Differences in Sensitivity to DNA-damaging Agents between *XRCC4-* and *Artemis*-deficient Human Cells

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Artemis/DNA damage/Gene targeting/HCT116/XRCC4.

Non-homologous end-joining (NHEJ) is the predominant pathway for the repair of DNA double-strand breaks (DSBs) in human cells. XRCC4 is indispensable to NHEJ and functions together with DNA ligase IV in the rejoining of broken DNA ends. Artemis is a nuclease required for trimming of some, but not all, types of broken DNA ends prior to rejoining by the DNA ligase IV/XRCC4 complex. To better understand the roles of these factors, we generated *XRCC4-* and *Artemis*-deficient cells from the human colon adenocarcinoma cell line HCT116 by gene targeting and examined their cellular responses to several DNA-damaging agents including X-rays. As anticipated, kinetic analyses of γ-H2AX foci and chromosomal aberrations after ionizing radiation (IR) demonstrated a serious incompetence of DSB repair in the *XRCC4*-deficient cells, and relatively moderate impairment in the *Artemis*-deficient cells. The *XRCC4*-deficient cells were highly sensitive to etoposide and 5-fluoro-2'-deoxyuridine as well as IR, and moderately sensitive to camptothecin, methyl methanesulfonate, cisplatin, mitomycin C, aphidicolin and hydroxyurea, compared to the parental HCT116 cells. The *Artemis*-deficient cells were not as sensitive as the *XRCC4*-deficient cells, except to cisplatin and mitomycin C. By contrast, the *Artemis*-deficient cells were significantly more resistant to hydroxyurea than the parental cells. These observations suggest that Artemis also functions in some DNA damage response pathways other than NHEJ in human cells.

INTRODUCTION

DNA double-strand breaks (DSBs) are the most serious threat to the maintenance of genomic integrity among the many different DNA lesions. There are two primary pathways for DSB repair in mammalian cells, homologous recombination (HR) and non-homologous DNA end-joining (NHEJ).[1] HR requires homologous DNA sequences to rejoin the broken ends precisely and occurs in the late S to G2 phases of the cell cycle when the sister chromatid is in close proximity. In contrast, NHEJ, which is often associated with nucleotide loss, can function throughout the cell cycle

and is the major pathway for DSB repair in mammalian cells. It has been well established that NHEJ is also responsible for V(D)J recombination in developing B and T lymphocytes.[2] In general, NHEJ proceeds through three fundamental steps.[3] The first step is the detection of a DSB by the Ku heterodimer (Ku70 and Ku80). Once bound to the DSB, Ku recruits DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and stimulates its catalytic activity. In the second step, DNA termini with damaged or non-ligatable groups are trimmed by DNA processing enzymes such as Artemis, DNA polymerases μ and λ and polynucleotide kinase. The final step, ligation of the broken DNA ends, is exclusively carried out by DNA ligase IV with XRCC4 and XLF/Cernunnos. Other DNA ligases (I and III) are unable to substitute for this function.[4]

XRCC4 was isolated as a factor complementing impaired radioresistance, DSB repair and V(D)J recombination in a CHO mutant cell line, XR-1.[5] XRCC4 has no known enzymatic activity, but is absolutely required for stabilization and activation of its binding partner DNA Ligase IV.[6] XRCC4-deficient mice exhibit mostly similar phenotypes to DNA Ligase IV-deficient mice including embryonic lethality and neuronal degradation caused by p53-dependent apoptosis.[7,8] To date, there have been no observations suggesting the par-

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ticipation of XRCC4 other than in NHEJ.

Artemis was originally identified as a causative factor in a small subset of patients with B cell- and T cell-negative severe combined immunodeficiency (SCID), radiation sensitive-SCID and Athabascan SCID.[9,10] Artemis has 5'-to-3' exonuclease activity, and when associated with and phosphorylated by DNA-PKcs, acquires endonuclease activity towards a variety of single/double-stranded DNA structures.[11] In the process of V(D)J recombination, Artemis is essential for opening hairpin structures of the coding end, but is dispensable for signal joint formation, which is assumed not to require the processing of broken DNA ends.[9–11] Artemis-deficient mouse embryonic stem or embryonic fibroblast cells are less sensitive to ionizing radiation (IR) than isogenic XRCC4- or DNA Ligase IV-deficient cells.[12,13] Thus, in contrast to the DNA Ligase IV-XRCC4 complex whose presence is an absolute requirement for NHEJ, Artemis is most likely to be required for the processing of some, but not all, types of broken DNA ends prior to ligation. Artemis was proved to be phosphorylated by ATM and ATR, master controllers of cell cycle checkpoint signaling pathways,[12,14–16] as well as by DNA-PKcs, and has been recently suggested to have additional roles in response to DNA damage other than NHEJ such as regulation of the cell cycle, apoptosis, or HR.[14–19]

To better understand the roles of XRCC4 and Artemis in response to DNA damage in humans, we inactivated the XRCC4 and Artemis loci by gene targeting in the human adenocarcinoma somatic cell line HCT116, and assessed cellular responses to various DNA-damaging agents including IR. We found that the XRCC4-deficient cells were seriously incompetent in the repair of DSBs, while impairment of the DSB repair function in Artemis-deficient cells was limited. We also obtained results implying additional roles for Artemis in DNA-damage response pathways other than NHEJ.

MATERIALS AND METHODS

Cell lines and culture conditions

The human colon adenocarcinoma cell line HCT116, normal in p53 status but with a defective mismatch repair gene *hMLH1*, and *Artemis*^{−/−} and *XRCC4*^{−/−} derivatives of HCT116 were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and 100 μg/ml streptomycin in a humidified 5% CO_2 incubator at 37°C.

Targeting of the Artemis and XRCC4 loci

A pair of targeting vectors for the XRCC4 locus were constructed by inserting a promoterless puromycin resistance gene or promoterless neomycin resistance gene into exon 2 of the XRCC4 gene (Fig. 1A). A pair of targeting vectors for the Artemis locus were constructed by inserting a poly(A)-

signalless puromycin resistance gene or promoterless neomycin resistance gene between exons 7 and 8 of the *Artemis* gene (Fig. 1B). The 5'- and 3'-targeting elements were obtained by PCR amplification from isogenic DNA of HCT116 cells. The primers for the 5'-targeting element of *XRCC4* were 5'-ACTTGTCGACTCCTACTGACAAGCAA-AGTGCTGTGGTAAG-3' and 5'-GCTTAGCGGCCGCTC-CATTTCTTAATACCTGTAATGGGAG-3'. The primers for the 3'-targeting element of *XRCC4* were 5'-GGTTTGCG-GCCGCCACTTACTGATGGTCATTCAGCATGGACTG-3' and 5'-CTTTTGGCGCGCCATGTCATCAGCTTCTTGG-GAAATCTCTG-3'. The primers for the 5'-targeting element of *Artemis* were 5'-CAGGTCGACCCCTTGCCGAACAG-TATGATGTGCAG-3' and 5'-TGGAATGCGGCCGCATC-TTGGATCACAGAACGTAGTATCC-3'. The primers for the 3'-targeting element of *Artemis* were 5'-CTTATGCGGCCG-CATATCTGTTCACCAACCTTAGTGAAG-3' and 5'-AGA-GTTGGCGCGCCACTCTATGAGCTGTGGACCTACCTG-3'. The neomycin and puromycin resistance gene cassettes were prepared from pIRESneo2 and pIRESpuro2 (Clontech, Mountain View, CA, USA), respectively. Amplified targeting elements and antibiotic resistance marker genes were assembled as shown in Fig. 1A and 1B and the resulting targeting vectors were introduced into cells by electroporation. Genomic DNA from G418 or puromycin-resistant cells was screened for gene targeting by PCR using the primer sets specific for respective targeting constructs. To confirm the gene targeting, genomic DNA from candidate clones was digested with *Bam*H I plus *Hind* III or *Hind* III plus *Sph* I and subjected to a Southern blot analysis.

Western blotting

Cell lysate was prepared in 1 × SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol and 1% 2-mercaptoethanol), resolved by 10% SDS-PAGE and electrophoretically transferred to PVDF filters. The blot was probed with antibody against Artemis (Abcam, Cambridge, UK), XRCC4 (Serotec Ltd., Oxford, UK), DNA ligase IV (Protein Tech Group, Inc., Chicago, IL, USA), or G3PDH (TREVIGEN, Gaitherburg, MD, USA) and visualized with an LAS-1000 image analyzer (Fuji Film, Tokyo, Japan).

Growth rate analysis

Cells were cultured in a 96-well plate at a density of 1–5 × 10^2 per well. The relative cell number was determined every 24 hours with a CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) according to the manufacturer's directions.

Cell survival assay

Cells were plated at a density of 3 × 10^2 to 1 × 10^4 per 60-mm dish, cultured for 15–18 h and irradiated with X-rays. In other cases, cells were cultured for 15–18 h and then the indicated dose of etoposide, camptothecin, methyl meth-
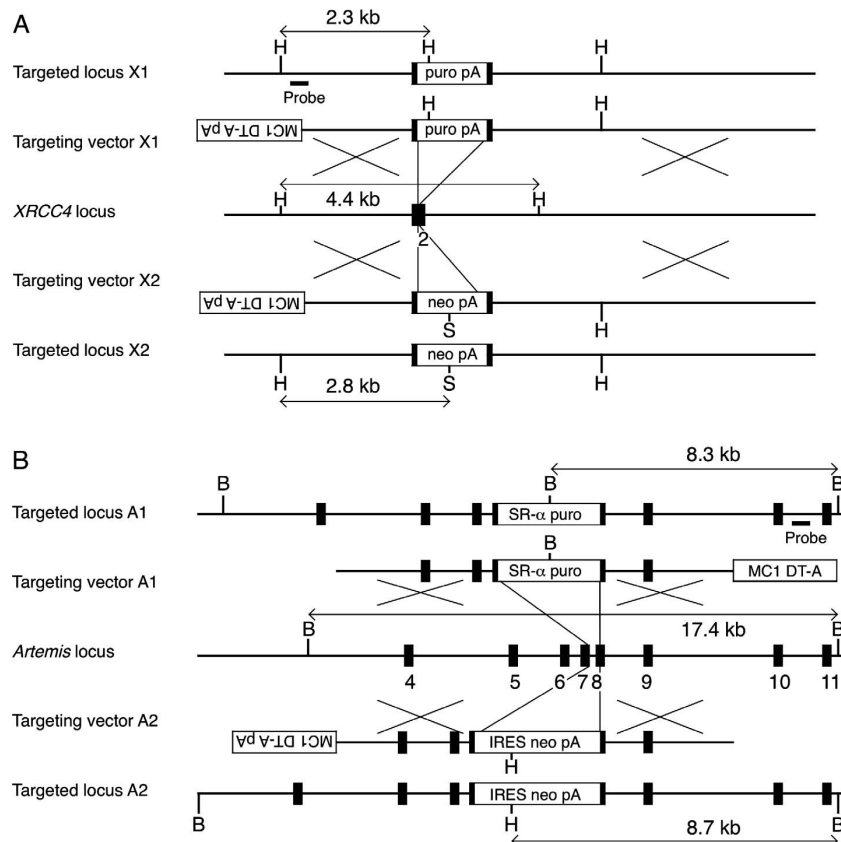
Fig. 1. Schematic representations of the homozygous targeting of the *XRCC4* (A) and *Artemis* (B) loci. The respective wild-type loci, the disruption constructs, and the configurations of the targeted loci are shown. Black boxes indicate the positions of exons. Relevant restriction enzyme sites and the positions of the probes used for Southern blot analyses are shown. B, *Bam*H I; H, *Hin*d III; S, *Sph* I; IRES, internal ribosomal entry site; SR-α, SR-alpha promoter; MC1, MC1 promoter; puro, puromycin resistance gene; neo, neomycin resistance gene; DT-A, Diphtheria toxin gene; pA, poly (A) additional signal.

anesulfonate (MMS), cisplatin, mitomycin C (MMC), aphidicolin, 5-fluoro-2'-deoxyuridine (FdUrd), or hydroxyurea was added to the medium. The cultures were continued for an additional 9 to 14 days and colonies were stained with methylene blue and counted. All the experiments were performed at least 3 times in duplicate or triplicate.

Analysis of phosphorylated H2AX (γ-H2AX) foci after X-ray irradiation

Cells grown on glass coverslips were irradiated with 2.0 Gy of X-rays at a dose rate of 0.6 Gy/min. The cells were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.5% Triton X-100 in PBS for 5 min, and blocked with 10% goat serum for 1 h to eliminate nonspecific reactions as described in our previous report.[20] The specimens were then treated with an anti-γ-H2AX mouse monoclonal antibody (Upstate Biotechnology, Lake Placid, NY, USA) diluted in A-buffer (20 mM Tris-HCl, pH7.4, 137 mM NaCl, 0.1% Tween20, and 5% non-fat milk) for 1 h at 37°C, and visualized with tetramethylrhodamine B isothio-

cyanate-conjugated secondary antibody. γ-H2AX foci were observed under a fluorescence microscope, Biozero (Keyence, Osaka, Japan).

Analysis of chromosomal aberrations induced by X-ray irradiation

Cells were plated in a 60-mm dish at a density of 1.5–2 × 10^6, grown for 24 h and irradiated with 1.4 Gy X-rays at a dose rate of 0.6 Gy/min. The cells were then harvested at various times by trypsinization after treatment with 0.1 μg/ml of colcemid for 1 h. Chromosomes were prepared according to an air-drying method described previously.[21] Chromosomal aberrations were scored in 22–100 cells at each post-irradiation time point, depending on aberration frequencies, according to the Human Cytogenetic Nomenclature ISCN 1985.[22] Briefly, chromosomal aberrations were categorized as chromatid-type (gap, break and exchange) and chromosome-type (gap, break, dicentric, ring, fragment, double minute and pulverization) aberrations. Translocations were omitted from the scoring because of

ambiguity. Gaps were excluded from the calculation of the number of aberrations per cell because of ambiguity between true DNA breaks and aberrant chromatin packaging. All types of aberrations were scored as one event.

RESULTS

Generation of human cell lines deficient in XRCC4 or Artemis

HCT116 is a well-characterized human colon adenocarcinoma cell line that has a stable near-diploid karyotype, and apparently intact DNA damage-dependent and spindle-dependent checkpoints.[23] To inactivate *XRCC4* or *Artemis* homozygously, a pair of targeting vectors similar in structure except for the selection marker, a puromycin-resistance (*puro*) or neomycin-resistance (*neo*) gene, were constructed for each locus (Fig. 1A and 1B). HCT116 cells were transfected with either first targeting vector, X1 or A1 (Fig. 1A and 1B), and puromycin-resistant cells were examined first by genomic PCR then by Southern blotting to isolate heterozygous *XRCC4*^{+/−} and *Artemis*^{+/−} mutant clones (data not shown). Next, one of the two resulting *XRCC4*^{+/−} clones and one of the three resulting *Artemis*^{+/−} clones were transfected with second targeting vectors, X2 and A2 (Fig. 1A and 1B), respectively, and G418-resistant cells were screened by genomic PCR and Southern blot analyses to isolate homozygously targeted clones. We obtained two independent *XRCC4*^{−/−} mutant clones (*XRCC4*^{−/−}#14 and *XRCC4*^{−/−}#54) and one *Artemis*^{−/−} mutant clone. Results of Southern and Western blot analyses of targeted cells are shown in Fig. 2A

and 2B. In the *XRCC4*^{−/−} mutant clones, a 4.4-kb germline band disappeared and 2.8 and 2.3-kb targeted bands appeared (Fig. 2A upper panel). The *Artemis*^{−/−} mutant clone lost a 17.4-kb germline band and gained 8.7 and 8.3-kb targeted bands (Fig. 2B upper panels). Western blot analyses revealed that these homozygous mutant clones did not express the mature XRCC4 or Artemis protein, respectively (Fig. 2A and 2B, lower panels). It is important to note that DNA ligase IV was mostly missing in the *XRCC4*^{−/−} human cells (Fig. 2A, lower panel) as reported previously in an *XRCC4*-deficient rodent cell line.[6] Rates of growth were slightly reduced in the mutant clones as compared to HCT116 cells (Fig. 2C). Population doubling times of the *XRCC4*^{−/−}#14, *XRCC4*^{−/−}#54, *Artemis*^{−/−}, and HCT116 cells were 24, 26, 30 and 23 h, respectively.

Responses of XRCC4^{−/−} and Artemis^{−/−} human cells to X-rays

We examined the responses of the *XRCC4*^{−/−} and *Artemis*^{−/−} human cells to IR. The radiosensitivity of the two *XRCC4*^{−/−} mutant clones was virtually identical and 3.2-fold higher than that of HCT116 cells, based on the dose required to reduce the cell survival to 10%. The *Artemis*^{−/−} mutant showed a 1.9-fold increase in radiosensitivity compared to the parental cells (Fig. 3A). The doses required to reduce the cell survival to 10% of *XRCC4*^{−/−}, *Artemis*^{−/−} and HCT116 cells were 1.3, 2.2 and 4.1 Gy, respectively.

To analyze the induction of DSBs and repair kinetics in these mutants, we examined the formation and loss of γ-H2AX foci, a surrogate marker for DSBs, after X-irradiation
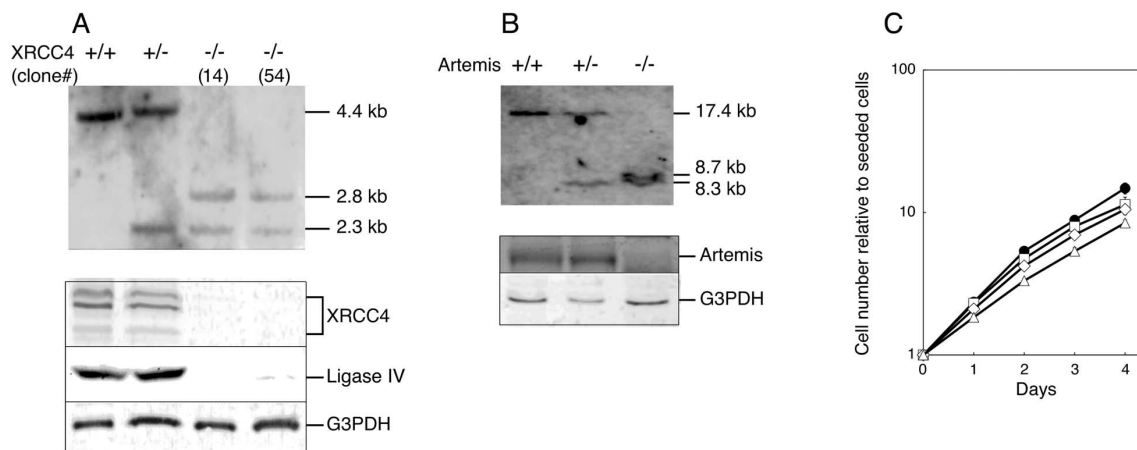

Fig. 2. Generation of *XRCC4*^{−/−} and *Artemis*^{−/−} cells. A and B, Representative Southern (upper panels) and Western (lower panels) blots of *XRCC4* (A) or *Artemis* (B) targeted cells. Genomic DNA from cells with indicated genotypes was digested with *Hind* III plus *Sph* I (upper panel in A) or *Bam*H I plus *Hind* III (upper panel in B). Whole-cell extract from indicated genotypes was probed with either anti-XRCC4, anti-DNA ligase IV, anti-Artemis, or anti-G3PDH antibody as indicated. C, Cell proliferation of homozygous mutant clones. Relative cell numbers were monitored by MTS assay and presented as the mean ± standard error obtained from at least 3 independent experiments. Closed circles, HCT116; open squares, *XRCC4*^{−/−}#14; open diamonds, *XRCC4*^{−/−}#54; open triangles, *Artemis*^{−/−}.
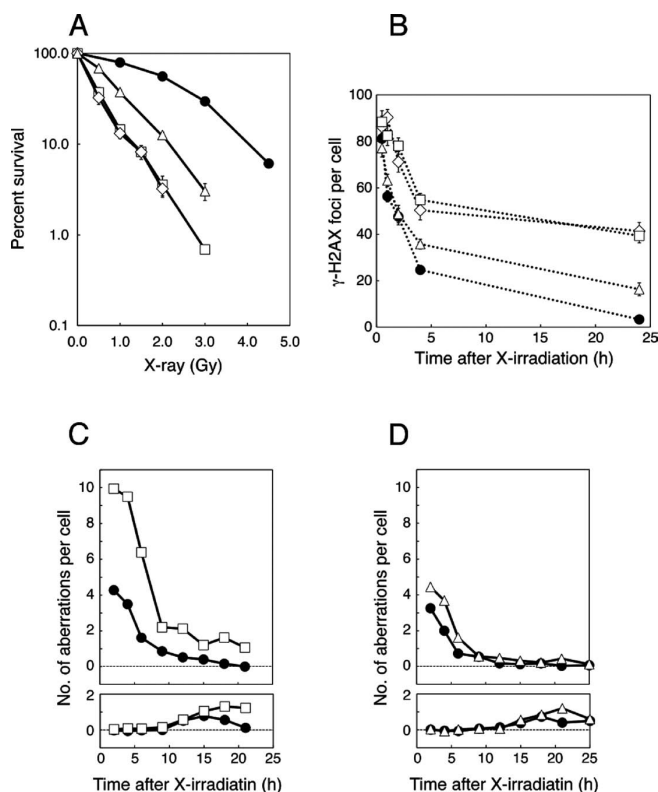
Fig. 3. Radiosensitivity of *XRCC4⁻/⁻*, *Artemis⁻/⁻* and parental HCT116 cells. A, Clonogenic cell survival assay after X-irradiation at the indicated doses. Values represent the mean ± standard error obtained from at least 3 independent experiments. Closed circles, HCT116; open squares, *XRCC4⁻/⁻*#14; open diamonds, *XRCC4⁻/⁻*#54; open triangles, *Artemis⁻/⁻*. Where absent, error bars fall within symbols. B, Kinetics of the formation and loss after X-irradiation of γ-H2AX foci. Cells were exposed to 2 Gy of X-rays and fixed at 0.5, 1, 2, 4 and 24 h after the irradiation for immunostaining γ-H2AX. Values represent the mean number of foci per cell ± standard error obtained from more than 30 nuclei. Background numbers of foci observed in non-irradiated parental HCT116, *XRCC4⁻/⁻*#14, *XRCC4⁻/⁻*#54 and *Artemis⁻/⁻* cells were 5.0 ± 0.7, 5.9 ± 1.4, 3.5 ± 0.6 and 5.1 ± 0.9, respectively, and were subtracted from the numbers in the corresponding X-irradiated cells. Symbols are the same as above. C and D, Frequencies of chromosomal aberrations in X-irradiated cells. *XRCC4⁻/⁻*#14 (open square), *Artemis⁻/⁻* (open triangle), and HCT116 (closed circle) cells were irradiated with 1.4 Gy of X-rays and chromosomes were prepared at the indicated time points post-irradiation. The frequency of X-ray-induced chromatid-type (upper panel) or chromosome-type (lower panel) aberrations was expressed after subtraction of the frequency of spontaneously occurring aberrations.

(Fig. 3B). The numbers of γ-H2AX foci observed at 30 min after irradiation (2 Gy) in HCT116, *XRCC4⁻/⁻* and *Artemis⁻/⁻* cells were similar to each other. There was however a significant difference in the rate at which the foci disappeared among the cell lines later on. In HCT116 cells, the foci disappeared rapidly, 70% of them within 4 h, and the number

had diminished to nearly the basal level 24 h after irradiation. In sharp contrast, the γ-H2AX foci that formed in irradiated *XRCC4⁻/⁻* mutant cells disappeared much more slowly, with about half still remaining at 24 h after irradiation. In *Artemis⁻/⁻* cells, the rate at which the foci disappeared was similar to that in HCT116 cells up to 2 h after X-irradiation but markedly reduced at later time points. About 20% of foci remained in *Artemis⁻/⁻* cells at 24 h after X-irradiation.

We also examined the frequency of spontaneously occurring and radiation-induced chromosomal aberrations in *XRCC4⁻/⁻* and *Artemis⁻/⁻* cells. A deficiency of either *XRCC4* or *Artemis* has an impact on both types of aberrations. The frequency of spontaneously occurring chromosomal aberrations in *XRCC4⁻/⁻*#14 and *Artemis⁻/⁻* mutant cells was 0.24 (the mean obtained from 600 metaphase cells) and 0.29 (the mean obtained from 100 metaphase cells) per cell, respectively, while that in parental HCT116 cells was 0.12 (the mean obtained from 700 metaphase cells) per cell. To compare frequencies of radiation-induced chromosomal aberrations, exponentially growing cells were exposed to X-rays (1.4 Gy) and chromosomal aberrations in metaphase cells were examined at various intervals. Most of the chromosomal aberrations generated by X-rays were of the chromatid-type until 9 h postirradiation, after which chromosome-type aberrations increased, reflecting post- and pre-DNA replication at the time of irradiation (Fig. 3C and 3D). The frequency of chromatid-type aberrations was highest at 2 h and decreased thereafter in all cell lines examined. *XRCC4⁻/⁻*#14 cells showed considerably elevated frequencies of both chromatid- and chromosome-type aberrations throughout the cell cycle as compared to HCT116 cells (Fig. 3C), while mildly increased frequencies were also found in *Artemis⁻/⁻* cells (Fig. 3D). Taken together, the rate of radiation-induced chromosomal aberration was higher in *XRCC4⁻/⁻* cells than in *Artemis⁻/⁻* cells.

Sensitivity of *XRCC4⁻/⁻* and *Artemis⁻/⁻* human cells to various DNA-damaging chemicals

To elucidate the function of XRCC4 and Artemis in the cellular response to various types of DNA damage, different classes of DNA-damaging chemicals were selected and the cellular responses to the chemicals were examined by evaluating clonogenic survival.

XRCC4⁻/⁻ mutant clones were extremely hypersensitive to etoposide, a DNA topoisomerase II inhibitor inducing DSBs, but *Artemis⁻/⁻* cells did not show a prominent increase in sensitivity to etoposide as compared to the parental HCT116 cells (Fig. 4A). In contrast, *XRCC4⁻/⁻* and *Artemis⁻/⁻* mutant cells showed a similar moderate increase in sensitivity to camptothecin, a DNA topoisomerase I inhibitor causing mostly single strand breaks (SSBs), at least at low doses up to 1 nM, although *XRCC4⁻/⁻* cells exhibited greater sensitivity at higher concentrations of camptothecin (Fig. 4B).
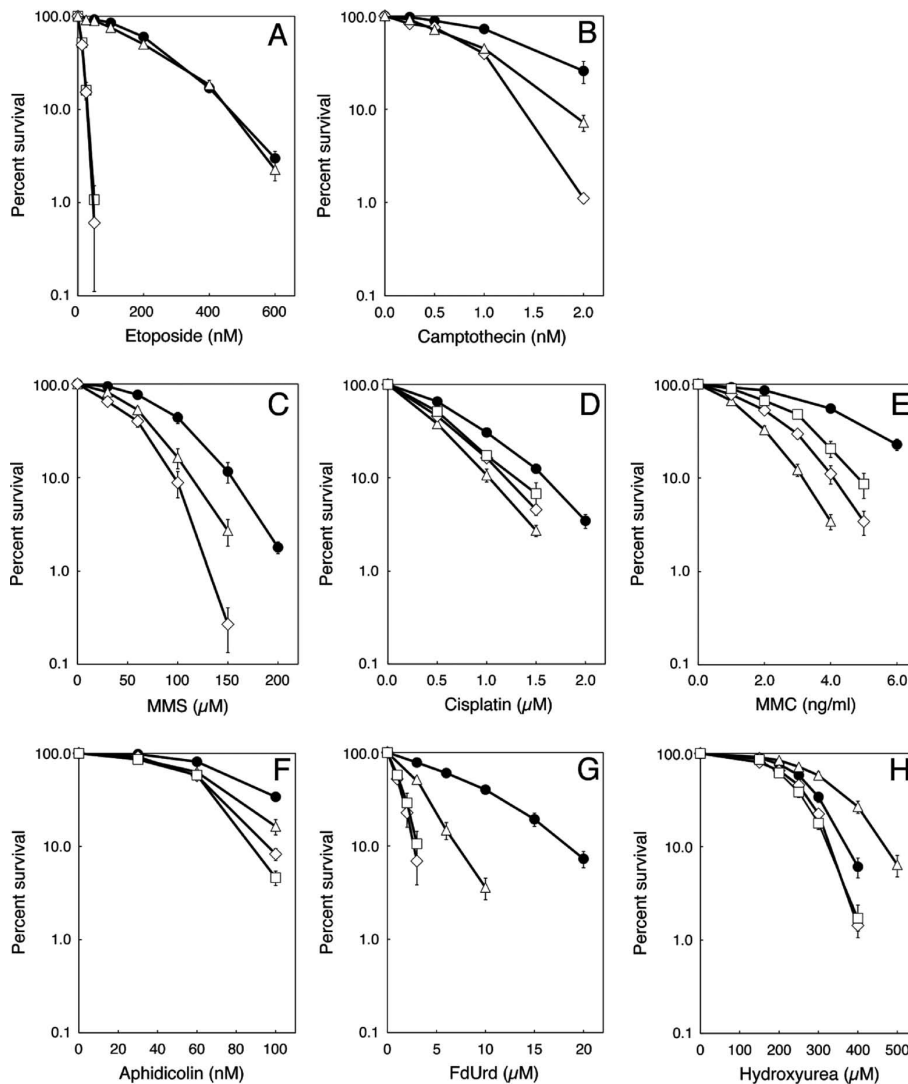
Fig. 4. Sensitivity of *XRCC4*$^{-/-}$, *Artemis*$^{-/-}$ and HCT116 cells to genotoxic agents. The sensitivity of cells to eto-poside (A), camptothecin (B), MMS (C), cisplatin (D), MMC (E), aphidicolin (F), FdUrd (G) and hydroxyurea (H) was determined with a clonogenic survival analysis. Closed circles, HCT116; open squares, *XRCC4*$^{-/-}$#14; open diamonds, *XRCC4*$^{-/-}$#54; open triangles, *Artemis*$^{-/-}$. The values represent the mean ± standard error obtained from at least 3 independent experiments. Where absent, error bars fall within symbols.

Both *XRCC4*$^{-/-}$ and *Artemis*$^{-/-}$ cells showed prominent increases in sensitivity to alkylating agents such as MMS, cisplatin and MMC in comparison with the HCT116 cells (Fig. 4C, 4D and 4E). The *Artemis*$^{-/-}$ mutation had less of an impact on sensitivity to MMS than the *XRCC4*$^{-/-}$ muta-tion (Fig. 4C), while it caused a larger increase in sensitivity to cisplatin and MMC than the *XRCC4*$^{-/-}$ mutation (Fig. 4D and 4E).

Finally, we examined chemical agents inducing DNA rep-lication stress such as aphidicolin, FdUrd and hydroxyurea. The *XRCC4*$^{-/-}$ and *Artemis*$^{-/-}$ mutations had little impact on cellular sensitivity to aphidicolin at low doses, although *XRCC4*$^{-/-}$ cells were slightly more sensitive to 100 nM of aphidcolin than *Artemis*$^{-/-}$ cells (Fig. 4F). On the other hand, FdUrd was strikingly toxic to these mutant cells. The sensi-tivity of *XRCC4*$^{-/-}$ and *Artemis*$^{-/-}$ cells to FdUrd was 6.2- and 2.6-fold higher than that of the parental cells, respectively, based on the dose required to reduce the cell survival to 10% (Fig. 4G). Again, the *XRCC4*$^{-/-}$ mutation had only a small impact on the sensitivity to hydroxyurea (Fig. 4H). Unex-pectedly, however, *Artemis*$^{-/-}$ mutant cells were clearly resis-tant to hydroxyurea as compared to HCT116 cells (Fig. 4H). This result suggested an accelerative role of Artemis in the response to damage by hydroxyurea leading to cell death (see discussion).

DISCUSSION

XRCC4 and Artemis are core factors of the canonical NHEJ pathway. We disrupted each locus homozygously by gene targeting in the human cell line HCT116 and obtained two $XRCC4^{-/-}$ mutant clones and one $Artemis^{-/-}$ mutant clone. As compared to the parental HCT116 cells, the $XRCC4^{-/-}$ and $Artemis^{-/-}$ cells were approximately 3- and 2-fold more susceptible to X-irradiation in cell survival assays, respectively (Fig. 3A). Because of a lack of DNA ligase IV due to the elimination of XRCC4 (Fig. 2A), the final rejoining process of the NHEJ-mediated DSB repair pathway would be markedly impaired in $XRCC4^{-/-}$ cells. In fact, the cells were severely hampered in the ability to recover from DSBs with about half of the γ-H2AX foci persisting until 24 h after the irradiation (Fig. 3B). On the other hand, $Artemis^{-/-}$ cells recovered from DSBs as efficiently as the parental HCT116 cells up to 2 h after X-irradiation, but the rate at which γ-H2AX foci disappeared slowed down later (Fig. 3B). It is most likely that the majority of IR-induced DSBs are repaired within the first 2 to 4 h by Artemis-independent NHEJ and the rest by Artemis-dependent NHEJ with slow kinetics, probably depending on the complexity of features of the broken ends, as suggested in previous reports.[12,24,25]

DSBs are directly responsible for chromosomal aberrations, and NHEJ as well as HR plays a role in maintaining genomic integrity.[26] The $XRCC4^{-/-}$ and $Artemis^{-/-}$ cells showed increase in both spontaneously occurring and radiation-induced chromosomal aberrations (Fig. 3C and 3D) as mouse embryonic stem cells which are deficient for each homologous gene.[13] Apparently larger increase in radiation-induced chromosomal aberrations in $XRCC4^{-/-}$ cells than in $Artemis^{-/-}$ cells might reflect more serious defect in repairing radiation induced DSBs in $XRCC4^{-/-}$ cells than in $Artemis^{-/-}$ cells. Major causes for spontaneous DSBs, which result in spontaneous chromosomal aberrations, might be reactive oxygen species (ROS), stalled replication forks and, in particular cells, V(D)J recombination. It should be noted that little or no increase in spontaneous chromosomal instability was observed in Ku80-deficient Chinese hamster ovary cell lines, xrs-5, xrs-6 and xrs-7 and a Ku80-deficient Chinese hamster lung fibroblast cell line XR-V15B.[27–29] However, an $XRCC4$-deficient mouse lymphoma cell line M10[30] as well as primary fibroblast cells from NHEJ-deficient knockout mice showed obvious increase in spontaneous chromosomal instability. Difference in cellular activities generating and/or removing ROS depending on the cell types and culture conditions might be a most probable cause for this discrepancy.

In contrast to IR, which generates a broad spectrum of DNA damage, DNA topoisomerase inhibitors, etoposide and camptothecin, introduce a relatively homogeneous popula-tion of enzymatically induced DSBs and SSBs, respectively.[31,32] As expected, etoposide, which induces DSBs directly by inhibition of topoisomerase II, was highly toxic to $XRCC4^{-/-}$ cells, while camptothecin was moderately toxic (Fig. 4A and 4B). DNA topoisomerase I-camptothecin-DNA covalent complexes can be converted into replication-mediated DSBs.[33] This is probably why camptothecin exhibited cytotoxicity to some extent in the $XRCC4^{-/-}$ cells. In contrast, $Artemis^{-/-}$ cells did not show an apparent increase in sensitivity to etoposide (Fig. 4A). The DSBs generated by etoposide would be rejoined mostly via Artemis-independent NHEJ as proposed in previous studies using $Artemis$-deficient human fibroblasts from radiation sensitive-SCID or Athabascan SCID patients.[12,24] On the other hand, some proportion of replication-mediated DSBs produced by camptothecin must be repaired by Artemis-dependent NHEJ, because $Artemis^{-/-}$ cells showed hypersensitivity to camptothecin compared with the parental HCT116 cells (Fig. 4B). Thus, the responses exhibited in our $XRCC4^{-/-}$ and $Artemis^{-/-}$ human cells to IR and DNA topoisomerase inhibitors corroborate the notion that XRCC4 is essential for NHEJ-mediated DSB repair but Artemis is not.

We further compared the sensitivity of $XRCC4^{-/-}$ and $Artemis^{-/-}$ cells to other chemicals which damage DNA. MMS is a monofunctional alkylating agent primarily causing DNA monoadducts, mostly 7-methylguanine and 3-methyladenine.[34] Cisplatin and MMC are bifunctional alkylating agents which cause primarily intrastrand cross-links, though also interstrand cross-links to a lesser extent.[35] $XRCC4^{-/-}$ mutant cells were apparently sensitive to these alkylating agents as compared to the parental HCT116 cells (Fig. 4C, 4D and 4E). The damage from the alkylation of bases might be converted to DSBs through DNA replication and/or repair processes, and so $XRCC4^{-/-}$ cells exhibited hypersensitivity to these agents.[34,36] Interestingly, $Artemis^{-/-}$ cells were more sensitive to cisplatin and MMC, but less sensitive to MMS, than $XRCC4^{-/-}$ cells. If Artemis only mediates broken end trimming in NHEJ-mediated DSB repair, $Artemis^{-/-}$ cells would be less sensitive than $XRCC4^{-/-}$ cells to any agent inducing DSBs. Thus, the relatively greater sensitivity to cisplatin and MMC of $Artemis^{-/-}$ cells than $XRCC4^{-/-}$ cells implies an additional function of Artemis in DNA damage responses other than NHEJ. Artemis shares homology with yeast SNM1/PSO2 which functions in DNA interstrand cross-link repair.[37] Hence, it seems probable that $Artemis^{-/-}$ cells may have defect in interstrand cross-link-repair and exhibit hypersensitivity to interstrand cross-linkers such as cisplatin and MMC. However, it was shown that two vertebrate SNM1 family proteins, SNA1A and SNM1B, play a role in interstrand cross-link-repair but SNM1C/Artemis does not.[38] Recently, it was reported that Artemis is involved in the HR-mediated repair of DSBs.[18] HR is one of the major pathways for the repair of DNA damaged by bifunctional alkylating agents.[36] An

impairment of the HR-mediated repair of bifunctional alkylation damage due to the *Artemis* deficiency is the most probable cause for the relatively serious effect of cisplatin and MMC on *Artemis*$^{-/-}$ cells compared to *XRCC4*$^{-/-}$ cells. In previous studies, however, *Artemis*-deficient human, mouse and chicken cells had not shown remarkable increases in sensitivity to cisplatin and MMC as compared to matched *Artemis*-proficient cells.[13,25,38,39] Differences in genetic background would be a likely cause of the discrepancy regarding the sensitivity of Artemis-deficient cells to cisplatin and MMC. Defective mismatch repair (MMR) in HCT116 cells caused by a hemizygous nonsense mutation in the *hMLH1* gene is one possible explanation for the discrepancy.[40] MMR-deficient cells are more resistant to some chemotherapeutic agents including cisplatin and MMC than matched MMR-proficient cells and thus MMR is thought to be involved in the signaling and/or the inappropriate processing of DNA damage induced by such agents.[41] Therefore, the increase in sensitivity to cisplatin and MMC observed in our *Artemis*$^{-/-}$ cells derived from the HCT116 line would be due to the combined effects of the *Artemis* deficiency and concomitant nonfunctional mutation of *hMLH1*. In any event, the relatively greater sensitivity to cisplatin and MMC of our *Artemis*$^{-/-}$ mutant cells than that of isogenic *XRCC4*$^{-/-}$ mutant cells implies an additional XRCC4-independent function of Artemis in DNA damage responses other than NHEJ.

Aphidicolin is a DNA polymerase inhibitor that disturbs replication.[42] Hydroxyurea and FdUrd also disturb DNA replication by affecting cellular pools of deoxynucleotide.[43–45] Aphidicolin and hydroxyurea had only a small impact on *XRCC4*$^{-/-}$ cells, while FdUrd was highly toxic (Fig. 4F, 4G and 4H). DSBs induced at stalled DNA replication forks might be responsible for the susceptibility of *XRCC4*$^{-/-}$ cells to these inhibitors.[45,46] FdUrd not only inhibits DNA replication but also mediates the incorporation of unusual bases such as 5-florouracil into DNA.[47] The combined effects of the dNTP pool imbalance and incorporation of unusual bases into DNA may cause numerous DSBs, making FdUrd remarkably toxic to *XRCC4*$^{-/-}$ cells as compared to other replication inhibitors. As anticipated, the deleterious effects of aphidicolin and FdUrd on *Artemis*$^{-/-}$ cells were no greater than those on *XRCC4*$^{-/-}$ cells (Fig. 4F and 4G). Unexpectedly, the deficiency of *Artemis* was advantageous to the cells treated with hydroxyurea (Fig. 4H). The most likely explanation for this is that Artemis functions in some cellular response by which hydroxyurea causes cell death and that a defect in this pathway caused by the absence of Artemis prevents hydroxyurea-induced cell death. Suspended DNA replication and/or DSBs are not likely triggers for this Artemis-dependent cellular response, because cell death was not prevented in *Artemis*$^{-/-}$ cells exposed to other DNA replication inhibitors, aphidicolin and FdUrd. Hydroxyrea generates ROS and reactive nitrogen species (RNS), which are not only detrimental but also act as second messengers probably depending on their amount, and the duration and site of their production.[48–50] There are several reports suggesting the participation of Artemis in signaling pathways of oxidative stress response, cell-cycle checkpoints, and apoptosis.[14–17,19] It is therefore plausible that Artemis responds to ROS or RNS produced by hydroxyurea and then accelerates a prolonged cell-cycle arrest and/or apoptosis. In any event, a detailed comparison of the responses of *XRCC4*$^{-/-}$ and *Artemis*$^{-/-}$ human cells to hydroxyurea will provide further insight into the involvement of Artemis in cellular stress response pathways other than NHEJ-mediated DSB repair.

ACKNOWLEDGEMENTS

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