Radiation-induced delayed cell death in a hypomorphic Artemis cell line

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Received January 13, 2006; Revised and Accepted March 3, 2006

Null mutations in Artemis confer a condition described as RS-SCID, in which patients display radiosensitivity combined with severe combined immunodeficiency. Here, we characterize the defect in Artemis in a patient who displayed progressive combined immunodeficiency (CID) and elevated lymphocyte apoptosis. The patient is a compound heterozygote with novel mutations in both alleles, resulting in Artemis proteins with either L70 deletion or G126D substitution. Both mutational changes impact upon Artemis function and a fibroblast cell line derived from the patient (F96-224) has greatly reduced Artemis protein. In contrast to Artemis null cell lines, which fail to repair a subset of DNA double strand breaks (DSBs) induced by ionizing radiation, F96-224 cells show slow but residual DSB rejoining. Despite showing intermediate cellular and clinical features, F96-224 cells are as radiosensitive as Artemis null cell lines. We developed a FACS-based assay to examine cell division and cellular characteristics for 10 days following exposure to ionizing radiation (2 and 4 Gy). This analysis demonstrated that F96-224 cells show delayed cell death when compared with rapid growth arrest of an Artemis null cell line, and the emergence of a cycling population shown by a control line. F96-224 cells also display elevated chromosome aberrations when compared with control cells. F96-224 therefore represents a novel phenotype for a hypomorphic cell line. We suggest that delayed cell death contributes to the progressive CID phenotype of the Artemis patient.

INTRODUCTION

DNA damage poses a major threat to multicellular organisms as it can lead to genomic instability and cancer. DNA double-strand breaks (DSBs) are particularly significant and potentially lethal lesions that result from damage by exogenous agents such as ionizing irradiation and potentially by reactive oxygen species (ROS) generated as by-products of cellular metabolism. DSBs also occur during programmed DNA rearrangement in V(D)J recombination, a process by which immunoglobulin and T-cell receptor genes are assembled in the mammalian immune system to generate B- and T-cell antigen specificity (1).

The DNA non-homologous end joining (NHEJ) pathway is the predominant mechanism for repairing DSBs in mammalian cells (2,3). The core NHEJ apparatus includes the DNA-dependent protein kinase (DNA-PK), which is composed of the heterodimeric Ku protein (Ku70 and Ku80) and its catalytic subunit, DNA-PKcs and a ligation complex that encompasses DNA ligase IV and XRCC4. However, ends generated by DNA damage are rarely directly ligatable and recent studies have identified Artemis as a nuclease involved in processing DNA ends prior to ligation by NHEJ (4,5). Artemis is required to repair ~10% of the DSBs induced by γ-irradiation, which represent those rejoined with slow kinetics in mammalian cells (4). It has been argued that these represent ends that require processing prior to rejoicing (6). In addition, Artemis is required to cleave the hairpin end generated during V(D)J recombination (7). Artemis nuclease activity requires DNA-PKcs consolidating its role in NHEJ (7).

Patients with mutations in Artemis have been identified and designated radiosensitive-severe combined immunodeficiency (RS-SCID) because of their associated radiosensitivity.
and immunodeficiency, both representing outcomes of the characterized roles of Artemis in end-processing (5,8,9). The majority of Artemis patients display marked T-B-SCID and have genomic deletions causing loss of protein function (null mutations) (5,10). Patients displaying combined immunodeficiency (CID) with point mutational changes in Artemis have also been reported (11). As these patients displayed CID rather than the SCID observed in patients with genomic deletions, the mutations identified were considered to be hypomorphic. Interestingly, two of the patients developed EBV lymphoma. One patient displaying Omenn’s syndrome, another ‘leaky’ form of T-B-SCID with immunodeficiency, erythroderma, lymphadenopathy and hepatosplenomegaly, has also been shown to have Artemis mutations, which again were considered to be hypomorphic (12).

We previously described a patient with progressive CID characterized by lymphopenia, whose lymphocytes showed elevated apoptosis and whose fibroblasts displayed radiosensitivity (13). We were unable to detect cell cycle checkpoint defects, V(D)J recombination defects or impaired DSB repair in a fibroblast line (F96-224) derived from the patient. Using more sophisticated assays for detecting DSB repair than were available at the time of our initial analysis, we re-examined F96-224 cells and identified a subtle DSB repair defect consistent with impaired but not ablated Artemis function. We, therefore, report a sensitive procedure capable of detecting impaired but residual Artemis function. Sequencing verified the patient to be an Artemis compound heterozygote. Surprisingly, although the DSB repair kinetics and V(D)J recombination defect are distinct from cells with null Artemis mutations, the level of radiosensitivity is indistinguishable between F96-224 and an Artemis null cell line. Examination of cell cycle progression post-irradiation demonstrated that whereas Artemis null cells fail to recover from G2/M arrest after exposure to 2 or 4 Gy and control cells continue progression, a significant population of F96-224 cells progress through six to seven divisions before arresting as small cells. Our findings, therefore, provide evidence for delayed cell death in this cell line, which we relate to the progressive CID observed in the patient. We consider our findings in terms of a breakage bridge fusion model.

RESULTS
F96-224 is DSBR deficient because of compound heterozygous mutations in ARTEMIS

We previously used pulsed field gel electrophoresis (PFGE) to examine DSB repair in F96-224 cells and reported a normal rate of repair following exposure to 30 Gy (13). Recent studies have demonstrated that analysis of the rate of loss of γ-H2AX foci post-irradiation represents a markedly more sensitive DSB repair assay (4,14). Analysis of F96-224 cells following exposure to 3 Gy γ-rays, revealed a subtle but reproducible DSB repair defect detectable at 24 and 48 h post-IR (Fig. 1A). However, DSBs were repaired slowly such that by 4–6 days post-irradiation no elevated unrepaird DSBs were detectable (Fig. 1A). This defect is distinct to that displayed by F02/385, an Artemis null cell line, where ~10% of the DSBs remain unrepaird for long times post-irradiation.

Figure 1. F96-224 is a hypomorphic Artemis deficient cell line. (A) F96-224 cells show impaired but residual DSB rejoining after irradiation. γ-H2AX foci were counted at the indicated times following exposure to 3 Gy γ-rays; h represents hours and D, days. WT1 is a control cell line (48BR), Ligase IV is a DNA ligase IV deficient cell line (180BR) and Artemis Null is a full Artemis mutant line (F02/385). 180BR cells show impaired rejoining of all DSBs; F02/385 cells show a fraction (~10%) of unrepaird DSBs that persist for long periods (upto 8 days shown); F96-224 cells show slow rejoining of the Artemis-dependent fraction of DSBs. The results represent the mean and SD of three experiments. (B) F96-224 cells have reduced but detectable Artemis protein. Although no residual protein was detectable in Artemis null (F02/385) cells, F96-224 cells show low residual protein. WT2 is a control line (1BR3). Western blotting was carried out using α-Artemis antibodies. (C) ΔGTT207-9 and 377G>A are impacting Artemis mutations. cDNAs expressing wild-type myc-tagged Artemis cDNA (Art Null + mycART), ΔGTT207-9 mycArt cDNA (Art Null + ΔGTT) or 377G>A myc-H2AX cDNA (Art Null + G377A) were transfected into Artemis null, CJ179 hTERT cells. After 24 h, cells were exposed to 10 Gy γ-rays and the number of 53BP1 foci scored 16 and 24 h later in those cells expressing Myc. Control cells were untransfected CJ179 hTERT (Art Null-DNA) or normal fibroblasts (48BR hTERT) examined either without transfection (WT1-DNA) or lowering transfections with a GFP only vector DNA (WT1-GFP). When GFP-expressing vectors were used, γ-H2AX foci were scored in GFP-expressing cells rather than Myc-expressing cells. Whereas wild-type myc-ART cDNA substantially corrects the DSB repair defect in CJ179 hTERT cells, no substantial correction was observed with either mutant cDNA demonstrating that the two mutational changes impact upon Artemis function. The results represent the mean and SD of three experiments.
LIG4 syndrome patient (180BR). These findings demonstrate that, in contrast to our previous conclusion, F96-224 cells show a subtle DSB repair defect and that γ-H2AX foci analysis represents a valuable assay to detect DSB repair defects in patients.

The DSB repair profile of F96-224 cells raised the possibility that the cells might have a mutation in a component required for Artemis-ATM dependent DSB repair (4). Candidate defects include Artemis and components of the MRN complex. Our previous studies demonstrated that F96-224 cells are proficient for radiation-induced G1/S, intra-S and G2/M checkpoint arrest (13). NBS and ATLD deficient cells display intra-S phase checkpoint defects suggesting that this aspect of the MRN complex is functional in F96-224 cells. Sequencing, moreover, revealed a normal Nbs1 sequence. In contrast, sequencing of Artemis revealed a 3 bp deletion (ΔGTT207-9) in exon 3 in one allele and a base substitution 377G>A in exon 6 on the other allele, which result in the loss of a single amino acid (L70) and an amino acid substitution (G126D), respectively (data not shown). To confirm that the patient was a compound heterozygote, full length Artemis from F96-224 was cloned and individual colonies sequenced, 10 clones showed the ΔGTT mutation alone, 12 showed 377G>A, none showed both, demonstrating unequivocally that each allele contained only one mutation.

Examination of Artemis expression in F96-224 cells by western blotting revealed markedly reduced levels of Artemis protein but, in distinction to Artemis null cells, a low level of residual protein is detectable (Fig. 1B). Artificial expression of both mutant Artemis alleles in 293T and HCT116 human cell lines produced nuclear localized protein (data not shown). In this system, the G126D mutant appeared to be less stable, however, it is not clear in the F96-224 cells which allele is responsible for residual protein expression.

To verify that the mutations observed in F96-224 represent impacting mutational changes rather than polymorphisms, we introduced ΔGTT207-9 and 377G>A into myc-tagged Artemis cDNA by site-directed mutagenesis and examined the ability of the mutant cDNAs to complement the DSB repair defect in CJ179 hTERT (Artemis null) cells using 53BP1 foci analysis as a monitor of DSB repair. We have previously shown that γ-H2AX and 53BP1 foci not only co-localize but are present in equal numbers and are lost with identical kinetics (4). 53BP1 foci were examined rather than γ-H2AX because phospho-53BP1 and myc antibodies require distinct secondary antibodies and can be used in conjunction. As most human cell lines including CJ179 hTERT cells undergo inefficient transfection, we monitored DSB repair solely in those cells expressing Myc. The wild-type Artemis cDNA substantially corrected the DSB repair defect in Artemis null CJ179 hTERT cells, whereas ΔGTT207-9 and 377G>A Artemis cDNAs failed to provide any substantial level of correction (Fig. 1C). The lack of full correction by the wild-type cDNA likely represents the fact that Artemis may not be efficiently expressed. Taken together these results provide strong evidence that F96-224 cells have impacting mutational changes in Artemis that underlie their DSB repair deficiency.

Figure 2. Radiosensitivity of Artemis fibroblast lines. F96-224 cells show a similar level of radiosensitivity to an Artemis null cell line, CJ179. Two control lines, WT 1 (48BR) and WT2 (1BR3) are shown for comparison. Results show colony survival 3 weeks following irradiation and represent the mean and SD of at least three independent experiments.

F96-224 cells display delayed cell death after irradiation

Our previous analysis of V(D)J recombination in F96-224 cells using a plasmid assay demonstrated nearly normal V(D)J recombination proficiency (13). Despite this and the fact that DSBs were repaired to normal levels, albeit with slow kinetics, we previously observed significant radiosensitivity in F96-224 cells. Here, we show that the magnitude of radiosensitivity of F96-224 cells is similar to that shown by an Artemis null cell line (Fig. 2). To gain insight into the mechanism of cell death in F96-224 cells, we devised a procedure (the CFSE assay) to couple the analysis of cell division with cell characteristics (granularity and cell size). Our aim was to identify the time and cell state at which control, F96-224 and Artemis null cells cease division after exposure to irradiation. Carboxy-fluorescein diacetate succinimidyl ester (CFSE) is a fluorescent dye that is stably maintained in the cytoplasm of cells. Following each cell division the intensity of fluorescence halves (15). Thus, the intensity of fluorescence signal (represented by the position on the X-axis) provides a monitor of cell division. Using FACS analysis to monitor the intensity of CFSE, we were able to estimate the number of divisions a cell has passed through since labelling (Fig. 3A). Immediately after labelling, control cells have a high CFSE content (Fig. 3A). Following 6 days of incubation without irradiation, the CFSE content is reduced to a level indicative of the number of cell divisions (Fig. 3A). Irradiated normal cells analysed at 6 days post-treatment show a profile indicating a mixed population of arrested and dividing cells. We also used forward- and side-scatter FACS analysis to separate cells based on cell size and granularity. Cell size provides an indicator of cell cycle state, with G1 cells being small, cells in late G2 being larger and more granular and senescent cells being generally very large and granular (Fig. 3B). For analysis of CFSE, we divided the cells into two populations: those with normal cell size and granularity (low forward and side scatter depicted by the left-hand
panels in Fig. 3B) and larger, granular cells (high forward and side scatter; right-hand panel in Fig. 3B). The CFSE content was monitored by FACS following gating for each population to determine whether the cells with low or high CFSE content represent actively dividing or arrested cells.

Initially, this assay was applied to control, F96-224 and Artemis null (CJ179) cells that were pulse-labelled with CFSE prior to irradiation. Following removal of the dye, cells were irradiated with 2 or 4 Gy IR, then analysed by FACS for CFSE content at 6 days post-treatment. Irradiated control cell populations show significant early recovery from both doses of IR. Six days after irradiation 70% (2 Gy) and 31% (4 Gy) of 1BR3 cells had completed five divisions (Fig. 4A). Irradiated Artemis null cells, in contrast, show highly reduced cell division (high CFSE levels), with only 16% (2 Gy) and 8% (4 Gy) completing five cycles. Surprisingly, F96-224 cells showed significant levels of cells passing through five divisions during this early post-irradiation period (49% at 2 Gy and 20% at 4 Gy) (Fig. 4B). When the cells were plotted according to CFSE content and cell size it was clear that IR-induced all but a small proportion of Artemis null CJ179 cells to arrest as large cells after no more than one division (Fig. 4B). In contrast, a significant proportion of F96-224 cells were present as small dividing cells, resembling recovering wild-type cells. This was unexpected given that F96-224 and CJ179 show similarly low survival following exposure to 4 Gy when assayed by colony survival. We, therefore, extended the analysis to identify the point at which F96-224 cells cease to divide.

CFSE fluorescence diminishes to undetectable levels after six or seven divisions. To determine the fate of F96-224 cells dividing beyond 6 days post-IR, irradiated cells (4 Gy) were incubated for 6 days, then labelled with CFSE. Although the majority of control cells continue to cycle, and Artemis null cells remain arrested, the F96-224 cells still dividing after 6 days progress through a further one to two cell divisions and then arrest as small cells (Fig. 5A). A much smaller proportion of Artemis null cells can also be identified in this late arresting category. Size analysis and staining for senescence-associated β-gal (SAβ-gal) indicated that these are non-dividing, non-senescent cells (Fig. 5B) distinct from the SAβ-gal positive arrested population.
A significant proportion of F96-224 cells continue to cycle for six to seven divisions post-irradiation. This suggested that F96-224 cells are able to escape from cell cycle checkpoint arrest, while maintaining damage that causes delayed cell death. One potential model we considered is that F96-224 cells harbour elevated chromosome aberrations. An important aberration in this context is a dicentric chromosome, which will lead to breakage bridge fusion events at subsequent cell divisions. We, therefore, examined chromosome breakage and particularly dicentric formation in F96-224, CJ179 (Artemis null) and 1BR3 (control) cells. Our analysis revealed that F96-224 and Artemis null (CJ179) displayed similar levels of total chromosome damage (2.9 and 2.7 breaks per metaphase; \( P < 0.05 \)), which was significantly elevated when compared with WT2 (1BR3, 1.6 breaks per cell) (Table 1). However, F96-224 cells showed a 4-fold elevated frequency of dicentric chromosomes when compared with control cells, with 21% of the metaphase cells displaying dicentric chromosomes. The number of acentric chromosome fragments correlated or exceeded dicentric scores in metaphases containing a dicentric. Dicentric formation in F96-224 cells also appeared elevated when compared with Artemis null (CJ179) cells. However, it is noteworthy that it was difficult to obtain high numbers of metaphase chromosomes from the Artemis null irradiated population most likely because the cells remained arrested at the G2/M checkpoint because of the significant DSB repair defect, making it difficult to assess the significance of the difference in dicentrics observed in F96-224 relative to Artemis null cells.

DISCUSSION

F96-224 is a cell line derived from a patient who displayed progressive CID (13). A sibling of the patient displayed a similar condition. Previously, we demonstrated that the patient’s lymphocytes showed elevated spontaneous apoptosis and the fibroblasts displayed elevated radiosensitivity (13).

Here, we show that the patient is an Artemis-deficient compound heterozygote with novel Artemis mutations; in one allele the mutation results in a G126D amino acid change and in the other allele the mutational change causes loss of a single amino acid (L70). Both of these mutations lie within the β-Casp domain, which has been shown to be important for function (16,17). We demonstrate that expression of cDNAs carrying either mutational change fails to complement the DSB repair defect of an Artemis null cell line demonstrating that they represent impacting mutational changes. Furthermore, we show that there is dramatically reduced, although detectable, Artemis protein in F96-224 cells. Our findings are summarized in Table 2. Together, these findings provide strong evidence that deficiency in Artemis function underlies the progressive CID observed in the patient.

The majority of Artemis defective patients have genomic deletions causing loss of Artemis function (5). Such patients normally display T-B-SCID, consistent with an obligatory role for Artemis in hairpin cleavage during V(D)J recombination (5,7). The slightly milder immunodeficiency observed in the patient and sibling suggests that one or both of the mutational changes identified in patient F96-224 are hypomorphic, a notion consistent with the mutational analysis and ability to detect residual Artemis protein in F96-224 cells. This notion is further substantiated by our DSB repair analysis, which demonstrates reduced but residual DSB rejoining in F96-224 cells when compared with the defect observed in an Artemis null cell line. Three other Artemis null cell lines show a similar persistent DSB repair defect (4). In addition, in our complementation analysis, the ΔGT207-9 myc Art cDNA, reproducibly showed a slight ability to complement Artemis null cells suggesting that it may represent a hypomorphic allele. However, because of insensitivity of this assay, this difference was on the borderline of significance. To date, we have been unable to express stable mutant Artemis protein for activity analysis in primary cells. However, we previously found normal V(D)J recombination in F96-224 cells following transfection of V(D)J recombination substrate plasmids which is in striking contrast to the near complete defect in coding join formation in Artemis null cells.
Table 1. Irradiation-induced chromosome aberrations

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Metaphases scored</th>
<th>Chromosome breaks per metaphase</th>
<th>Total dicentrics</th>
<th>Metaphases with a dicentric</th>
<th>Metaphases with a dicentric (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1BR3</td>
<td>40</td>
<td>1.6 ± 0.23</td>
<td>2</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>F96/224</td>
<td>53</td>
<td>2.9 ± 0.28</td>
<td>12</td>
<td>11</td>
<td>21</td>
</tr>
<tr>
<td>CJ179</td>
<td>30</td>
<td>2.7 ± 0.37</td>
<td>4</td>
<td>3</td>
<td>10</td>
</tr>
</tbody>
</table>

Cells were irradiated with 3 Gy, cell cycle was arrested using colcemid and metaphases were analysed from the presence of chromosome damage. Breaks are the average per metaphase ± SEM.

Table 2. Comparison of defects in Artemis mutants

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Artemis null</th>
<th>Patient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblast lines</td>
<td>48BR, 1BR3</td>
<td>F02/385, CJ179</td>
<td>F96/224</td>
</tr>
<tr>
<td>Artemis protein</td>
<td>++</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Artemis mutations</td>
<td>-</td>
<td>Genomic deletions</td>
<td>ΔL70, G126D</td>
</tr>
<tr>
<td>DNA repair</td>
<td>Normal</td>
<td>Defective</td>
<td>Delayed</td>
</tr>
<tr>
<td>Cell cycle checkpoints</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Cell division post-damage</td>
<td>Recovers</td>
<td>Immediate arrest</td>
<td>Arrest delayed 5–7 divisions</td>
</tr>
<tr>
<td>Cell phenotype post-damage</td>
<td>Normal</td>
<td>Senescent</td>
<td>Small, non-senescent</td>
</tr>
<tr>
<td>Lymphocyte apoptosis</td>
<td>ND</td>
<td>+</td>
<td>Elevated</td>
</tr>
<tr>
<td>Chromosome breaks (3 Gy)</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Dicentric formation (3 Gy)</td>
<td>+</td>
<td>++</td>
<td>++++</td>
</tr>
<tr>
<td>Immunodeficiency</td>
<td>-</td>
<td>T/B severe</td>
<td>T/B Progressive</td>
</tr>
</tbody>
</table>

evidence for residual Artemis function in F96-224 cells (13). Taken together, our findings provide strong evidence that F96-224 has hypomorphic Artemis mutation(s), which significantly reduce, but crucially do not abolish, Artemis function.

We also demonstrate that γ-H2AX foci analysis is a powerful assay for identification of Artemis defects that allows hypomorphic mutants to be identified and distinguished from null mutants. The analysis of V(D)J recombination in F96-224 cells, in contrast, failed to detect the defect (13). Similarly, LIG4 syndrome patients show only a subtle defect in the plasmid based V(D)J recombination assay despite displaying a pronounced DSB repair defect (18). The insensitivity of the plasmid-based V(D)J recombination assay is surprising given that F96-224 and LIG4 syndrome patients display pronounced immunodeficiency but perhaps reflects the small number of plasmids transfected into each cell.

Although F96-224 cells show an intermediate DSB repair defect when compared with Artemis null cells, they display a similar level of radiosensitivity. Examination of events post-irradiation demonstrated that, while the vast majority of Artemis null cells fail to divide even after 2 Gy, the majority of F96-224 cells progress through five to seven cell divisions before finally arresting as small cells, which are negative for SA-β-gal. In contrast, control fibroblast populations are able to recover from 2 and 4 Gy and proceed through multiple cell divisions. Although a previous report suggested that Artemis defective cells have impaired G2/M checkpoint arrest, our analysis has demonstrated proficient G2/M checkpoint arrest and, in fact, a prolonged arrest compared with control cells, consistent with the characterized repair defect [4,19; Deckbar et al., submitted for publication]. Our findings, therefore, demonstrate that F96-224 cells can escape cell cycle arrest, which is not surprising given their ability to rejoin DSBs with slow kinetics. However, their delayed cell death suggests that the DSBs might be repaired inaccurately.

One potential model to explain our findings is that the DSB repair defect in F96-224 cells promote inaccurate rejoining either via residual Artemis activity or via an alternative mechanism, leading to telomeric break fusions and/or dicentric formation. Coupled with the residual DSB repair defect, cells with such lesions could escape from G2/M checkpoint arrest but undergo subsequent breakage-bridge fusion events and delayed cell death. Chromosome analysis of irradiated F96-224 cells demonstrated that 21% of the F96-224 metaphase cells formed 8–24 h following irradiation with 3 Gy harboured dicentric chromosomes, however the total number observed was relatively small, and further experiments will be required to conclusively support the model.

The failure of Artemis null cells to display delayed cell death after the doses examined is most likely because of the greater number of persistent unrepaired DSBs triggering permanent cell cycle arrest. Thus, the residual DSB repair activity in F96-224 cells provide only a transient benefit in response to 2 and 4 Gy irradiation. Our findings are potentially important in considering the impact of hypomorphic mutations. Whereas loss of protein function may confer immediate cell death, residual DSB repair may allow progression through further cell divisions with the potential emergence of cycling cells with additional mutational changes. It will also be interesting to determine whether the novel phenotype described is unique to F96-224 cells or is a feature common to other hypomorphic repair-deficient cell lines. Further work is also required to carefully monitor chromosome aberrations at differing times.
post-irradiation. However, as only a single unrepair d DSB may be sufficient to trigger cell cycle arrest, elevated damage may not be readily detectable. It is tempting to speculate that delayed cell death may underlie the unusual clinical phenotype of progressive CID shown by patient F96-224 and his sibling. At an early age, patient F96-224 had nearly normal lymphocyte counts which decreased dramatically during childhood (13). Thus, it is possible that DSBs induced during V(D)J recombination also generate dicentric chromosomes or other abnormal rejoining events which allow cell cycle progression and hence lymphocyte development but underlie subsequent or delayed cell death. Such an explanation might also explain the elevated spontaneous apoptosis previously observed in F96-224 lymphocytes (13). Interestingly, a previous study demonstrated elevated N-myc amplification in Artemis null mouse cells which was proposed to result from dicentric formation and amplification via breakage bridge fusion events (20).

In conclusion, we describe a novel clinical manifestation of impaired but not ablated Artemis activity, namely progressive CID. We describe a valuable assay suitable for clinical diagnosis that can detect loss or impaired Artemis function and distinguish between the two. Our analysis of the cellular impact of impaired Artemis function has shown that it can lead to delayed cell death after DSB formation. We provide evidence that this correlates with escape from cell cycle checkpoint arrest because of delayed DSB rejoining and suggest that these findings could be explained by a breakage bridge fusion model. We suggest that delayed cell death might underlie the progressive immune deficiency displayed by the patient.

MATERIALS AND METHODS

The patient

Case reports of the patient and his sibling have been described previously (13). In brief, the patient’s sibling displayed progressive immunodeficiency with lymphopenia of T- and B-cells. The patient was investigated from the age of 15 months to 6 months of age he had minor infections. As a result of the decreasing lymphocyte counts, he underwent bone marrow transplant at 4 years of age and died from transplant-related complications.

Cell lines and cell culture conditions

F96-224 is a primary fibroblast cell line established from the patient. CJ179 and F02/385 are primary fibroblast cell lines established from Artemis null patients described previously (4). CJ179 and F02/385 cells have genomic deletions in Artemis and no detectable Artemis transcript using primers to either the 3’ or 5’ region of the gene. CJ179 hTERT, used for complementation analysis, is a hTERT immortalized derivative of CJ179. Control fibroblast lines, 1BR3 and 48BR were also used. Fibroblasts were cultured in MEM (Gibco/Invitrogen Ltd, Paisley, UK) supplemented with 15% FCS, 0.2% sodium bicarbonate, 2 mM glutamine, 100 U ml−1 penicillin G and 100 μg ml−1 streptomycin at 37°C, 5% CO2.

Irradiation and radiosensitivity assay

Fibroblasts were irradiated using a 137Cs γ-ray source at a dose rate of 0.09 Gy s−1. Radiosensitivity was assessed by colony survival assay following 0, 1, 2, 4 and 6 Gy irradiation. Dilutions of 200, 400, 800 and 1600 cells were plated and left for 3 weeks prior to colony staining (Methylene Blue) and counting.

Artemis sequencing

Total RNA was extracted from F96-224 fibroblasts and cDNA was synthesized using random hexamer primers and SUPERSCRIPT II reverse transcriptase (Invitrogen). PCR amplification of Artemis cDNA was performed in two separate reactions (yielding overlapping products) with Pfx polymerase (Invitrogen). 5’ Ar temis (1160 bp) was amplified with primers (upper and lower) 5’-GATCGGCGGCGCATATGAGTT-3’ and 5’-GGTGAAGTCGTTCTAGCTTC-3’. 3’ Artemis (1177 bp) was amplified with primers 5’-CCTCTCACAGTGAGATTAAA-3’ and 5’-TGTCATCTCTGAGCGT-3’ (94°C for 2 min, then 35 cycles of 94°C, 15 s; 54°C for 30 s; 68°C for 1 min 30 s; then 68°C for 10 min). Each fragment was amplified for three times in separate PCR reactions, gel-purified and sequenced in both directions using ABI BigDye Terminator Cycle Sequencing Kit V1.1 (Applied Biosystems, Foster City, CA). To confirm compound heterozygosity, full length Artemis from 1BR and F96-224 was amplified (2083 bp) and cloned into pcDNA3.2/V5/GW/D-TOPO using the Gateway TOPO cloning system (Invitrogen). Twenty-five clones from 1BR and 22 F96-224 clones were sequenced. The primers employed in the initial PCR reactions were used in combination with the following sense (U) and antisense (L) primers to sequence amplified Artemis cDNA: 5’ fragment (U2: 5’-AGGAGACTTTCAGATGGCG-3’; U3: 5’-TGTTTTGGAGAAAGGAGC-3’; L4: 5’-TGTCGATTATGTGGAGTGAGTGG-3’; L5: 5’-CTCTCCTCTTCTCTTGATGCC-3’). 3’ fragment (U4: 5’-TGAGCAACAGCCCCAGGATG-3’; U5: 5’-CCCGAGGAAAAAGTTTGCC-3’; L2: 5’-GGAGTAAATCTTCCCTTTGCG-3’; L3: 5’-TCTCAGTTTTCCAGCTGC-3’).

Western blotting

For analysis of Artemis by western blotting, 50 μg of cell extracts were resolved on a 7.5% PAGE gel. Following transfer to nitrocellulose and electroblotting, blocking with 5% skim milk powder in TTBS buffer (20 mM TrisBase–HCl pH 7.5; 150 mM NaCl; 0.1% Tween-20), the blots were incubated overnight at 4°C with anti-Artemis antibody (Orbigen, San Diego; Insight Biotechnologies, London) at 1:2000 dilution. Following washing, the blots were incubated with secondary antibody (Anti-Rabbit HRP) (Dakocytemation Ltd, Cambridge, UK) at 1:2000 dilution. Amersham ECL reagent (Biosciences, Buckinghamshire, UK) was used for detection.

DSB repair analysis and complementation

γ-H2AX foci analysis was performed using non-dividing, confluent cultures (at least 98% of the cells in G1). This is important to ensure that the foci analysed represent DSBs.
Following irradiation at the indicated dose, cells were processed for immunofluorescence using anti-pH2AX antibodies as described previously (21,22). For complementation analysis, CJ179 hTERT cells were transfected with wild-type or mutant Artemis cDNA cloned into pCI-neo-c-Myc using the AMAXA transfection protocol for adherent cell lines (Solution R, program A-24) (Amaca Biosystems, Koeln Germany). Twenty-four hour post-transfection, cells were exposed to 10 Gy γ-rays and analysed for 53BP1 foci 16 and 24 h later. Foci were scored into the Myc positive cells representing those that have been successfully transfected. Approximately, 10–20% transfection efficiency was obtained; a-myc antibody (BL181) was from Bethyl, CA, USA. Mutations in Artemis were introduced using Quickchange XL site-directed mutagenesis kit (Stragene, La Jolla, CA, USA).

Analysis of post-irradiation cell division using CFSE

CFSE (Molecular Probes, Leiden, Netherlands) was dissolved in dimethylsulphoxide (DMSO) at a concentration of 5 mM as a stock solution and stored in aliquots at −20°C. Fibroblasts were seeded at a density of 5 × 10⁴ in 10 cm tissue culture plates and incubated overnight to allow them to adhere. Duplicate plates were prepared for each dose and time-point. Cells were washed once with 5 ml Opti-MEM (Invitrogen), CFSE stock was diluted 1/1000 in Opti-MEM (5 μM final concentration) and 5 ml of this was added to label each cell culture. Cells were incubated with CFSE for 10 min at 37°C, 5% CO₂, washed twice with 5 ml Opti-MEM, followed by the addition of fibroblast culture medium. Cells were then irradiated and incubated at 37°C, 5% CO₂. FACS analysis was then performed counting 15 000 events per sample.

Two protocols were followed. For early post-IR division experiments, CFSE-labelled cells were treated with 0, 1, 2 or 4 Gy γ-radiation on day 0. Control cells were trypsinized immediately after labelling on day 0, fixed in 1% paraformaldehyde (PFA) and stored at 4°C. FACS stock was diluted 1/1000 in Opti-MEM (5 μM final concentration) and 5 ml of this was added to label each cell culture. Cells were incubated with CFSE for 10 min at 37°C, 5% CO₂, washed twice with 5 ml Opti-MEM, followed by the addition of fibroblast culture medium. Cells were then irradiated and incubated at 37°C, 5% CO₂. FACS analysis was then performed counting 15 000 events per sample.

Chromosome analysis

Exponentially growing cells were irradiated or mock-irradiated with 5 Gy using a 137Cs γ-source at a dose rate of 0.09 Gy s⁻¹. After incubation (8 h, 37°C), 150 μl of Colcemid solution (10 μg ml⁻¹, Invitrogen) were added and flasks incubated overnight. Cells arrested at metaphase were harvested by partial trypsinization for 2 min at room temperature. Cells were re-suspended in 5 ml preheated (37°C) 1% sodium citrate, incubated (37°C, 15 min), centrifuged (1000g, 5 min) and re-suspended in 10 ml of fix solution (3:1, methanol:acetic acid). Fixed cells were washed three times in 5 ml of fix solution, re-suspended in 1 ml of fix solution and dropped onto clean glass slides using a Pasteur pipette. After drying, cells were stained with Giemsa (10 min). Metaphase spreads were visualized by light microscopy at 1000× magnification using a Zeiss Axioskop microscope (Photometrics, Tucson, USA) and the number of unrepairied chromosome and chromatid breaks were counted per metaphase. Chromosome breaks were defined as acentric chromosome fragments and chromatid breaks were defined as chromatid gaps (larger than a chromatid width) or fragments. We also noted the number of dicentric chromosomes observed.

ACKNOWLEDGEMENTS

We wish to thank Amanda Heywood for undertaking work on F96-224 as part of her MSc project, and Helena Kempski for help with chromosome analysis. PME was supported by an MRC Studentship. Work in the PAJ laboratory is supported by the Medical Research Council, the Leukaemia Research Fund, the Human Frontiers Science Program, the International Agency for Cancer Research and the European Community Grant Number FIGH-CT-200200207. A.R.G. is supported by the Bubble Foundation UK.

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