A DNA Double-Strand Break Defective Fibroblast Cell Line (180BR) Derived from a Radiosensitive Patient Represents a New Mutant Phenotype

Christophe Badie, Michele Goodhardt, Alastair Waugh, Noëlle Doyen, Nicolas Foray, Patrick Calsou, Belinda Singleton, David Gell, Bernard Salles, Penny Jeggo, Colin F. Arlett, and Edmond-Philippe Malaise


ABSTRACT

The 180BR cell line was derived from an acute lymphoblastic leukemia patient who overresponded to radiation therapy and died following radiation morbidity. 180BR cells are hypersensitive to the lethal effects of ionizing radiation and are defective in the repair of DNA double-strand breaks (DSBs). The levels and activity of the proteins of the DNA-dependent protein kinase complex are normal in 180BR cells. To facilitate a measurement of V(D)J recombination, we have characterized 180BRM, a SV40-transformed line derived from 180BR. 180BRM retains the radiosensitivity and defect in DSB repair characteristic of 180BR. The activities associated with DNA-dependent protein kinase are also normal in 180BRM cells. The ability to carry out V(D)J recombination is comparable in 180BRM and a reference control transformed human cell line, MRC5V1. These results show that 180BR and 180BRM differ from the rodent mutants belonging to ionizing radiation complementation groups 4, 5, 6, and 7 and, therefore, represent a new mutant phenotype, in which a defect in DNA DSB rejoining is not associated with defective V(D)J recombination. Furthermore, we have shown that 180BR can arrest at the G1-S and G2-M cell cycle checkpoints after irradiation. These results confirm that 180BR can be distinguished from ataxia telangiectasia.

INTRODUCTION

Several radiosensitive cell lines have been derived from cancer patients who have responded severely to radiotherapy (1). Many of these cell lines display only modest radiosensitivity, but the cell line 180BR is unusual in being extremely hypersensitive (2). 180BR was derived from a normal skin explant from a 14-year-old patient suffering from acute lymphoblastic leukemia. The patient dramatically overresponded to radiation therapy and, subsequently, died as a result of severe radiation morbidity. He showed no clinical symptoms of either AT3 or Nijmegen breakage syndrome and, indeed, was clinically normal until the onset of leukemia. The fibroblasts derived from this patient are as sensitive to ionizing radiation as those from AT patients in both growth and plateau phase (2, 3). They are also hypersensitive after low-dose rate irradiation (4). Unlike AT cells, which display radioresistant DNA synthesis (5, 6), 180BR cells show a normal level of radiation-induced inhibition of DNA synthesis (2). Furthermore, in contrast to AT cells, 180BR cells exhibit a major defect in their ability to repair DNA DSBs (3). They also display an elevated frequency of radiation-induced chromosome breaks and chromosomal aberrations (translocations and dicentrics) compared to normal cell lines (7, 8). 180BR, therefore, represents the first characterized radiosensitive human fibroblast cell line with a major defect in its ability to repair DNA DSBs. Another human cell line, MO59J, derived from a malignant glioma, is similarly highly radiation sensitive and defective in DSB rejoining (9). MO59J cells have been shown to lack expression of DNA-PKcs, a subunit of the DNA-PK, which is known to play a major role in the repair of DNA DSBs in mammalian cells.

A range of radiosensitive rodent cell lines have been described and represent at least 11 complementation groups (10, 11). Mutants belonging to three of these groups (ionizing radiation groups 4, 5, and 7) are defective in DNA DSB rejoining. The genes defined by these groups (XRC4, XRCC5, and XRCC7) and XRCC6 have recently been identified. XRCC4, which is defective in the hamster XR-1 cell line, is of unknown function (12). XRCC5, XRCC6, and XRCC7 encode components of the protein complex, DNA-PK. DNA-PK consists of DNA-PKcs and a DNA-targeting component, Ku. Ku is a heterodimer comprising two monomers, one of M, 70,000 and the other of M, 86,000. Ku86 (Ku80; XRCC5) is defective in xrs-6, XR-V15B (13-16), and xci-3 (17), and the repair defects of these rodent mutants can be partially complemented by human Ku80 cDNA and fully complemented by hamster Ku80 cDNA. None of the rodent mutants isolated on the basis of their radiosensitivity have been proven to be defective in Ku70, but recently, ES cell lines that are defective in Ku70 have been derived and show radiosensitivity. DNA-PKcs is defective in the hamster V-3 and the mouse scid cell lines (18-21). All these rodent cell lines are unable to carry out effective V(D)J recombination, and scid and Ku80 knockout mice manifest a SCID phenotype (Ref. 22; for reviews see Refs. 23 and 24). These results show, therefore, that the DNA-PK complex plays a key role in the repair of ionizing radiation-induced DSBs, as well as DSBs arising during V(D)J recombination. Wiler et al. (25) have also shown that a SCID defect in thoroughbred foals, which is associated with hypersensitivity to radiation as well as V(D)J recombination deficiency, results from a defect in DNA-PKcs, demonstrating that DNA-PK plays the same role in other mammals.

Here, we have further characterized 180BR cells for their ability to carry out V(D)J recombination, for activities associated with DNA-PK, and for their ability to arrest at cell cycle checkpoints. On the basis of our results, 180BR is shown to differ from AT, from the rodent mutants, and from the glioma cell line MO59J, and, thus, represents a new class of DSB repair-defective mutant.
MATERIALS AND METHODS

Cell Lines. 180BRM is a SV40-transformed cell line derived from 180BR (kindly provided by Dr. Iliakis, Thomas Jefferson University, Philadelphia), which is not immortalized and stops growing after 20–25 passages. MRC5VI is a SV40-transformed and immortalized cell line derived from the normal human fibroblast cell line, MRC5 (26). 1BR3 is a normal primary human fibroblast that was used as a control for the cell cycle studies. Cells were cultured in MEM supplemented with 15 or 20% FCS without antibiotics.

Irradiation and Cell Survival. Cells were irradiated with a 137Cs γ-ray source at a dose rate of 1.45 Gy·min⁻¹. For pulsed-field gel electrophoresis studies, the culture flasks were placed on ice for 30 min prior to irradiation, and the culture medium was kept cool (2°C) throughout the period of irradiation. For cell survival, cells in exponentially growing phase were trypsinized and irradiated 4 h after plating. Colonies were fixed and stained after 9–12 days of incubation. The survival curves were fitted with the linear-quadratic model. The parameters (mean inactivation dose) and the surviving fraction at 2 Gy were calculated and used to characterize the intrinsic radiosensitivity (27).

Measurement of DNA DSBs. The DNA DSB rejoining protocol is detailed elsewhere (7). Briefly, 2 × 10⁶ exponentially growing cells were grown for 4 days in MEM plus 20% FCS before labeling and irradiation. The fraction of cells in S phase, which was routinely 30–50%, was measured by cytofluorometry. Experiments measuring repair kinetics were fitted using the formula described by Foray et al. (28).

Preparation of Cell Extracts. Cells were collected in midexponential phase of growth. Nuclear extracts were prepared according to a published protocol (29, 30), except that the final dialysis was performed for 3 h in an excess volume of 50 mM Tris-HCl (pH 7.5), 10% glycerol, 100 mM potassium glutamate, 1 mM EDTA, and 1 mM DTT. Whole-cell extracts were prepared as described previously (30). After preparation, all of the extracts were immediately frozen and stored at −80°C.

DNA End Binding (Mobility-Shift Gel Electrophoresis Assay). These assays were carried out in two laboratories using slightly different protocols. Assays using the transformed cells were carried out using a probe prepared from an aliquot of the 123-bp DNA ladder digested with Aval (both from Life Technologies, Inc.) to generate 123-bp monomers. These were separated by pulsed-field gel electrophoresis (PFGE) (8) and were visualized using ethidium bromide staining. The bands were cut out from the Pulsed Field Inventory (PFIX) gel, and the 123-bp fragments were end-labeled with [γ-32P]ATP using DNA polymerase I Kloneo fragment (Life Technologies, Inc.).

RESULTS

Survival Curves. The radiosensitivities of the primary (180BR) and transformed cell line (180BRM) were compared with those of a control cell line MRC5 and its transformed counterpart, MRC5V1 (Fig. 1). Both transformed cell lines were less radiosensitive than the untransformed lines from which they were derived, but 180BRM remained hypersensitive (Fig. 1). The surviving fractions at 2 Gy for MRC5V1 and MRC5 were 52 and 43%, respectively, compared with 14 and 6% for 180BRM and 180BR, respectively. The ratio of the mean inactivation dose (D), which quantifies the decrease of radiosensitivity associated with transformation, was slightly greater for 180BR [D (180BRM/180BR) = 1.46] than it was for MRC5 [D (MRC5V1/MRC5) = 1.22].

DISB Repair. Induction of the DSBs in the four cell lines was not significantly different (data not shown). However, the DSB rejoining

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capacities of 180BR and 180BRM were similar and significantly reduced compared to those of MRC5 and MRC5VI (Fig. 2). In a previous publication (3) using two primary cell lines 180BR and HF19 (a control cell line) in G0 phase, we analyzed DSB rejoining at multiple times after irradiation and showed that 180BR has a decreased rate and extent of DSB repair.

DNA End-binding Activity. Rodent mutants with defects in DSB rejoining have been shown previously to have mutations in components of DNA-PK (22, 23, 40). The DNA end-binding activity of Ku was examined by the electrophoretic mobility shift assay using extracts of primary and transformed 180BR cells, 1BR3 cells, and MRC5VI cells, together with extracts of HeLa cells, rodent CHO-K1 cells, and the mutant xrs-6, which is known to lack Ku protein. Bands B1 and B2 corresponded to the Ku binding activity that is specific for double-stranded DNA ends because both bands were competed with excess linearized plasmid DNA (data not shown) and were absent from extracts of xrs-6 cells. 180BR and 180BRM cells had end-binding activity that was comparable to that of the control cell lines (Fig. 3).

DNA-PK Activity. The kinase activity of the DNA-PK complex was analyzed in primary and transformed 180BR and control human fibroblasts (Fig. 4A). Background DNA-PK-independent kinase activity resulted in a low level of phosphorylation of the peptide in the absence of DNA (Lanes 3 and 6). However, there were similar marked increases in peptide labeling in both 180BRM and MRC5VI (Lanes 2 and 5) and in 180BR and 1BR3 in the presence of linear DNA, indicating similar levels of DNA-PK activity. Quantification of DNA-PK activity by scintillation counting of the excised gel slices, corresponding to the peptide substrate, indicated a similar activity in extracts from both 180BRM and MRC5VI transformed lines (Fig. 4B) as well as in extracts from both 1BR and 180BR untransformed cells (Fig. 4C). Differences in levels of incorporation that were observed between the primary and transformed lines are due to differences in the protocols used to examine the pair of primary lines compared with the pair of transformed lines.

Analysis of Protein Levels by Immunoblot Analysis. The expression of the components of DNA-PK (Ku70, Ku80, and DNA-PKcs) was also examined by Western blotting using the primary cell lines and antibodies cross-reacting with these proteins. Normal protein expression was observed in every case (Fig. 5). Similar data have also been obtained using the transformed cell lines (data not shown).

V(D)J Recombination. Because all mammalian cell lines with pronounced defects in DNA DSB rejoining described to date have a concomitant defect in ability to carry out V(D)J recombination, we examined whether 180BR had a similar defect. The above experiments confirm that 180BRM maintains the DSB rejoining defect and...
strates were completely intact with no observed deletions (data not shown). Introduction of RAG expression vectors into the 180BRM line resulted in V(DJ) recombination at frequencies (mean, 0.7%) 3-fold lower than that obtained for the control MRC5V1 line (2.3%; Table 1). Analysis of the structure of recombined pH2 Rec plasmid recovered showed that the recombination junctions were essentially normal, in that recombination had occurred at the end of the recombination heptamer without major deletions (Fig. 6). We conclude that the frequency of V(DJ) recombination in 180BRM cells is in the same range as that of normal cells and that there is no change in the fidelity of the junctions formed. 180BRM, therefore, does not manifest the marked defect in V(DJ) recombination that is characteristic of the rodent mutants belonging to complementation groups 4, 5, and 7.

Analysis of the Ability of 180BR to Arrest at Cell Cycle Checkpoints. Sensitivity to ionizing radiation in AT cell lines is associated with inability to arrest at cell cycle checkpoints (7, 41-43). In contrast, the DNA-PK-defective hamster and ES cell lines, which more closely resemble 180BR in the magnitude and kinetics of their defect in DSB rejoining, are able to effect cell arrest at cycle checkpoint (44, 45). Therefore, to determine whether 180BR had associated cell cycle checkpoint defects, we examined cell cycle progression by FACS analysis of cells labeled with BrdUrd for 30 min immediately prior to irradiation and sampled at varying times postirradiation. The unlabeled and labeled populations that represent cells predominantly in G1/G2 or S phase, respectively, at the time of irradiation are separated on the X axis (sample profiles are shown in Fig. 7). The S-phase cells represent a diffuse band of a size that is intermediate between those of G1 and G2 cells. These cells will remain at that position on the X axis, and their progression through the cell cycle at times after irradiation can be determined from their position on the Y axis.

To examine the integrity of the G1-S checkpoint, the movement of

the radiosensitivity described for 180BR. Because 180BR cells have a low transfection frequency, 180BRM cells were therefore used to measure their capacity to carry out V(DJ) recombination following transfection of the recombination activating genes, pRAG1 and pRAG2. The transient V(DJ) recombination substrate pH2 Rec was used to analyze coding junction formation. In the absence of cotransfected RAG expression vectors, no measurable V(DJ) recombination activity was detected (Table 1), and recovered recombination sub-

![Fig. 4. A, in vitro DNA-PK activity assay. Nuclear extracts (45 μg) from 180BRM and MRC5V1 cells were incubated with a p53-derived peptide in the presence of [γ-32P]ATP, and the amount of peptide phosphorylation was determined. Reactions were conducted in either the absence (−) or presence (+) of sonicated calf thymus DNA or peptide substrate as indicated. B, DNA-PK activity in nuclear extracts of MRC5V1 and 180BRM cells. The peptide was incubated with nuclear extract (45 μg) and [γ-32P]ATP. Peptide phosphorylation was measured by liquid scintillation counting. Reactions were performed in both the absence (−) and presence (+) of sonicated calf thymus DNA. The error bars indicate the standard error of the mean. C, whole-cell extracts (30 μg) from 180BR and IBR cells were incubated with a wild-type p53-derived peptide ( ), which is recognized by DNA-PK, and with a mutated peptide ( ), which is not an effective DNA-PK substrate, in the presence of [γ-32P]ATP and the level of peptide phosphorylation determined as described above. It should be noted that it is not valid to compare the levels of activity in the extracts from transformed cells with those from the primary cells (i.e., B with C) because different peptides and assay conditions were used.

![Fig. 5. Immunoblot analysis of extracts from IBR3 and 180BR. Whole-cell extracts from IBR3 and 180BR cells (2 mg for Ku80, 10 mg for Ku70, and 15 mg for DNA-PKcs) were examined by Western blotting using antibodies Ku80-4, Ku70-5, and FLA.

<table>
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<sup>a</sup> AmpR, ampicillin-resistant colonies. The results were obtained from four independent transfections for each cell line.

<sup>b</sup> R, recombination frequency [(no. of blue clones × 3)/total no. of clones].
Fig. 6. Nucleotide sequence of pH2 Rec recombination junctions recovered from 180BRM mutant cell line following cotransfection with RAG1 and RAG2 expression vectors. Sequences of independent recombinant clones are aligned with that of the native plasmid. —¿, nucleotide deletions. Boldface letters, recombination signal heptamers.

CCGCTCTAGAACTAGTGGATCC-CACAGTG-(12)
(23)-CACTGTG-GTCGACCTCGAGGGG

Fig. 7. FACS analysis of 1BR3 and 180BR following exposure to ionizing radiation. Exponentially growing cells were labeled with BrdUrd for 30 min, the medium was changed, and cells were washed to remove the BrdUrd and irradiated with the doses indicated. Control cultures received no irradiation. Cells were incubated for the times indicated and then subjected to FACS analysis. The sample profiles show the progression of cells through the cell cycle after 12 h incubation with or without 3-Gy irradiation. A, unlabeled G1 cells; B, unlabeled G2 cells; C, labeled S and G2 cells; D, labeled G1 cells; and F, unlabeled S phase cells.

the unlabeled cells into S phase (gate F) was estimated (fraction of unlabeled cells in F; sample profiles are shown in Fig. 7 and the data from these and other profiles are summarized in Fig. 8). In unirradiated 1BR3 and 180BR cells, a peak number of unlabeled cells had entered S phase after 12 h. The peak was smaller and less pronounced for 180BR, probably due to the decreased proportion and rate of cycling of these cells. Nevertheless, a pronounced dose-dependent G1-S arrest was observed in both the normal cell line 1BR3 and in 180BR, and it was seen even at the lowest dose examined (1 Gy; Fig. 8).

To examine the integrity of the G2 checkpoint, the ability of the labeled cells (i.e., those cells that had traversed the G1-S boundary prior to irradiation) to enter a labeled G1 position (gate D) was estimated. In the unirradiated control population, the majority of labeled cells entered G1 phase 12 h after being released from labeling. At 18 h, the percentage of cells in G1 has decreased because they have progressed into the subsequent S phase (Fig. 8). This cycling time is approximately the same for 1BR3 and 180BR. Following radiation with increasing doses, both cell lines exhibited a delay in entry into G1, indicating an accumulation of cells at the G2-M checkpoint. 1BR3

1BR3 0 Gy; 0 hr
1BR3 0 Gy; 12 hr
1BR3 3 Gy; 12 hr

180BR 0 Gy; 0 hr
180BR 0 Gy; 12 hr
180BR 3 Gy; 12 hr

BrdUrd FITC
DISCUSSION

The radiosensitivities of the two transformed cell lines that were examined here (180BRM and MRC5V1) were lower than those of the untransformed counterpart, in agreement with previous findings of Arlett et al. (46), who showed that the introduction of SV40 T antigen reduced the intrinsic radiosensitivity in cell lines. However, transformation did not change the relative hypersensitivity of 180BR cells. Because the 180BRM cells are transformed but not immortalized (precrisis), whereas the MRC5V1 cells are immortalized (postcrisis), our findings support previous observations that it is transformation rather than immortalization that reduces radiosensitivity (46).

180BRM cells also display the DSB repair defect that is characteristic of 180BR cells (3). Therefore, the reduction in radiosensitivity that is linked to transformation does not correlate with the DSB repair capacity.

180BR and 180BRM cells express wild-type levels of the three components of DNA-PK (Ku70, Ku80, and DNA-PKcs) and have normal levels of DNA end binding and DNA-PK activity. Recently, using antibodies supplied by S. Critchlow and S. P. Jackson (Cambridge University, Cambridge, United Kingdom), we have also shown that XRCC4, another protein functioning in the DNA-PK-dependent DSB rejoining mechanism, is expressed at normal levels in 180BR (data not shown). We, therefore, conclude that 180BR is not defective in XRCC4 or one of the components of DNA-PK. This conclusion is strengthened by our unpublished observations that, in contrast to SCID cell lines (47), 180BR is not sensitive to the DNA cross-linking agent mechloretamine.

Members of the four complementation groups of rodent mutants with major defects in DNA DSB repair are also defective in their ability to carry out V(D)J recombination (22–24, 40, 48, 49). Our results here show that 180BRM cells differ from these cell lines because they are proficient in their ability to carry out V(D)J recombination following introduction of Rag expression vectors. Hence, they do not seem to lack the factors necessary to produce a correctly repaired coding junction following initial cutting at the recombination signal sequences. The recombination rate is about 3 times lower in 180BRM than in the control cell line, but this difference is within the range observed between normal pre-B lymphocytes (ratio of 4.3; Ref. 35). Moreover, this small difference is much less dramatic than the 30–500-fold decrease in recombination frequency reported for the hitherto characterized DSB repair-defective cell lines (12, 14, 17, 18, 22, 50, 51). The normal V(D)J recombination in 180BRM cells is in agreement with the clinical observations that the patient from whom the cell line was derived had no identifiable immune defect (2). In this phenotype, 180BRM cells resemble AT cell lines, which are also proficient in their ability to carry out V(D)J recombination (52).

To gain further insight into the nature of the defect in 180BR, we found that irradiated 180BR cells were able to arrest at both the G1-S and G2-M checkpoints. Previously, we have also shown that 180BR cells inhibit DNA synthesis following irradiation (52). These combined results distinguish 180BR from AT cell lines, which fail to arrest at the G1-S checkpoint (53), and display radioresistant DNA synthesis.

In its cell cycle arrest phenotype, 180BR resembles the other DSB repair-defective cell lines. The Ku80-defective ES cell line and primary scid cells both show a radiation induced G1-S arrest (this is not seen in the rodent mutants or their parents due to changes in their p53 status; Refs. 44 and 45). Additionally, the Ku80 ES cells, as well as scid cells and xrs-6 cells show a more pronounced G2-M arrest than do parental cells (44, 45, 54). At the same dose, the magnitude of G2 delay is more pronounced in 180BR compared with 1BR3. These results indicate that the G1-S and G2-M checkpoints are intact in 180BR and that the G2-M arrest is triggered by a lower dose, which is consistent with an elevated number of unrejoined DSBs.

In conclusion, our results show that 180BR represents a new mutant phenotype that is characterized by hypersensitivity to ionizing radiation and defective DNA DSB rejoining but also by proficiency in its ability to carry out effective V(D)J recombination and to arrest at G1-S and G2-M cell cycle checkpoints. In addition to the members of groups 4–7, one other DSB repair-defective rodent mutant, V-C8, has been described, but this cell line differs from 180BR in displaying radioresistant DNA synthesis (55). This suggests that 180BR carries a mutation in a currently unidentified gene involved in DSB repair. Because both the patient and his fibroblast cells were radiosensitive, it appears likely that he carried the mutation in his germ line. Surprisingly, the patient was normal.
until the onset of leukemia. It, therefore, appears that this defect does not confer a major growth or developmental problem but may impart a predisposition to cancer.

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