Restoration of G1 chemo/radioresistance and double-strand-break repair proficiency by wild-type but not endonuclease-deficient Artemis

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ABSTRACT

Deficiency in Artemis is associated with lack of V(D)J recombination, sensitivity to radiation and radiomimetic drugs, and failure to repair a subset of DNA double-strand breaks (DSBs). Artemis harbors an endonuclease activity that trims both 5'- and 3'-ends of DSBs. To examine whether endonuclease trimming of terminally blocked DSBs by Artemis is a biologically relevant function, Artemis-deficient fibroblasts were stably complemented with either wild-type Artemis or an endonuclease-deficient D165N mutant. Wild-type Artemis completely restored resistance to c-rays, bleomycin and neocarzinostatin, and also restored DSB-repair proficiency in G0/G1 phase as measured by pulsed-field gel electrophoresis and repair focus resolution. In contrast, cells expressing the D165N mutant, even at very high levels, remained as chemo/ radiosensitive and repair deficient, as evidenced by persistent c-H2AX, 53BP1 and Mre11 foci that slowly increased in size and ultimately became juxtaposed with promyelocytic leukemia protein nuclear bodies. In normal fibroblasts, overexpression of wild-type Artemis increased radioresistance, while D165N overexpression conferred partial repair deficiency following high-dose radiation. Restoration of chemo/radioresistance by wild-type, but not D165N Artemis suggests that the lack of endonuclease trimming of DNA ends is the principal cause of sensitivity to double-strand cleaving agents in Artemis-deficient cells.

INTRODUCTION

Artemis nuclease is a phosphoprotein that has been shown to play a role in hairpin opening in V(D)J recombination (1,2) and more recently in the regulation of G2/M, and S phase cell cycle checkpoints (3,4). Artemis is also required for the repair of a subset of chemo/radiotherapy-induced DNA double-strand breaks (DSBs) that are rejoined very slowly in normal cells. These DSBs may largely overlap the fraction of DSBs whose repair requires ATM- and 53BP1-dependent phosphorylation of the heterochromatin maintenance protein KAP-1 (5–7). Previous in vitro studies with oligomeric substrates have shown that Artemis nuclease activity is DNA-PK-dependent at DNA ends and that this activity can remove 3'-PG blocking lesions commonly found at DSB termini (8,9). Thus, it is reasonable to propose that lack of such endonuclease trimming accounts for both the repair deficiency and the increased cytotoxicity of radiation and radiomimetic agents toward Artemis-deficient cells. Yet, the resulting repair deficiency is subtle, affecting only 10–20% of DSBs, raising the question of whether cell cycle or other regulatory functions of Artemis might be equally or more important determinants of chemo/ radiosensitivity.

Artemis belongs to SNM1 family of nucleases and possesses metallo-β-lactamase and β-CASP domains at its amino terminus. Mutation of an aspartic acid residue to asparagine (D165N) selectively abrogates the endonuclease function of Artemis without affecting its exonuclease activity or phosphorylation status (8,10). Though D165 is not found in sequences of the available metallo-β-lactamase crystal structures, the abrogation of endonuclease activity by this mutation suggests that it may be located in the active site of Artemis (10).

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To investigate whether the endonucleolytic activity of Artemis functions in chemo/radioresistance, patient-derived CJ179 cells defective for Artemis were complemented with lentiviral vectors expressing wild-type or D165N Artemis. The D165N mutation eliminates Artemis-mediated endonucleolytic processing of 3′-PG DSBs in vitro. To establish the role of Artemis nuclease activity in DNA repair and cellular survival after DNA damage, clonogenic and DSB-repair assays were carried out with these cells, following treatment with radiation or radiomimetic drugs. Earlier studies (5,6) investigating complementation of Artemis defect by exogenous protein expression were carried out with transiently expressing cell lines due to difficulties in expressing Artemis in cells (11). In contrast to these studies, we have been successful in stably complementing Artemis-deficient fibroblasts with wild-type or D165N mutant Artemis, allowing us to explore the effect of such expression on the critical endpoint of survival following radiation or genotoxic chemical treatment. In the current study, low-level Artemis expression was found to be sufficient for complementing the survival/repair defect of Artemis-deficient cells while even high levels of the D165N Artemis failed to do so. Moreover, the residual DNA DSBs that remained unrejoined due to Artemis deficiency juxtaposed with promyelocytic leukemia (PML) nuclear bodies. Taken together, these results indicate that the endonucleolytic end-processing activity of Artemis is essential for promoting DSB repair and cell survival.

MATERIALS AND METHODS

Cell lines and complementation

Normal 48BR and patient-derived Artemis-deficient CJ179 hTERT-immortalized human fibroblasts originally from Dr Penny Jeggo, were obtained from Dr Lynn Harrison, Louisiana State University, and were cultured in minimum essential medium (MEM-α) supplemented with 10% fetal bovine serum and antibiotics (GIBCO). Cells were constantly maintained at 37°C in 5% CO₂ and a humidified atmosphere. Normal Epstein–Barr virus-transformed human lymphoblastoid cells (patient 1646) were from Dr James Lupski, Baylor College of Medicine (12).

Lentiviral constructs were prepared in the 693-2 lentiviral backbone harboring the hygromycin resistance gene (13) carrying either wild-type or D165N mutant Artemis cDNA fused to a c-myc epitope tag on the carboxyl terminus. Artemis wild-type, D165N and empty lentivector DNAs were transfected along with packaging plasmids, pLP1, pLP2 and pLP-VSVG into 293FT cells with Lipofectamine 2000 (Invitrogen). Medium containing lentivector DNAs were transfected along with packaging carboxyl terminus. Artemis wild-type, D165N and empty gene (13) carrying either wild-type or D165N mutant MEDicine (12).

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Western blots

Direct western blotting of cell lysates was attempted with a variety of antibodies to Artemis but was found to be insufficiently sensitive and specific for Artemis detection; therefore, an immunoprecipitation (IP) step was added as previously described (14). Briefly, ~2.5 million cells were harvested and sonicated on ice with three 30-s pulses in 750 μl of TBST (25 mM Tris–HCl pH 7.4, 130 mM NaCl, 3 mM KCl, 0.1% (v/v) Tween-20) supplemented with 1 μM leupeptin, 1 μM pepstatin A and 1 μM aprotinin protease inhibitors.

Lysates were cleared by centrifugation at 14 000 rpm for 15 min at 4°C and incubated with equilibrated protein A/G beads (Protein A/G UltraLink Resin, Thermo Scientific) for 1 h, then the extract was transferred to a new tube. Extracts were incubated with 1 μl of SCIDA1024 rabbit antiserum (14) for 1 h at 4°C, then 20 μl of equilibrated A/G beads were added and tumbled overnight at 4°C. The A/G beads were pelleted in a microfuge at 5000 rpm for 30 sec at 4°C and washed three times with 500 μl extract buffer. Fifty micro-liter Laemmli buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.004% bromphenol blue, 0.125 M Tris–HCl pH 6.8) was added to the washed beads and heated for 10 min at 98°C and centrifuged at 14 000 rpm for 15 min. Approximately one million cell equivalents (20 μl) was loaded onto 8% SDS–PAGE gels and resolved at 150 V and transferred to a nitrocellulose membrane with 100 V for 90 min in

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Selection of clones

From each derivative cell line, approximately 20 clones were isolated. Total RNA was extracted from each clone using Qiagen RNeasy minikit and converted to cDNA (Applied Biosystems) following the protocols provided by the manufacturer. Amplification of cDNA was performed on an ABI 7900HT Real time q-PCR instrument using SYBR green detection (Applied biosystems). Relative Artemis levels were determined after normalizing to β-actin levels using SDS 2.2.2 software. The primers used were 5′-ACAGAGGCTCGCCTTTGC-3′, 5′-CACATCA CGCCTCTGGTCC-3′ for β-actin and 5′-AGTACGGA GCCAAAGTATAACCACT-3′, 5′-TCCGGGTATGCC ACCCTTGTGTC-3′ for Artemis cDNA amplification (Synthesized by IDT). Highest and lowest Artemis expressing clones among those screened were identified and the protein levels were confirmed by western as described below. The selected clones were further used in survival/repair assays.

Western blots

Direct western blotting of cell lysates was attempted with a variety of antibodies to Artemis but was found to be insufficiently sensitive and specific for Artemis detection; therefore, an immunoprecipitation (IP) step was added as previously described (14). Briefly, ~2.5 million cells were harvested and sonicated on ice with three 30-s pulses in 750 μl of TBST [25 mM Tris–HCl pH 7.4, 130 mM NaCl, 3 mM KCl, 0.1% (v/v) Tween-20] supplemented with 1 μM leupeptin, 1 μM pepstatin A and 1 μM aprotinin protease inhibitors.

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methanol transfer buffer, then incubated in TBST, 10% (w/v) powdered milk (blocking buffer), 1 h at 22°C, with rocking. Membranes were probed with chicken IgY anti-human Artemis antibody (Abcam, ab14289) at a 1:2000 dilution in 8 ml blocking buffer overnight at 4°C with tumbling, then washed in TBST 10 min × 3, and incubated in peroxidase-conjugated donkey anti-chicken IgY (H+L) (Gallus Immunotech Inc.) at 1:5000 dilution in 8-ml blocking buffer, 1 h, RT, tumbling then washed in TBST 10 min × 3 and developed with ECL plus (GE Healthcare).

Cell survival assays

Confluent cells were further maintained in 0.5% serum for 5 days. The cells were then irradiated [MDS Nordion GammaCell 40 research irradiator (ON, Canada), with a 137Cs source delivering a dose rate of 1.05 Gy/min] or treated with either bleomycin or neocarzinostatin (NCS) for 4 h. Following treatment, cells were washed with PBS and 500–20 000 cells were seeded into 10-cm dishes in fresh medium. Cells were then incubated for 12 days before they were fixed with methanol (100%) and stained with 1% crystal violet, rinsed with water and air-dried. Visible colonies were counted manually.

Immunofluorescence

Cells were grown to confluence on tissue culture-treated 4-well chamber slides (Nunc lab tek) and further maintained at 0.5% serum for 24 h. Cells were then either irradiated (2 or 4 Gy) or treated with NCS (6 nM) for 1 h. After treatment, cells were fixed with 3% paraformaldehyde and permeabilized with 0.5% Triton X-100/PBS. Blocking was done using Casein blocker (Bio-Rad). Primary antibodies used for immunostaining were anti-γ-H2AX (Upstate), anti-53BP1 (gift of Dr David Gewirtz, Virginia Commonwealth University, originally from Dr Thanos Halazonetis, University of Geneva) at 1:500 dilution, anti-PML (PG-M3, Santacruz biotech) at 1:200 dilution, anti-γ-H2AX rabbit polyclonal (Novus biologicals) at 1:400 dilution and anti-hMre11 rabbit polyclonal (Novus biologicals) 1:200 dilution. The secondary antibodies were Alexa Fluor 488/568 goat anti-mouse/anti-rabbit (Invitrogen Molecular Probes) at 1:500 dilutions. Slides were mounted with Vectashield mounting medium containing DAPI (4',6-diamidino-2-phenylindole). Images were captured using Olympus fluoview 500 confocal microscope, using a 430 nm diode laser with a 605 nm band pass filter, a 510 nm laser with a 530 nm band pass filter and a 660 nm laser with 605 nm band pass filter. Foci from approximately 100 cells were scored for each time point in 2–3 independent experiments for each cell line. Focus diameter for the five largest foci in each cell was measured in one direction parallel to the equatorial plane of the image field.

Fraction of activity released DSB-repair assay

Pulsed-field gel electrophoresis (PFGE) was used to quantify DSB repair as described previously (15) with minor variations. Briefly, subconfluent cells were cultured on a 150 cm dish and labeled for 24 h with 0.2 μCi/ml [methyl-3H]thymidine (20 Ci/mmol, Perkin-Elmer). Confluence-arrested cells were serum starved (0.5%) for 24 h prior to irradiation. After irradiation samples were incubated for noted repair times and then trypsinized and resuspended in L buffer (0.1 M EDTA, 0.01 M Tris–Cl, 0.02 M NaCl) at a concentration of 2 × 107 cells/ml. Of this suspension, 250 μl was mixed with 250 μl of 2% low-melting agarose (Nusieve GTG) in a 15 ml conical tube maintained at 45°C. About 60 μl of cell agarose mixture was transferred to a sample CHEF disposable plug mold (Bio-Rad) and allowed to solidify at 4°C. The plugs were removed and incubated in the digestion mixture (0.1 M EDTA, 0.01 M Tris–Cl, 0.02 M NaCl, 1% w/v Sarkosyl, 0.1 mg/ml proteinase K). The plugs were incubated for 24 h at 50°C with a change of digestion mixture after 3 h. They were then washed with TE (Tris–EDTA) over a period of 3 h. The plugs were further incubated with 40 μg/ml PMSF for 30 min at 50°C followed by three washes over a period of 3 h. The plugs were then incubated with 3×10^7 cell/ml at 1.5 V/cm with switch times varying between 60 and 3600 s. The temperature was maintained at 14°C throughout the run. Each lane of the plug was cut into five equal slices and the fraction of radioactivity released fraction of activity released (FAR) from the plugs and remaining in the plugs was measured by liquid scintillation.

RESULTS

D165N mutant Artemis fails to rescue chemo/radiosensitivity

Previous in vitro studies have shown that in the presence of DNA-PK, Artemis has endonucleolytic activity toward both the 3’ and 5’ termini of DNA ends (2,8), which may participate in the slow processing of 3’-PG-terminated DSBs (9). To investigate the biological relevance of Artemis endonucleolytic activity, wild-type and D165N mutant Artemis were expressed in patient-derived CJ179 cells, as well as in normal 48BR cells, using lentiviral vectors. In the absence of recombinant DNA vectors, the CJ179 cells are Artemis-null and fail to express any Artemis transcript (5).

Artemis expression levels were first evaluated by western blots of Artemis immunoprecipitated from extracts of the complemented and mock-complemented lines (Figure 1A). In cells harboring Artemis-encoding viral constructs, levels of wild-type and D165N Artemis were comparable, and were much greater than the level of native Artemis in normal fibroblasts, which was below the level of detection. However, the presence of an Artemis transcript in normal 48BR cells was verified by gel electrophoresis following real-time q-PCR, which showed a product of the expected size that was not evident in the Artemis-deficient CJ179 cells (Supplementary Figure 1).

Radiation, bleomycin and NCS are known to induce free radical-mediated DSBs of diverse structure, many of which bear 3’-PG termini (16–18). The toxicity of these agents was therefore evaluated in Artemis-deficient
CJ179 cells carrying integrated viral constructs expressing wild-type Artemis, D165N mutant Artemis or no protein. The Artemis-deficient CJ179 fibroblasts showed significant hypersensitivity to all three agents (Figure 1B–D), as reported previously for other, unrelated Artemis-deficient cell lines (9). Complementation with wild-type Artemis but not D165N mutant Artemis rescued this sensitivity. Furthermore, this overexpression of wild-type Artemis increased the chemo/radioresistance of normal 48BR cells, as compared to that of 48BR cells carrying the empty vector. Taken together, these results suggest a direct role of Artemis endonucleolytic activity in survival following radiation or radiomimetic drug treatment.

**Endonucleolytic activity of Artemis is essential for DNA DSB rejoining in cells**

To more directly assess the role of Artemis’ endonuclease activity in DSB repair, γ-H2AX and 53BP1 foci were quantified as surrogate markers for residual DNA DSBs in Artemis-deficient/proficient cell lines following irradiation or NCS treatment. These assays were performed using non-replicating G0/G1 cells (Supplementary Figure S2) to avoid spontaneous focus formation at stalled replication forks. The formation and loss of γ-H2AX and 53BP1 foci was similar in all cell lines at 30 min and 2 h post-irradiation. However, at 6–18 h, a significant fraction of foci were seen to persist in Artemis-deficient CJ179 fibroblasts, while nearly all of the foci resolved in normal 48BR cells (Figure 2A and B and Supplementary Figure S3). Stable complementation of CJ179 with wild-type Artemis corrected this defect, resulting in wild-type focus levels, while the D165N mutant Artemis completely failed to rescue the DSB repair defect, indicating that resolution of γ-H2AX and 53BP1 foci following radiation requires Artemis endonucleolytic activity. Essentially identical complementation results were obtained in cells treated with NCS (Figure 2C). Although the kinetics of γ-H2AX and 53BP1 foci were nearly identical, a higher radiation dose was required to induce a comparable number of 53BP1 foci. Staining with a mouse monoclonal antibody to γ-H2AX (data not shown) confirmed that some γ-H2AX foci did not contain detectable 53BP1, as has been previously reported (19).
The above results were confirmed by a PFGE assay that measures DSBs directly but requires use of higher doses of radiation (40 Gy, Figure 2D and E). Consistent with the repair focus studies above, 10–20% of the DSBs remained unrejoined in Artemis deficient and D165N mutant-complemented CJ179 cells. Taken together, these data show that repair of this subset of DNA DSBs requires Artemis endonucleolytic activity at both low and high doses.

**Low-level Artemis expression is sufficient to restore radiosurvival**

A possible confounding factor in interpreting these studies, as well as other studies using transient expression (6), is the elevated level of Artemis transgene expression, which is much higher than endogenous levels in normal fibroblasts. To address this concern, clonal isolates having various levels of Artemis expression were derived from cultures of 48BR and CJ179 cells transduced with vectors encoding wild-type or D165N Artemis.

Relative Artemis expression levels were first evaluated by real-time q-PCR and then confirmed by IP/western. All transgene-expressing clones produced detectable levels of Artemis protein, which in all cases correlated with Artemis mRNA levels. While the Artemis levels in all clones were still higher than the undetectable level in 48BR cells, they were comparable to and in some cases lower than the endogenous level in normal lymphoblastoid cells (Figure 3A and B). Moreover, the CJ179 clone expressing the lowest level of wild-type Artemis was as radioresistant (Figure 3D) and as repair-proficient (Figure 3E) as the highest expressing clone (showing 7-fold higher expression by q-PCR), and both were more radioresistant than normal 48BR Vect cells. In contrast, even high levels of Artemis D165N expression (~8-fold higher than the level in CJ Arte+ clone 1 by q-PCR; Figure 3A) conferred no detectable radioresistance (Figure 3D).
Overall, these results show that a relatively modest amount of wild-type Artemis is sufficient to correct the repair defect in Artemis-deficient cells and increase radioresistance, and suggest that the restoration of radioresistance is dependent on endonuclease activity and is not an artifact of overexpression.

**Persistent radiation-induced foci in Artemis-deficient cells grow larger with time and juxtapose with PML-nuclear bodies**

In addition to a larger number of persistent foci, cells lacking functional Artemis showed a significant time-dependent increase in the average diameter of the residual foci, from ~0.8 μm at 30 min to 1.4 μm at 12–18 h (Figure 4). This increase was seen for both 53BP1 and γ-H2AX foci, and for both Artemis-deficient and Artemis D165N-complemented cells. These results are consistent with hypersensitivity in these cells being due to defective repair processes. Moreover, these data suggest that the unrepaired DSBs promote persistent ATM activation and continued accumulation of repair factors such as Mre11 in the vicinity of the break site (Supplementary Figure S4).

PML protein is a tumor suppressor that along with Daxx, SP100 and CBP has been identified as the main constituent protein within sub-nuclear compartments also referred to as nuclear bodies (20,21). PML is phosphorylated by ATM (22) and PML-NBs have also been shown to co-localize with Mre11 and p53 at the sites of radiation-induced foci specifically at later time points (21).

To investigate whether PML NBs and associated proteins may be recruited to the residual DNA DSBs,
PML and γ-H2AX interaction was analyzed in cells by double immunostaining and confocal microscopy. As shown in Figure 5 and Supplemental Figure S5, a subset PML NBs were found to be juxtaposed to the persisting DNA DSBs in Artemis-deficient cell lines 12–18 h after irradiation. These results indicate that such positioning occurs even at relatively low levels of initial DNA damage, and suggest that the determining factor for co-localization is the persistence of a significant number of unresolved DSBs.

Overexpression of D165N mutant Artemis renders normal cells repair deficient

If, as proposed, the primary role of Artemis in chemo/radioresistance is endonucleolytic trimming of DSB ends, then overexpression of mutant Artemis might be expected to displace the normal enzyme and prevent such trimming. At 2 Gy, or at equally toxic concentrations of bleomycin or NCS, no such dominant-negative effect was seen, as D165N-overexpressing 4BR cells were as chemo/radioresistant as empty-vector controls (Figure 2C and Supplementary Figure S6), and were fully competent in resolution of repair foci (Figure 2A). However, in FAR assays, overexpression of D165N mutant Artemis conferred upon normal fibroblasts a slight repair defect at high doses of γ-radiation (40 Gy, Figure 2B). Conversely, overexpression of wild-type Artemis rendered normal cells more radioresistant (Figure 2C), but did not produce a detectable change in repair, with all measurable DSBs being rejoined within 12 h according to both focus formation (Figure 2A) and FAR assays (Figure 2B).

DISCUSSION

Artemis deficiency has pleiotropic effects in human cells following exposure to DNA damaging agents, including defects in regulation of cell cycle checkpoints (3,4) and in apoptotic DNA fragmentation (23). However, other studies suggest that Artemis is epistatic with ATM in promoting radiosurvival and DSB repair, even in growth-arrested cells that should not be subject to cell cycle effects (5). Specifically, in ATM- or Artemis-deficient cells a small fraction of DSBs (10–20%) remains unrejoined, even after several days (5,14).

Experiments using defined substrates and purified enzymes have shown that in the presence of DNA-PK, Artemis gains an endonucleolytic activity toward DNA ends that is inherently capable of resolving DSBs bearing terminal blocking groups such as 3'-PGs (8–10,24,25). PFGE as well as focus-formation assays in noncycling contact-arrested fibroblasts (Figure 2) show that wild-type but not endonuclease-deficient Artemis completely restores DSB-repair proficiency, in agreement with recent observations in G2 cells where Artemis was expressed transiently (6). Taken together, these results strongly suggest that the primary function of Artemis in DSB repair is the endonucleolytic processing of DNA ends.

More importantly, survival assays with stably complemented Artemis cell lines (Figure 1) show that only endonuclease-proficient Artemis is able to restore chemo/radioresistance. These data suggest that lack of endonucleolytic activity, and by inference DSB end processing, is the principal cause of chemo/radiosensitivity in Artemis-deficient fibroblasts. Although an effect of the D165N mutation on recruitment to damaged DNA (26),
or on other unknown functions of Artemis, cannot be strictly excluded, the mutant enzyme retains its 5'→3' exonuclease activity (8), and is phosphorylated by DNA-PK as efficiently as the wild-type protein (10). These data suggest that D165N Artemis has intact secondary structure and retains proficiency in assembling with DNA-PK at DNA ends. Recently, it was shown that most (but not all) of the exonuclease activity in His6-affinity-purified Artemis could be eliminated by ion-exchange chromatography, suggesting that the exonuclease might be a contaminant (27). However, other studies showed that extensively purified Artemis protein retained exonuclease activity (28), and that antibodies raised against Artemis protein gel-purified from *Escherichia coli* inactivate the exonuclease, suggesting that this exonuclease activity is intrinsic (8).

The finding that endonuclease-deficient Artemis, even when overexpressed, has no effect at all on chemo/radiosurvival (Figure 1), combined with evidence that Artemis-deficient fibroblasts retain functional G1 and G2 checkpoints (14,29), would also appear to exclude a checkpoint defect as a major contributor to the chemo/

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**Figure 5.** PML and γ-H2AX partial co-localization and juxtapositioning after 2 Gy γ-radiation exposure. Double PML/γ-H2AX staining was performed after paraformaldehyde fixation. Confocal analysis of representative cells is shown. Green fluorescence: γ-H2AX (polyclonal anti-γ-H2AX, Novus biologicals); red fluorescence: PML (monoclonal anti-PML, Santa Cruz, PGM-3). IR Ctrl = no irradiation.

**Figure 6.** Overexpression of D165N mutant Artemis confers high-dose DSB-repair deficiency. (A) γ-H2AX focus assay was performed after 2 Gy γ-irradiation. (B) PFGE analysis of normal fibroblasts overexpressing wild-type or D165N mutant Artemis after exposure to 40 Gy γ-rays. The panel displays FAR versus repair time (for 48BR Vect versus 48BR Arte− $P > 0.3$ at all times; for Vect versus Endo− $P = 0.04$ at 12 h and $P = 0.16$ at 18 h, by t-test). (C) Clonogenic survival was determined following treatment with radiation (for Vect versus Arte− $P = 0.04$ at 4 Gy and $P = 0.07$ at 6 Gy; for Vect versus Endo− $P > 0.3$ at all doses). The data for 48BR Vect and 48BR Arte− are same as shown in Figures 1 and 2, and are shown here separately for the sake of clarity and comparison with 48BR Endo−. Error bars represent the SEM from three independent experiments except for (A), which shows data from two experiments.
radiosensitivity of Artemis-deficient fibroblasts. Although checkpoint deficiencies have been reported for tumor cells in which Artemis expression has been knocked down with siRNA (3,4), there is no evidence that these deficiencies account for radiosensitivity.

Previous studies have shown that a small number of DSBs remain unrejoined in irradiated cells lacking Artemis (5,14), but the exact nature of these repair-resistant breaks remains incompletely defined. The three Artemis (5,14), but the exact nature of these repair-DSBs remain unrejoined in irradiated cells lacking account for radiosensitivity.

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Although
once a critical level of Artemis expression is reached in cells, additional Artemis confers no added survival advantage (Figure 3). This result suggests that in the Artemis transgene-expressing cells, the concentration of Artemis at DSBs is no longer a limiting factor in repair.

Conversely, overexpression of D165N mutant Artemis in normal cells made them slightly repair deficient as judged by PFGE (Figure 6), suggesting that the mutant Artemis can effectively compete with endogenous Artemis for DNA binding. The defect in repair appears to be expressed only when the repair system is saturated by very high levels of DNA damage. Although there was a slight trend toward greater radiosensitivity when very high levels of mutant Artemis were expressed in normal cells, it was not statistically significant (Figure 3C). Nevertheless, the dominant-negative effect on repair as well as the increase in radioresistance with overexpression of Artemis is most consistent with Artemis functioning directly in the repair process.

Taken together, the results suggest that a fraction of DSBs with chemically modified termini strictly require Artemis endonuclease activity for repair in G1. The complete failure of endonuclease-deficient Artemis to improve survival suggests that lack of end processing by this endonuclease can fully account for the chemoresistance conferred by Artemis deficiency.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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