huichen Wang,[1] Simon N. Powell,[2] George Iliakis,[3] and Ya Wang[1]

[1]Department of Radiation Oncology, Kimmel Cancer Center of Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania; [2]Laboratory of Molecular and Cellular Radiation Biology, Department of Radiation Oncology, Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts; and [3]Institute of Medical Radiation Biology, University of Duisburg-Essen Medical School, Essen, Germany

ABSTRACT

ATR is one of the most important checkpoint proteins in mammalian cells responding to DNA damage. Cells defective in normal ATR activity are sensitive to ionizing radiation (IR). The mechanism by which ATR protects the cells from IR-induced killing remains unclear. DNA double-strand breaks (DSBs) induced by IR are critical lesions for cell survival. Two major DNA DSB repair pathways exist in mammalian cells: homologous recombination repair (HRR) and nonhomologous end joining (NHEJ). We show that the doxycycline (dox)-induced ATR kinase dead (ATRkd) cells have the similar inductions and rejoining rates of DNA DSBs compared with cells without dox induction, although the dox-induced ATRkd cells are more sensitive to IR and have the deficient S and G_2 checkpoints. We also show that the dox-induced ATRkd cells have a lower HRR efficiency compared with the cells without dox induction. These results indicate that the effects of ATR on cell radiosensitivity are independent of NHEJ but are linked to HRR that may be affected by the deficient S and G_2 checkpoints.

INTRODUCTION

Double-strand breaks (DSBs) induced in DNA by ionizing radiation (IR) are critical lesions, which, if not repaired, can lead to cell killing, mutation, or transformation. Two major complementary DSB repair pathways exist in mammalian cells: nonhomologous end joining (NHEJ) and homologous recombination repair (HRR). NHEJ repair is a fast process that requires Ku80, Ku70, DNA-dependent kinase catalytic subunit (DNA-PKcs), ligase IV, and XRCC4 (1). HRR repair is a slow process that requires Rad51, Rad52, Rad54, and the Rad51 paralogs, including XRCC2, XRCC3, Rad51B, Rad51C, and Rad51D (2). Deficiencies in either NHEJ or HRR in animals lead to increased sensitivity to IR, indicating that both NHEJ and HRR are important to genomic integrity (1). After exposure to IR, proliferating cells slow progression through the cell cycle (G_1, S, and G_2 phases) by activating DNA damage-induced checkpoints that are regulated by protein kinase pathways (3, 4). It is believed that checkpoint activation promotes DNA repair and benefits genomic integrity (5, 6). However, the relationships between checkpoint activation and the two DNA DSB repair pathways, NHEJ and HRR, remain to be elucidated. Our results recently suggest that NHEJ is a checkpoint-independent process (7) and that multi-checkpoint activation may affect HRR (8).

ATR is one of the most important checkpoint proteins of cells for responding to DNA damage inducers, including IR and UV (9, 10). This response differs from ATM, another important checkpoint protein that mainly responds to IR but not to UV-induced DNA damage. The role of ATM in cellular radiosensitivity is linked to HRR (11, 12), but which pathway, HRR or NHEJ or both, is responsible for the effects of ATR on the sensitivity of cells to IR remains unclear. Our results previously suggested that DNA damage-induced checkpoint facilitates HRR but not NHEJ (7, 8). To test this hypothesis that the role of ATR in cellular radiosensitivity is linked to HRR but does not affect NHEJ, we used an ATR kinase dead (ATRkd) cell line (9) to study the relationship between checkpoint response and DNA repair in these cells after exposure to IR. We show that the ATRkd cells with doxycycline (dox) induction have similar inductions and rejoining rates of DNA DSBs compared with the cells without dox induction, although the dox-induced ATRkd cells are more sensitive to IR and have the deficient S and G_2 checkpoints. We also show that the dox-induced ATRkd cells have a lower HRR efficiency compared with the cells without dox induction. These results indicate that the effects of ATR on cell radiosensitivity are independent of NHEJ but are linked to HRR that may be affected by the deficient S and G_2 checkpoints.

MATERIALS AND METHODS

Cell Line and Irradiation. ATRkd cells (provided by Dr. Karlene A. Cimprich, Stanford University School of Medicine, Palo Alto, CA) are transformed human fibroblast cell lines expressed with kinase-dead ATR regulating under an inducible tetracycline system (Tet-On) (9). These cells were grown in DMEM supplemented with 10% bovine calf serum. At the start of each experiment, cells were grown in the absence or presence of 1 μg/mL dox for 24 to 48 hours in the growth medium. Addition of dox to the culture results in expression of kinase-dead ATR of the ATRkd cells, which inhibits normal ATR function (9). High-energy particles with high linear energy transfer (LET) exposure were carried out using the alternating gradient synchrotron (Fe ions, 1 GeV/amu) at Brookhaven National Laboratory (Upton, NY). The high LET IR dose rate is ~1 Gy/min.

NHEJ Measurement. The induction and rejoining of DNA DSBs by NHEJ were measured by asymmetric field inversion gel electrophoresis (AFIGE) assay. AFIGE was performed as described previously (7, 13) with minor modification. Cells in cold medium were irradiated and returned to the incubator at 37°C. At various times thereafter, cells were collected and mixed with an equal volume of 1% agarose (InCert agarose; FMC Corporation, Philadelphia, PA) at a final concentration of 3×10^6 cells/mL to prepare 3- × 5-mm cylindrical blocks containing ~1×10^5 cells. A similar protocol also was used to determine induction of DNA DSBs, except that in this case cells were embedded in agarose blocks before irradiation. The blocks were placed in lysis buffer [10 mmol/L Tris, (pH 8.0), 50 mmol/L NaCl, 0.5 mol/L EDTA, 2% N-lauryl sarcosyl, and 0.1 mg/mL proteinase E] immediately after irradiation. Blocks in lysis buffer were incubated first at 4°C for 45 minutes and then at 50°C for 16 to 18 hours. The blocks were washed in a buffer containing 10 mmol/L Tris (pH 8.0) and 0.1 mol/L EDTA and treated at 37°C for 1 hour with 0.1 mg/mL RNase A in the same buffer. AFIGE was carried out in 0.5% Seakem agarose (FMC Corporation) in 0.5× TBE [45 mmol/L Tris (pH 8.2), 45 mmol/L boric acid, and 1 mmol/L EDTA] at 10°C for 40 hours. During this time, cycles of 1.25 V/cm for 900 seconds in the direction of DNA migration alternated with 5 V/cm for 75 seconds in the reverse direction. The agarose gels were stained with ethidium bromide (0.5 μg/mL) for 6 hours at room temperature and destained with H_2O for 1 hour. To quantitate DNA DSBs, the fraction of activity released from the well into the lane in irradiated and nonirradiated samples was calculated using a fluorescence image measured with a PhosphoImager (Typhoon 8600; Amersham Biosystems, Piscataway, NJ).

Received 4/13/04; revised 7/6/04; accepted 7/21/04.

Grant support: NASA grant NAG2–1628 and NIH grants T32-CA09137 and P30-CA56036.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Ya Wang, Thomas Jefferson University, Thompson Building, B-1, 1020 Sansom Street, Philadelphia, PA 19107. Phone: 215-955-2045; Fax: 215-955-2052; E-mail: ya.wang@mail.tju.edu.

Colony-Forming Assay. Cellular sensitivity to radiation was determined by loss of the colony-forming ability. Briefly, 2×10^5 cells were plated per T25 flask with 5 mL of medium. The cells were irradiated 48 hours later. The cells then were collected and plated, aiming at 20 to 200 colonies per flask. Two replicates were prepared for each datum point and were incubated for 2 weeks to allow colonies to develop. Colonies were stained with crystal violet (100% methanol solution) before counting.

DNA Synthesis. The measurement of DNA synthesis was similar to that described previously (14). Briefly, 2×10^5 cells from a growing culture were seeded in a T25 flask with 5 mL of medium. Forty-eight hours later, the cells were exposed to different doses of high LET particles and returned to 37°C for 3 hours. [^{3}H]thymidine at 1 μCi then was added to the cultures for another 1 hour. After the cells were trypsinized, 0.5 mL of suspension was taken from collected cultures (4 mL in total) for cell counting. The rest of the cultures were loaded onto the microfiber filters. The following procedures were performed as described (14). The rate of DNA synthesis for each sample was calculated as [^{3}H] dpm/cell number and is presented as a percentage of the control values obtained from sham-irradiated cells at the same time point.

Flow Cytometry Measurement. Briefly, 2×10^5 cells were plated in a T25 flask with 5 mL of medium. Forty-eight hours later, cells were exposed to 3 Gy high LET particles and returned to 37°C. At different times thereafter, cells were trypsinized and fixed in 70% ethanol. Cells were stained in a solution containing 62 μg/mL RNase A, 40 μg/mL propidium iodide, and 0.1% Triton X-100 in PBS buffer at room temperature for 1 hour. The distribution of cells in the cell cycle was measured in a flow cytometer (Coulter Epics Elite; Beckman Coulter, Fullerton, CA).

Western Blot Analysis. The ATRkd cells in T25 flasks with or without dox induction were irradiated with high LET IR and then were returned to 37°C. Three hours later, the cells were collected and counted. A total of 1×10^6 cells were lysed in 50 μL of RIPA lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL each aprotinin, leupeptin, and pepstatin, 1 mmol/L Na_3VO_4, and 1 mmol/L NaF] and mixed with 50 μL of 2× protein loading buffer. After boiling for 5 minutes, 40 μL (for phospho-CHK1) or 20 μL (for FLAG, ATR, regular CHK1, and CDC25A) of whole cell lyses were loaded into either 8% (for FLAG and ATR) or 10% (For CHK1 and CDC25A) polyacrylamide gel. The FLAG antibody (F4042) was purchased from Sigma-Aldrich Inc. (St. Louis, MO). The ATR antibody (*sc-1887*), CHK1 antibody (*sc-8404*), and CDC25A antibody (*sc-7389*) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The phospho-CHK1 (Ser345) antibody was purchased from Cell Signal Technology (Beverly, MA).

HRR Measurement. pDR-green fluorescent protein (GFP; obtained from Dr. Jasin's laboratory, Sloan-Kettering Institute, New York, NY) containing a mutated *GFP* gene with an 18 bp *I-SceI* site (15) was transfected into the ATRkd cells using Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA). The stable transfected cell lines were selected by growing in medium containing 5 μg/mL of puromycin. Puromycin-resistant colonies were screened by Southern blot analyses for an intact DR-GFP reporter. A 714-bp *GFP* coding fragment obtained by PCR system 9700 (Perkin Elmer Inc., Wellesley, MA) with primers forward 5′-ATGGTGAGCAAGGGCGAG-GAGCT-3′ and reverse 5′-CTTGTACAGCTCGTCCATGCCGA-3′ from template pDR-GFP labeled with ^{32}P as the probe. Ten micrograms of genomic DNA from puromycin-resistant colonies were digested by *Sal*I and *Hind*III and separated on a 0.7% agarose gel and transferred to a nylon membrane. Hybridization was carried out under standard conditions using the ^{32}P-labeled 714-bp probe to test whether these puromycin-resistant colonies had integrated an intact DR-GFP fragment. A radiosensitive screen exposed with the hybridized membrane was analyzed using a PhosphoImager (Storm 840; Amersham) with the ImageQuaNT analysis software (Amersham). The positive cell lines were named A-DRGFP; 20% of the analyzed puromycin-resistant ATRkd clones had randomly integrated an intact DR-GFP repair substrate.

To evaluate HRR of DNA DSBs, A-DRGFP cells were first transfected with pGFP (containing full-length cDNA of *GFP*) with or without dox induction to induce transient expression of *GFP* gene. GFP expression was assayed on different days after transfection by flow cytometry (XL/MCL; Beckman Coulter) using an argon ion laser emitting at 488 nm. GFP emission was collected at photomultiplier tube 1 (PMT1) using a 525-bp filter. Results were collected as dot plots of PMT1 (525 bp) to facilitate discrimination between GFP-positive and GFP-negative cells. Overall, 70 to 90% of the GFP-positive cells were observed in these pGFP-transfected A-DRGFP cells, and dox induction did not affect the transfection frequency. A-DRGFP cells then were transfected with pCMV3xnlsI-SceI plasmid (obtained from Dr. Nickoloff's laboratory, University of New Mexico, School of Medicine, Albuquerque, NM; ref. 16) to induce transient expression of I-SceI endonuclease and to generate a DSB at the integrated sequences of *GFP* gene with or without dox treatment. The frequency of recombination events was calculated from the frequency of GFP-positive cells in A-DRGFP cells transfected with *I-SceI*, subtracting the GFP-positive cells in A-DRGFP cells without transfection, and dividing by the GFP-positive cells in A-DRGFP cells transfected with pGFP.

RESULTS

ATRkd Cells Show Normal NHEJ Repair after Exposure to High LET IR. IR-induced DNA DSBs result in the most severe damage affecting cell survival. NHEJ and HRR are the two major pathways for repairing DNA DSBs in mammalian cells. ATRkd cells are sensitive to IR-induced killing (9). However, to which pathway the radiosensitivities of ATRkd cells are related, NHEJ or HRR or both, needs to be clarified. For this purpose, we examined the DNA DSB induction and rejoining by using AFIGE assay in ATRkd cells with or without dox induction. The cells with or without dox induction showed the same levels of induction and rejoining of DNA DSBs (Fig. 1), although they showed a great difference in their survival (Fig. 2*A*). The cells with or without dox induction also showed the same levels of induction and rejoining of DNA DSBs after exposed to low LET X-ray, although the repair time is much shorter than for cells exposed to the high LET high-energy particle (data not shown). These results suggest that ATR does not affect induction of DNA DSBs and NHEJ repair.

ATRkd Cells Are Sensitive to High LET IR-Induced Killing with Impaired S and G_2 Checkpoint Responses. Overexpression of ATRkd protein results in the cells being sensitive to γ-ray, the low

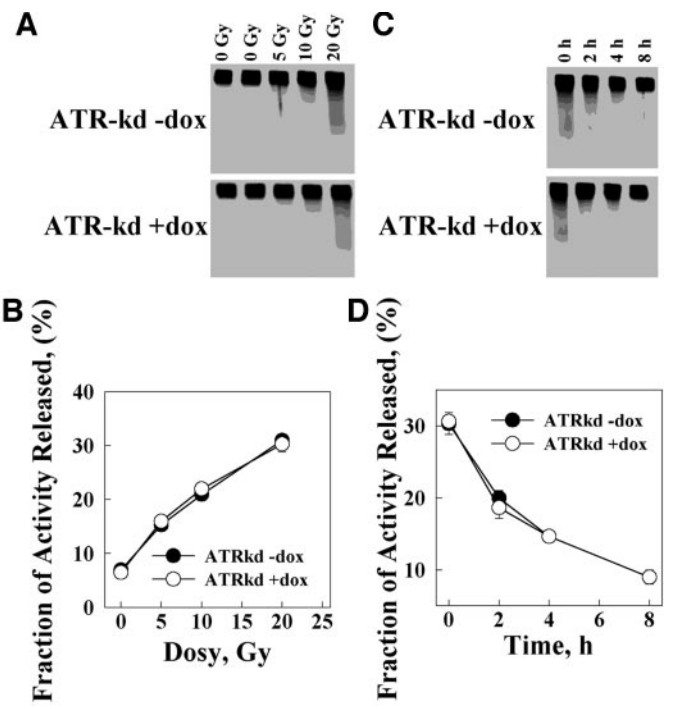

Fig. 1. ATRkd cells show similar induction and rejoining rates of DNA DSBs. Induction and rejoining of IR-induced DNA DSBs in ATRkd cells with or without dox induction assayed by AFIGE as described in Materials and Methods. *A* and *B*, induction of DNA DSBs. *C* and *D*, kinetic rejoining of DNA DSBs after exposure to 20 Gy (high LET IR). *B* and *D* are the quantification of the gel results shown in *A* and *C*. Data shown are the average SE from three independent experiments.

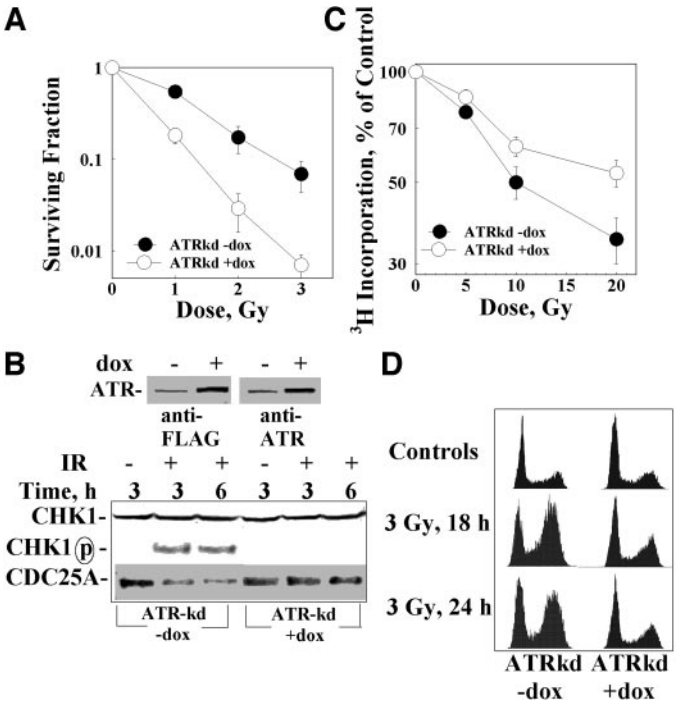

Fig. 2. ATRkd cells are sensitive to high LET IR-induced killing with impaired S and G_2 checkpoints. *A*, Clonogenic assay was performed as described in Materials and Methods. Three replicates were prepared for each datum point and incubated for 2 weeks. Data shown are the average and SE from three independent experiments. *B*, Western blot analysis. *Top*, inducible expression of ATRkd in ATRkd cells. Immunodetection of ATRkd in ATRkd cells with or without dox induction for 24 hours using anti-FLAG and anti-ATR antibodies. *Bottom*, ATR activity was examined by Western blot analysis to measure the levels of CHK1 phosphorylation (CHK1-p) and CDC25A degradation at 3 hours and 6 hours after IR. CHK1 protein as a control indicates the loading amount. Similar results were obtained from two independent experiments. *C*, S checkpoint was examined by measuring [^{3}H]thymidine incorporation at 3 hours following IR as described in Materials and Methods. Data shown are the average and SE from three independent experiments. *D*, G_2 checkpoint was examined by analyzing flow cytometry samples prepared at different times following IR as described in Materials and Methods.

LET IR-induced killing (9). The effects of ATR kinase on cellular response to high LET IR have not yet been investigated. To confirm that the results of DNA DSBs (Fig. 1) were derived from the cells with different radiosensitivity, we also examined the clonogenic ability after the ATRkd cells were exposed to high LET iron (^{56}Fe) at 1 GeV/nucleon with or without dox induction. Similar to the results derived from the cells exposed to low LET IR (9), dox induction results in the cells being sensitive to high LET IR-induced killing (Fig. 2*A*). To examine whether the radiosensitivity of the cells to high LET IR with dox induction was related to the mutant ATR (ATRkd) expression, we measured the levels of FLAG and ATR in the cells with and without dox induction because the cDNA of ATRkd was tagged with an amino-terminal FLAG epitope and placed under the control of the tet promoter (9). Similar to the results reported previously (9), the FLAG and ATR expression dramatically increased after dox induction (Fig. 2*B*). To confirm that the expressed ATR was ATRkd, we measured CHK1 phosphorylation and CHK1 activity in these cells because CHK1 is one of the most important substrates of ATR (17–20). Without dox induction, the cells showed a CHK1 phosphorylation signal after IR, but the cells with dox induction did not show any CHK1 phosphorylation signal (Fig. 2*B*). CDC25A is a major substrate of CHK1 to regulate checkpoints after IR (21). The level of CDC25 in the cells without dox induction was dramatically reduced after IR (31% of the control level at 3 hours and 22% of the control level at 6 hours), but the level of CDC25 in the cells with dox induction was only slightly reduced after IR (83% of the control level at 3 hours and 72% of the control level at 6 hours), indicating less degradation of CDC25A in the cells with dox induction (Fig. 2*B*). Besides CHK1, CHK2 also could phosphorylate CDC25A to respond to IR-induced damage (22). To identify which kinase, CHK1 or CHK2, was responsible for less degradation of CDC25A in the dox-induced ATRkd cells, we measured CHK2 phosphorylation. We did not observe any difference in CHK2 activity and in phosphorylation between the cells with or without dox induction after high LET IR (data not shown), indicating that the lower degradation of CDC25A shown in the cells with dox induction is because of CHK1 and confirming that dox induction does inhibit normal ATR activity and that this inhibition results in radiosensitivity of the cells to high LET IR.

HRR mainly occurs in S and G_2 phases (23), and it is reasoned that S and G_2 checkpoint will facilitate HRR. ATR is one of the most important checkpoint proteins to respond to DNA damage in mammalian cells. On the basis of our hypothesis that checkpoint response promotes HRR, we investigated the S- and G_2-phase checkpoint responses in high LET-irradiated ATRkd cells with or without dox induction. At 3 hours after IR, cells with dox induction showed less inhibition of DNA replication than cells without dox induction (Fig. 2*C*), suggesting that an impaired S-phase (S) checkpoint exists in the cells with overexpressed ATRkd protein. These results differ from those reported before, which show that the cells with overexpressed ATRkd protein do not show less inhibition of DNA replication following IR compared with the cells without dox induction (9). We believe that the different results are caused by different supplemental times of [^{3}H]thymidine into cell culture because there is no clear difference of DNA synthesis between the cells with dox induction and the cells without dox induction at 1 hour after IR (data not shown). We recently reported that there are two different but overlapping pathways: ATM and ATR regulated IR-induced S checkpoint in mammalian cells (14). The ATM-dependent pathway regulated the early response of DNA replication in cells after IR, and the ATR-dependent pathway regulated the late response in cells after IR. Adding [^{3}H]thymidine to the cell culture immediately after IR and collecting these cells 4 hours later (9) will reflect the responses regulated by the ATM- and ATR-dependent pathways. Such measurement in the cells may hide the impaired ATR-dependent S checkpoint regulation because ATRkd cells have normal or overactivated ATM function. Therefore, we added [^{3}H]thymidine to the cell cultures 3 hours after IR and collected the cells 1 hour later and observed the different S checkpoint responses between ATRkd cells with and without dox induction. Similar results also were observed with the cells exposed to X-ray, the low LET IR (data not shown).

There are two molecular distinct G_2-M checkpoints in DNA-damaged cells (24): one occurs early after IR, is transient, ATM dependent and dose independent, and represents the failure of cells that had been in G_2 at the time of irradiation to progress into mitosis; the other one, G_2-M accumulation, typically assessed by propidium iodide staining, begins to be measurable only several hours after IR, is ATM independent, dose dependent, and represents the accumulation of cells that had been in earlier phases of the cell cycle at the time of exposure to radiation (24). The ATR/CHK1 pathway contributes to the late G_2-M accumulation, G_2 arrest (25, 26). Besides the impaired S checkpoint response shown in dox-induced ATRkd cells, G_2 arrest also was reduced to much lower levels in these cells compared with those without dox induction after high LET IR (Fig. 2*D*). These results suggest that, similar to that in low LET-irradiated cells, ATR-regulated multi-checkpoints also play an important role in maintaining cell survival following high LET IR.

ATRkd Cells Show Lower HRR Efficiency after DNA DSBs. The pDR-GFP plasmid was transfected into ATRkd cells, and clones that had randomly integrated the substrate into the genome were

selected with puromycin. Two independently isolated clones were identified by Southern analysis to have undergone integration of an intact, single copy of this transfected substrate (Fig. 3*A*) and were named A-DRGFP4 and A-DRGFP8. Multiple digests were performed to confirm that the integrated recombination substrate was a single copy and that no gross changes in the integrity of the reporter substrate had occurred before integration (data not shown). To detect HRR of an induced chromosomal DSB, the pGFP or I-SceI expression vector pCMV3xnlsI-SceI was transiently transfected into A-DRGFP4 and A-DRGFP8 cells, and flow cytometry was used to quantify GFP-positive cells (Fig. 3*B*). pGFP containing full-length cDNA of GFP was transfected to these cells as a calibration control of GFP-positive cells (Fig. 3*B*). Because of the slow growth characteristics of ATRkd cells, flow cytometry was performed at different time points to determine the time after transfection for maximal detection of GFP-positive cells. Maximal GFP-positive cells were detected at 5 days after transfection of pCMV3xnlsI-SceI. Equal amounts of GFP-positive cells were observed in A-DRGFP4 (Fig. 3*B*) and A-DRGFP8 cells (data not shown) with or without dox induction at different days after transfection with pGFP, indicating dox induction does not affect the transfection efficiency. Approximately 1% of GFP-positive cells were detected without transfection of pCMV3xnlsI-SceI (Fig. 3*B*). Compared with the GFP-positive cells without *I-SceI* transfection from other reports (15, 27), the number that we obtained is relatively higher, indicating that more events of spontaneous intrachromosomal gene conversion occurred in the A-DRGFP cells. However, with *I-SceI* transfection, A-DRGFP cells without dox induction showed many more GFP-positive cells than the untransfected control (Fig. 3*B*), indicating that these increased GFP-positive cells from I-SceI expression were from DSB-induced recombination. At the same time, after *I-SceI* transfection, A-DRGFP cells with dox induction reduced their GFP-positive cells to half (Fig. 3*B* and *C*) at all of the days after transfection, which provides direct evidence that ATR affects the DSB-induced recombination.

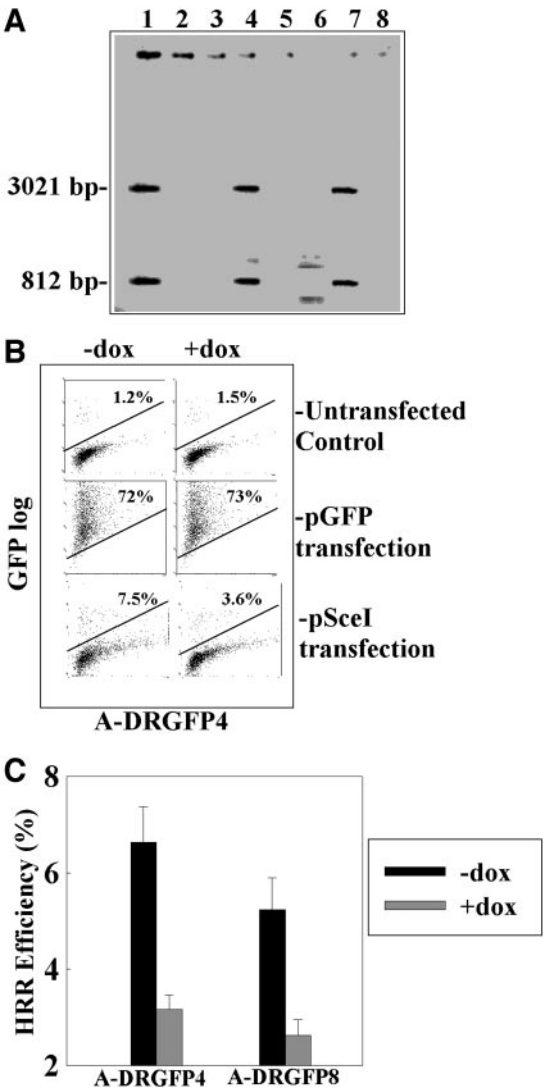

Fig. 3. ATRkd cells show impaired HRR. *A*, Southern blot analysis of ATRkd clones derived from transfection of the DR-GFP substrate. Genomic DNA from puromycin-resistant clones was digested with *Sal*I and *Hind*III, 3021 and 812 bp GFP coding fragments were detected with a radiolabeled GFP probe as described in Materials and Methods. *Lane 1* is the positive control obtained from Draa-40 (hamster cells) transfected with pDR-GFP (15). *Lanes 2* through *8* are clones 2, 3, 4, 6, 7, 8, and 9. Clones 4 and 8 contain the GFP fragments and are named as A-DRGFP4 and A-DRGFP8, respectively. *B*, representative flow cytometric analyses of A-DRGFP4 cells with or without dox induction following DSBs. *Top*, A-DRGFP4 cells without additional plasmid transfection. *Middle*, A-DRGFP4 cells 5 days following pGFP plasmid transfection. *Bottom*, A-DRGFP4 cells 5 days following pDR-GFP plasmid transfection. *C*, HRR efficiency was calculated by GFP-positive cells in I-SceI–transfected A-DRGFP cells, subtracting the background from untransfected controls and dividing by the GFP-positive number from pGFP-transfected A-DRGFP cells. Data shown are the average SE from three independent experiments.

DISCUSSION

We provide evidence in this report that ATR does not affect NHEJ but promotes HRR. ATR is one of the most important checkpoint proteins in mammalian cells. $Atr^{-/-}$ is lethal in mammalian cells (28), and the absence of ATR mutations in human diseases, which limits the function of ATR, is being widely studied. The cell line ATRkd expression of a dominant-negative ATR provides a useful tool to study ATR function. It is known that these cells are sensitive to almost all of the forms of DNA damage (9, 29, 30), showing the important role of ATR in maintaining cell survival after DNA damage. IR-induced DNA DSBs result in the most severe damage for cells to survive. NHEJ and HRR are critical for repair of DNA DSBs induced by IR in mammalian cells (1). ATR affects cell radiosensitivity, but by which pathway, NHEJ or HRR, remains unclear. In this study, we show that ATRkd cells with and without dox induction have equal inductions of DNA DSBs and similar rejoining rates of DNA DSBs following IR, although those cells with dox induction are more sensitive to IR-induced killing than the cells without dox induction. We also show that ATRkd cells with dox induction have less HRR efficiency compared with cells without dox induction. These results indicate that ATR affects cell radiosensitivity via the HRR pathway but not via the NHEJ pathway.

Compared with their wild-type counterparts, other gene mutant cell lines, such as XRCC3 (15), BRCA1 (31), and BRCA2 (27), show 4- to 10-fold reduction of I-SceI–induced DSB recombination. The ATR-kdDRGFP cells with dox induction show only a twofold reduction of I-SceI–induced recombination compared with the ATR-kdDRGFP cells without dox induction, which may be explained by the fact that the ATRkd mutant cell line still has some functions of ATR because $Atr^{-/-}$ mouse cells are lethal (28) and ATRkd cells with dox induction are viable with normal growth (9); therefore, the sensitivity of the ATRkd mutant cells to IR-induced killing is less severe than in the aforementioned mutant cell lines.

The mechanism by which ATR affects HRR remains unknown. We believe that this mechanism may be related to the checkpoint regulation of ATR. Checkpoint activation and DNA repair are independent processes. All of the checkpoints, including G_1, S, and G_2 checkpoints, are important for genomic integrity following exposure to DNA-damage inducers. However, S and G_2 checkpoints correlate more with radioresistance to killing. Besides ATM, ATR also con-

tributes to IR-induced S and G_2 checkpoint response in mammalian cells (9, 14, 25, 26). ATM and ATR have partly overlapping roles but have distinctive roles in the signaling pathways (32). Radiosensitivity can be dissociated from defection in single checkpoint (24, 33, 34), suggesting that the effect of single checkpoint defection on HRR is not strong enough to affect radiosensitivity of nonsynchronized cells. Either the ratio of S or that of G_2 generally is not $>30\%$ in nonsynchronized cells, and IR-induced DNA DSBs are not affected by cell cycle (35), indicating $<30\%$ DSBs in either S or G_2 phase. The effect of single checkpoint defection on HRR is small; therefore, the phenotype of radiosensitivity is not apparent. However, multi-checkpoint defection in ATM or ATR abnormal cells clearly shows radiosensitizing effects, which are believed to affect HRR. The results shown in this study provide additional evidence supporting the hypothesis that ATR-regulated checkpoints facilitate HRR.

IR could be used as a tool to manage tumors but also could induce normal tissue to become tumor. High LET IR with high energy is one of the more efficient tools in radiotherapy but also is one of the most dangerous factors affecting astronauts' health. To elucidate the biological effect of high LET IR, it is important for us to find efficient ways to improve radiotherapy on one hand and to reduce high LET IR-induced damage on the other. High LET IR sources are available only in some locations with special equipment. Our results suggest that we could use a low LET radiation source to evaluate the effects of high LET IR on cellular responses. The factor of relative biological effectiveness of high LET IR in our experiments for dose equivalent to low LET IR, such as X-ray, is approximately two. However, different cell lines may show different quality factors; therefore, we need to test more cell lines to draw a final conclusion.

In summary, we provide direct evidence that the effects of ATR-regulated checkpoints on cellular resistance to IR-induced killing is linked to HRR but not NHEJ, which, for the first time, clarifies the effect of ATR on the HRR pathway. The mechanism of ATR affecting HRR may be via the ATR-regulated S and G_2 checkpoints, which provide time for efficient HRR.

ACKNOWLEDGMENTS

We thank Drs. Karlene A. Cimprich, Maria Jasin, and Jack A. Nickoloff for the reagents. We also thank the groups at Brookhaven National Laboratory for help in the high LET radiation, and Ms. Nancy Mott for help in the preparation of the manuscript.

REFERENCES

1. Pastink A, Eeken JCJ, Lohman PHM. Genomic integrity and the repair of double-strand DNA breaks. Mut Res 2001;480–481:37–50.
2. Thompson LH, Schild D. Homologous recombinational repair of DNA ensures mammalian chromosome stability. Mut Res 2001;477:131–53.
3. Paulovich AG, Hartwell LH. A checkpoint regulates the rate of progression through S phase in S. cerivisiae in response to DNA damage. Cell 1995;82:841–7.
4. Paulovich AG, Toczyski DP, Hartwell LH. When checkpoints fail. Cell 1997;88: 315–21.
5. Zhou B-BS, Elledge SJ. The DNA damage response: putting checkpoints in perspective. Nature 2000;408:433–9.
6. Iliakis G, Wang Y, Guan J, Wang H. DNA damage checkpoint control in cells exposed to ionizing radiation. Oncogene 2003;22:5834–47.
7. Wang X, Wang H, Iliakis G, Wang Y. Caffeine-induced radiosensitization is independent of non-homologous end joining of DNA double strand breaks. Radiat Res 2003;159:426–32.
8. Wang H, Wang X, Iliakis G, Wang Y. Caffeine could not efficiently sensitize homologous recombination repair deficient cells to ionizing radiation-induced killing. Radiat Res 2003;159:420–5.
9. Cliby WA, Roberts CJ, Cimprich KA, et al. Overexpression of a kinase-inactive ATR protein causes sensitivity to DNA-damaging agents and defects in cell cycle checkpoints. EMBO J 1998;17:159–69.
10. Bomgarden RD, Yean D, Yee M-C, Cimprich KA. A novel protein activity mediates DNA binding of an ATR-ATRIP complex. J Biol Chem 2004;279:13346–53.
11. Morrison C, Sonoda E, Takao N, et al. The controlling role of ATM in homologous recombinational repair of DNA damage. EMBO J 2000;19:463–71.
12. Golding SE, Rosenberg E, Khalil A, et al. Double strand break repair by homologous recombination is regulated by cell cycle-independent signaling via ATM in human glioma cells. J Biol Chem 2004;279:15402–10.
13. Hu B, Zhou XY, Wang X, et al. The radioresistance to killing of A1–5 cells derives from activation of the Chk1 pathway. J Biol Chem 2001;276:17693–8.
14. Zhou XY, Wang X, Hu B, et al. An ATM-independent S phase checkpoint response involves CHK1 pathway. Cancer Res 2002;62:1598–603.
15. Pierce AJ, Johnson RD, Thompson LH, Jasin M. XRCC3 promotes homology-directed repair of DNA damage in mammalian cells. Genes Dev 1999;13:2633–8.
16. Taghian DG, Nickoloff JA. Chromosomal double-strand breaks induce gene conversion at high frequency in mammalian cells. Mol Cell Biol 1997;17:6386–93.
17. Martinho RG, Lindsay HD, Flaggs G, et al. Analysis of Rad3 and Chk1 protein kinase defines different checkpoint responses. EMBO J 1998;17:7239–49.
18. Guo Z, Kumagai A, Wang SX, Dunphy WG. Requirement for Atr in phosphorylation of Chk1 and cell cycle regulation in response to DNA replication blocks and UV-damaged DNA in Xenopus egg extracts. Genes Dev 2000;14:2745–56.
19. Hekmat-Nejad M, You Z, Yee M, Newport JW, Cimprich KA. Xenopus ATR is a replication-dependent chromatin-binding protein required for the DNA replication checkpoint. Curr Biol 2000;10:1565–73.
20. Liu Q, Guntuku S, Cui XS, et al. Chk1 is an essential kinase that is regulated by Atr and required for the G_2/M DNA damage checkpoint. Genes Dev 2000;14:1448–59.
21. Zhao H, Watkins JL, Piwnica-Worms H. Disruption of the checkpoint kinase 1/cell division cycle 25A pathway abrogates ionizing radiation-induced S and G2 checkpoints. Proc Natl Acad Sci USA 2002;99:14795–800.
22. Falck J, Mailand N, Syljuasen RG, Bartek J, Lukas J. The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. Nature 2001;410: 842–7.
23. Takata M, Sasaki MS, Sonoda E, et al. Homologous recombination and non-homologous end-joining pathways of DNA double-strand break. EMBO J 1998;17:5497–508.
24. Xu B, Kim S-T, Lim D-S, Kastan MB. Two molecularly distinct G2/M checkpoints are induced by ionizing radiation. Mol Biol Cell 2002;22:1049–59.
25. Brown EJ, Baltimore D. Essential and dispensable roles of ATR in cell cycle arrest and genome maintenance. Genes Dev 2003;17:615–28.
26. Wang X, Khadpe J, Hu B, Iliakis G, Wang Y. An over-activated ATR/CHK1 pathway is responsible for the prolonged G2 accumulation in irradiated AT cells. J Biol Chem 2003;278:30869–74.
27. Moynahan ME, Pierce AJ, Jasin M. BRCA2 is required for homology-directed repair of chromosomal breaks. Mol Cell 2001;7:263–72.
28. Brown EJ, Baltimore D. ATR disruption leads to chromosomal fragmentation and early embryonic lethality. Genes Dev 2000;14:397–402.
29. Wright JA, Keegan KS, Herendeen DR, et al. Protein kinase mutants of human ATR increase sensitivity to UV and ionizing radiation and abrogate cell cycle checkpoint control. Proc Natl Acad Sci USA 1998;95:7445–50.
30. Cliby WA, Lewis KA, Lilly KK, Kaufmann SH. S phase and G2 arrests induced by topoisomerase I poisons are dependent on ATR kinase function. J Biol Chem 2002;277:1599–606.
31. Moynahan ME, Chiu JW, Koller BH, Jasin M. Brca1 controls homology-directed DNA repair. Mol Cell 1999;4:511–8.
32. Abraham RT. Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev 2001;15:2177–96.
33. Taniguchi T, Garcia-Higuera I, Xu B, et al. Convergence of the Fanconi anemia and ataxia telangiectasia signaling pathways. Cell 2002;109:459–72.
34. Zhang J, Willers H, Feng Z, et al. Chk2 phosphorylation of BRCA1 regulates DNA double-strand break repair. Mol Cell Biol 2004;24:708–18.
35. Metzger L, Iliakis G. Kinetics of DNA double strand breaks throughout the cell cycle as assayed by pulsed field gel electrophoresis in CHO cells. Int J Radiat Biol 1991;59:1325–39.

ATR Affecting Cell Radiosensitivity Is Dependent on Homologous Recombination Repair but Independent of Nonhomologous End Joining

Hongyan Wang, Huichen Wang, Simon N. Powell, et al.

Cancer Res 2004;64:7139-7143.