Reduced apoptotic response to camptothecin in CHO cells deficient in XRCC3

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Eukaryotic cells respond to DNA damage by activation of DNA repair, cell-cycle arrest and apoptosis. Several reports suggest that such responses may be coordinated by communication between damage repair proteins and proteins signalling other cellular responses. The Rad51-guided homologous recombination (HR) repair plays an important role in recognition and repair of DNA double-strand breaks (DSBs) and cells deficient in this repair pathway become hypersensitive to agents that induce DSBs. In the work reported here we investigated the possible role of the Rad51-like HR proteins XRCC2, XRCC3 and Rad51C in apoptosis following the induction of DSBs by camptothecin. We show that a hamster cell line (irs1SF) deficient in the HR repair gene XRCC3 exhibits altered death and cell-cycle checkpoint responses following treatment with growth inhibitory concentrations of camptothecin. In contrast, hamster cells defective in XRCC2 (irs1) or Rad51C (irs3) treated with equally toxic doses of this agent exhibit a rapid induction of apoptosis similar to that seen in the parental cell line or mutant cells corrected for the HR defect. These results suggest that XRCC3 activity may be necessary for efficient entry into apoptosis in response to DSBs.

Introduction

Recent studies provide evidence that proteins, typically associated with DNA damage recognition and repair, may also play a role in other cellular responses to genotoxic stresses. For example, the loss of the mismatch repair enzymes hMSH2 or hMLH1 leads to genomic instability and reduces G2 checkpoint and apoptotic responses to DNA damage recognized by that system (1–4). Attenuation of an apoptotic response has also been reported in primary human fibroblasts from Xeroderma Pigmentosum patients with mutations of the TFIH DNA helicases XPD or XPB (5), and in XPD lymphoblastoid cell lines (6).

DNA double-strand breaks (DSBs) are one of the most potent forms of DNA damage in cells as a single DSB may be sufficient to trigger cell-cycle checkpoints or apoptosis (7). Two distinct repair systems respond to DSBs, homologous recombination (HR) and non-homologous end joining (NHEJ) (8). Cellular responses to DSBs, including cell-cycle arrest and apoptosis, have been studied extensively [reviewed in ref. (9)]; however, the role of components of the DSB repair pathways in signalling such cellular events is unclear. There have been several studies concerning the involvement of DNA-PKcs in triggering the p53-mediated G1/S arrest and apoptosis. The consensus of these investigations is that DNA-PKcs deficient cells still induce p53 and the cell-cycle checkpoint (10,11); however, the effect on the induction of apoptosis remains unclear (11,12).

The involvement of the components of the HR repair pathway in the induction of cell-cycle checkpoints and apoptosis is unknown. The selective cleavage of recombination protein Rad51 (13) and the RECQ-like helicase BLM (14) during apoptotic cell death, and the specific suppression of the Rad51 recombinational pathway by the anti-apoptotic proteins Bcl-2 and Bcl-XL (15) provide some evidence of a link between HR and apoptosis. However, these events are likely to occur after commitment to apoptosis, rather than signal the induction of apoptosis.

As an initial approach to study the role of HR in the induction of apoptosis, we examined the apoptotic response of hamster cells deficient in the Rad51 paralogs XRCC2, XRCC3 or Rad51C to camptothecin (CPT). CPT is an alkaloid that exerts its cytotoxic action by interfering with the rescaling activity of DNA topoisomerase I (16). This interference stabilizes a normally transient DNA single-strand break (SSB) intermediate (17). These SSBs are converted into DSBs during replication via a run-off mechanism, producing a 5’ phosphorylated DSB only on the leading strand of DNA synthesis (18). Previously, it has been shown that these DSBs trigger HR repair, in accordance with the break-induced replication model for HR at replication forks (19). Here, we show that the apoptotic response of XRCC3 deficient cells is reduced relative to the parental line or cells corrected for the XRCC3 deficiency, despite the enhanced sensitivity of the deficient line to the cytotoxic effects of the drug. In contrast XRCC2 or Rad51C deficient cells respond similarly to parental or corrected cells.

Materials and methods

Cell culture

All cell lines in this study were grown in Dulbecco’s modified Eagle’s Medium (DMEM) with 10% fetal bovine serum and penicillin (100 U/ml) and streptomycin sulphate (100 µg/ml).

Treatments

Camptothecin (Sigma, Dorset, UK) was dissolved in DMSO and maintained at different stock concentrations so an equal volume of drug was added to each cell sample. Cells were plated onto 100 mm Petri dishes in 10 ml growth medium and allowed 4 h for attachment. Exposure to the drug was for 24 h, after which the cells were rinsed twice with PBS and fresh medium was added back to the plate. Time points after treatment refer to time beginning after the 24 h exposure to the drug, when fresh medium is restored. Staurosporine (Calbiochem, Nottingham, UK) was maintained in a stock solution (0.5 mM), and treatment was like that of CPT.

Colony formation assay

500 cells were plated onto 100 mm Petri dishes in triplicate and treated as described above. After growth for 1 week, colonies were stained with...
Table I. Genotype and origin of CHO cell lines used in this study and the concentration of CPT giving 10% survival

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Genotype</th>
<th>Defect</th>
<th>Origin</th>
<th>CPT (D_{10})</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA8</td>
<td>wt</td>
<td>wt</td>
<td>CHO</td>
<td>100 nM</td>
<td>21</td>
</tr>
<tr>
<td>Irs1SF</td>
<td>XRCC3−</td>
<td>XRCC3−, deficient in HR</td>
<td>AA8</td>
<td>10 nM</td>
<td>21</td>
</tr>
<tr>
<td>V3-3</td>
<td>XRCC7−</td>
<td>DNA-PKcs−, deficient in NHEJ</td>
<td>AA8</td>
<td>50 nM</td>
<td>28</td>
</tr>
<tr>
<td>CXR3</td>
<td>XRCC3− + hXRCC3</td>
<td>wt</td>
<td>irs1SF</td>
<td>50 nM</td>
<td>21</td>
</tr>
<tr>
<td>PXR3</td>
<td>XRCC3− + hXRCC3</td>
<td>wt</td>
<td>irs1SF</td>
<td>50 nM</td>
<td>21</td>
</tr>
<tr>
<td>V79-4</td>
<td>wt</td>
<td>wt</td>
<td>V79</td>
<td>120 nM</td>
<td>23</td>
</tr>
<tr>
<td>irs1</td>
<td>XRCC2−</td>
<td>XRCC2−, deficient in HR</td>
<td>V79-4</td>
<td>50 nM</td>
<td>23</td>
</tr>
<tr>
<td>irs3</td>
<td>RADS1C−</td>
<td>RADS1C−; presumably deficient in HR</td>
<td>V79-4</td>
<td>90 nM</td>
<td>29</td>
</tr>
<tr>
<td>irs1X2.1</td>
<td>XRCC2− + hXRCC2</td>
<td>wt</td>
<td>irs1</td>
<td>70 nM</td>
<td>23</td>
</tr>
<tr>
<td>irs1X2.2</td>
<td>XRCC2− + hXRCC2</td>
<td>wt</td>
<td>irs1</td>
<td>90 nM</td>
<td>23</td>
</tr>
</tbody>
</table>

0.4% methylene blue in methanol and those colonies containing >25 cells were counted.

Cell survival analysis

5×10^5 cells were plated onto 100 mm Petri dishes and treated in triplicate, as described above, with the D_{10} dose of CPT for 24 h, as determined by the colony formation assay (Table I). At various time intervals after treatment, the cells were trypsinized and counted with a haemocytometer.

Measurement of cell-cycle distributions

5×10^5 cells were treated as described above. At various time intervals after treatment, cells were trypsinized, rinsed with PBS and fixed in 1% paraformaldehyde in PBS for 5 min at room temperature. Following fixation, cells were rinsed in PBS, pelleted by centrifugation and resuspended in 70% ethanol for storage at −20°C for a minimum of 24 h. For cell-cycle analysis, fixed cells were centrifuged and rinsed once in PBS, before resuspension in PBS containing propidium iodide (PI, 50 µg/ml) and RNase-ONE (50 U/ml, Promega, Southampton, UK). Samples were analysed by flow cytometry (Becton-Dickenson FACSort, 488 nm laser, Becton-Dickenson, Oxford, UK), and cell-cycle distribution was determined by ModFit LT modelling software (Verity Software, Maine, USA).

Apoptosis measurements

5×10^5 cells were treated with CPT or staurosporine as described above. For Annexin-V analysis, attached cells were trypsinized and resuspended with medium containing any floating cells from that sample at various time points after exposure. The cells were pelleted by centrifugation and resuspended for apoptosis analysis with FITC-conjugated Annexin-V protein and PI for cell viability (ApoTarget, Biosource International, Nivelles, Belgium) according to manufacturer’s protocol. Samples were analysed by flow cytometry (Becton-Dickenson FACSort, 488 nm laser), and percentage of apoptotic cells was determined by the fraction of live cells (those that excluded PI) bound with Annexin-V. For apoptosis analysis based on nuclear morphology, 50 µl of Hoechst 33342 stain (Sigma) stock [in water (2 mg/ml)] was added to plates. After 30 min, cells were observed by fluorescence microscopy and a minimum of 500 nuclei were counted. Cells were counted as apoptotic if their nuclei were obviously bright and unambiguously condensed.

Results

Sensitivity of HR and NHEJ deficient cell lines to CPT

To determine the doses of CPT giving 10% survival (D_{10}) in HR proficient and deficient hamster cells (Table I), we measured their relative sensitivity to the drug, using a colony formation assay (Figure 1). In agreement with previous results, the XRCC3 deficient irs1SF strain and the XRCC2 deficient irs1, demonstrated a much greater sensitivity than either the parental lines (AA8 and V79, respectively) or the DNA-PKcs deficient V3-3 (19,20). The irs1SF cell line was the most sensitive of any of the tested cell lines to CPT. The increased sensitivity of irs1SF to CPT was partially corrected in two subclones stably expressing the human XRCC3 protein from either a plasmid (PXR3) or a library cosmid (CXR3) (21) (Figure 1A). Similarly the CPT sensitivity of irs1 was partially corrected in strains in which the wild-type XRCC2 gene was introduced (Figure 1B). The incomplete correction of the CPT sensitivity in the XRCC2 and XRCC3 complemented cell lines may be
the result of inability of the human gene product to interact efficiently with hamster proteins required for this repair pathway (21). Irs3 was not as sensitive to CPT as either irs1 or irs1SF.

**Apoptotic response of HR and NHEJ deficient hamster cell lines**

As CPT is known to be a potent inducer of apoptosis, we next determined whether the increased sensitivity of the lines was accompanied by an enhanced apoptotic response. Interestingly, analysis of apoptotic cells using the Annexin-V assay revealed that untreated irs1 and irs1SF cells showed a background level of apoptosis significantly higher (determined by t-test, $P < 0.05$) than the other cell lines. Notably, this elevated level of apoptotic cells was not present in irs1 or irs1SF cells complemented with XRCC2 or XRCC3, respectively. Irs3 cells also showed a consistently higher level of apoptotic cells, but the increase was not statistically significant. When parental hamster cells were treated with a dose of CPT giving 10% survival, there was a robust induction of apoptosis at 24 and 48 h after exposure (Figure 2A). HR deficient irs1 and irs3 and NHEJ deficient V3-3 cells also responded like the parental cells, with a strong induction of apoptosis. In contrast, irs1SF cells showed no significant increase in the proportion of apoptotic cells after a 24 h exposure to a dose of CPT giving 10% survival ($P = 0.0825$). A significant increase in the fraction of apoptotic cells in irs1SF cells was observed at 48 h; however, the response was much weaker than that seen in HR proficient Chinese hamster ovary (CHO) cells. Irs1SF cells complemented with the XRCC3 gene (PXR3 and CXR3) responded like the parental cells. The reduced apoptotic response in irs1SF was not due to a general defect in apoptosis as both the parental AA8 and irs1SF responded similarly to exposure to D$_{10}$ doses of the general protein kinase inhibitor, staurosporine (data not shown).

The results of the Annexin-V apoptosis assays were verified by staining similarly treated cultures with Hoechst 33342. The proportion of apoptotic cells was determined by counting cells showing nuclear condensation, resulting from the apoptosis-specific nuclear lamin cleavage, 24 h after CPT exposure. This analysis again revealed a much lower level of apoptotic cells in treated irs1SF cultures (Figure 2B). As in the previous experiment, the untreated irs1SF cells showed a higher level of apoptosis relative to the other cell lines tested. However, due to the high background of apoptotic cells present in all types of untreated cultures, the difference in background level was not significant (data not shown).

Counts of irs1SF cells treated with a D$_{10}$ level of CPT revealed that they persisted and remained attached to culture dishes, even though they were unable to form colonies under these conditions (Figure 3). Parental AA8 cells and the XRCC3 complemented PXR3 and CXR3 cell lines showed a substantial drop in cell numbers after 24 h, indicative of an immediate death response to the drug. This was also seen with the DNA-PKcs deficient V3-3 cells. Even after 48 h, the XRCC3 deficient irs1SF cells showed no substantial drop in cell count. The cell number for all cell lines began to recover after 48 h due to outgrowth of the subpopulation surviving the treatment.

**Irs1 SF cells show an altered cell-cycle response to cytotoxic levels of CPT**

Next we wanted to determine if the poor colony-forming ability and altered apoptotic response of irs1SF cells following CPT exposure, was accompanied by alterations in cell-cycle arrest cells. We treated CHO cells with CPT for 24 h and subsequently a 30 min treatment with bromodeoxyuridine (BrdU) before fixation. The DNA content of these cells was measured by PI staining and FACS analysis. Parental AA8 cells and irs1SF cells corrected for the XRCC3 deficiency (PXR3 and CXR3) showed a significant accumulation of cells in late S-phase and G$_2$/M (determined by t-test, $P < 0.05$) (Figure 4). NHEJ deficient V3-3 cells also showed a similar arrest (data not presented). The XRCC3 deficient irs1SF cells, however, showed no significant accumulation of cells in late S-phase and only a slight accumulation of cells in G$_2$/M. Analysis at 48 and 72 h post-treatment also showed no further
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Fig. 3. Cells remaining attached following CPT treatment. Percentage of attached cells remaining after treatment with D10 doses of CPT (100 nM for AA8, 10 nM for irs1SF, 50 nM for V3-3, PXR3 and CXR3). The percentage of attached cells remaining is the number of cells remaining 24 and 48 h after CPT treatment relative to the number of DMSO treated cells remaining after 24 h. The averages (columns) and standard deviations (bars) of three independent trials are presented. Stars denote statistical significance (using t-test) difference between measurements in irs1SF cells (open box) and other cell lines. One star denotes $P < 0.05$ and two stars denotes $P < 0.01$.

Discussion

The DNA-PKcs involved in NHEJ has been suggested to play a role in signalling after DNA damage (10,11). To determine whether proteins involved in HR may have a similar role, we used CHO cell lines defective in HR together with cell lines in which the specific defect have been corrected. We used CPT as a model substance to induce a DNA damage signal, as it is well established that genetic recombination is involved in the repair of CPT-induced lesions (19). Therefore, it was no surprise to find that the HR deficient cell line irs1SF was sensitive to this agent. However, despite the increased sensitivity to CPT, irs1SF lacked the immediate and robust apoptotic response or the G2 arrest seen in the XRCC3 proficient cell lines treated with similarly growth inhibitory doses of the drug. Thus, although the irs1SF cells form as few colonies as wild-type at D10 doses, the irs1SF cells appear to lack the signal that induces apoptosis. Given that the irs1SF cells corrected for the XRCC3 defect regain an apoptotic response, our data suggest that XRCC3 is necessary for induction of apoptosis following CPT treatment. This role may be direct in that XRCC3 [perhaps in combination with other Rad51 paralogs that interact with it (22)] may be pro-apoptotic and directly signal an apoptotic pathway. Thus far, however, there is no evidence that XRCC3 plays such a role as high level expression of this protein from constructs transfected into cells does not appear to induce cell death (21,23).

Alternatively XRCC3 may play an indirect role. XRCC3 may process DNA structures during repair that trigger the induction of apoptosis. Recent data suggest that the Rad51 paralogs act as a multimeric complex and facilitate Rad51 filament formation. It has been shown that several of the vertebrate Rad51 paralogs physically interact with each other (22) and cells in which these genes have been knocked out have similar phenotypes, including decreased levels of HR- and IR-induced Rad51 focus formation (24,25). Given the reports of an interaction between XRCC3 and Rad51C, it is puzzling that the irs3 cells, which are defective in Rad51C, show no alteration of the apoptotic response following treatment with a cytotoxic dose of CPT. However, it is important to note that irs3 shows only a slight sensitivity to CPT. Thus, the interaction between XRCC3 and Rad51C may not be necessary for the response to damage induced by this agent. Another reason for the inability of the XRCC3 deficient irs1SF cell line to enter apoptosis could be that the overall level of damage may not be sufficient to trigger an apoptotic signal. Given the HR repair defect in irs1SF cells, damage that leads to an arrest of growth but not to induction of apoptosis might persist. For any of the mechanisms proposed above, the consequence is that these cells do not enter apoptosis at concentrations of the drug that arrest growth.

Fig. 4. Effect of CPT on cell-cycle distribution of HR proficient and deficient CHO strains. (A) Dot plot for incorporation of BrdU and DNA content in AA8 and irs1SF cells in response to CPT. Distribution of AA8 (B), irs1SF (C), PXR3 (D) and CXR3 (E) cells in different phases of the cell cycle in control and after treatments with D10 doses of CPT. The mean and standard deviation (error bars) of four to eight experiments are depicted. Stars denote statistical significance (using t-test) difference between measurements in treated and untreated cells. One star denotes $P < 0.05$, two stars denotes $P < 0.01$, and three stars denotes $P < 0.001$.

accumulation of irs1SF cells in the G2/M phase of the cell cycle (data not shown).
The elevated level of spontaneous apoptosis occurring in the irs1SF cell line indicates that these cells still have the ability to undergo apoptosis and may be doing so in response to the accumulation of chromosome breaks or rearrangements that occur in these cells due to their HR deficiency (25). Indeed, chicken DT40 cells containing knockouts of the Rad51 paralogs (including the XRCC3) showed a similar high proportion of dead cells (25). Thus, there appear to be alternative apoptotic pathways in irs1SF that remain functional.

In this study, the proficient and deficient cell lines employed were created by one-step methods, allowing their genetic background to remain virtually identical except for the specific genetic deficiency of interest. These p53 deficient hamster lines provide a well-controlled model for studying the effects of chemotherapeutic agents on a genetic background similar to those of most tumour cells (26,27). p53 is known to play a key role in cell-cycle arrest and apoptotic responses to DNA damaging agents, and many studies have attempted to link the activity of a potential apoptotic signalling protein to its interactions or associations with this tumour suppressor. In the p53 deficient background of tumour cells, however, apoptosis can still occur, and understanding these pathways will be important to evaluate the potential of apoptosis-inducing chemotherapeutic agents.

The data presented in this report suggest that the gene product of XRCC3 in the p53 deficient hamster cells is important to maintain a functional DNA damage signal after CPT treatment. This could be due to a specific role of XRCC3 or a XRCC3-dependent HR pathway in the transmission of a DNA damage signal. As seen with other DNA repair pathways (e.g. mismatch and nucleotide excision repair), the disruption of an essential component of HR repair results in attenuation of both repair and apoptotic responses although a more detailed mechanism for the interaction between these pathways remains to be elucidated.

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References


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XRC3 and apoptosis