

Radiosensitive human tumour cell lines show misrepair of DNA termini

S N POWELL, FRCR, PhD, J MILLS, BA, MPhil and T J McMILLAN, PhD

Institute of Cancer Research, Downs Road, Sutton, Surrey SM2 5NG, UK

Abstract. Physical measures of the rejoining of radiation-induced breaks in DNA strands are limited in terms of sensitivity and the fact that they do not assess the fidelity with which the rejoining occurs. In this report, transfection of cleaved plasmid has been used as a probe for repair in three radiosensitive tumour cell lines and shown them to have low repair fidelity compared with resistant cells. Errors in the repair of linear plasmid were found by Southern analysis, in keeping with the measured repair fidelity. Radiosensitive tumour cells showed few errors in the uptake and integration of circular plasmid, in contrast to ataxia–telangiectasia (A–T) cells. In the neuroblastoma HX142, the repair of blunt-ended linear plasmid was associated with deletions of >1 kb; staggered-ended linear plasmid was repaired with small insertions and circular plasmid integration was intact in >60% of the copies. The neuroblastoma SKN.SH, processed staggered-ended plasmid by insertions of a variety of sizes, but processed circular plasmid largely error-free. In contrast, A–T cells (AT5BIVA) had the same spectrum of errors irrespective of the form of plasmid transfected. Cell fusion between HX142 and AT5BIVA showed complementation to a resistant phenotype, suggesting that misrepair in the tumour cell did not result from somatic mutation in the *ATM* gene. In conclusion, radiosensitive tumours show evidence of misrepair of DNA termini, with a mechanism which is functionally and genetically distinct from that in A–T cells.

There is considerable interest in identifying those factors which make tumour cells sensitive to DNA damaging agents such as ionizing radiation. The ultimate aim of studying mechanisms of radiosensitivity is to suggest means by which radioresistant tumours might be made more sensitive. In lymphoid malignancies and lymphoid tumour cell lines, apoptosis appears to be the dominant factor determining radiosensitivity. The difference between radiosensitive and radioresistant tumours, in a transformed fibroblast model, was also found to be due to the presence or absence of an intact apoptotic pathway [1]. However, apoptosis is not the universal explanation of radiosensitivity: there are many other radiosensitive tumour cell lines in which little induced apoptosis can be demonstrated [2].

The DNA repair mechanisms which are important determinants of sensitivity in tumours may have little in common with those determining sensitivity in radiosensitive mutants of normal cell lines. Human tumour cell lines have been studied

infrequently. Measurement of the level of DNA damage induction has sometimes found sensitive cells to sustain more damage [3–6]. The analysis of DNA double-strand break (dsb) rejoining has revealed large differences between radiosensitive mutants and wild-type normal cells [7] and isogenic sensitive and resistant tumour cells [8]. For human tumour cell lines, small differences in the rate of rejoining of dsb have been reported [9, 5], although not universally.

A correlation has recently been shown between the radiosensitivity of human tumour cell lines and the repair fidelity of linear plasmid [10]. It has also been shown that ataxia–telangiectasia (A–T) transformed fibroblasts, which are highly radiosensitive, have errors in the repair of linear plasmid [11, 12]. Genetic instability and chromosomal abnormalities are features of cancer cells, with no clear discrimination between sensitive and resistant tumours, and are also features of A–T cells. At a superficial level, there are features in common between radiosensitive tumours and A–T cells: similar survival after irradiation and low DNA repair fidelity. These similarities have not been extensively studied.

The purpose of this study was to analyse the repair defects in three radiosensitive tumour cells and to compare their processing of transfected plasmid DNA with that in A–T cells.

Received 4 August 1997 and in final form 24 June 1998, accepted 29 June 1998.

Address correspondence to Professor T J McMillan, Department of Biological Sciences, Institute of Environmental and Natural Sciences, Lancaster University, Lancaster, LA1 4YQ UK.

Materials and methods

Cell lines

Three sensitive tumour cell lines were studied: two neuroblastomas and one medulloblastoma. AT5BIVA, a transformed fibroblast cell line from a patient with ataxia–telangiectasia, was also used. Where investigated, these radiosensitive cell lines have been found to be cross-sensitive to cytotoxic drugs causing double-strand damage, in particular etoposide, bleomycin and cisplatinum. The sensitivity of the cell lines in this study is highly stable. The three radiosensitive tumour cell lines were: HX142 and SKN.SH (neuroblastomas [13–15]) and D283MED (medulloblastoma [16]). Clonogenic cell survival had been assessed previously in monolayer culture for all cell lines and soft agar [17] for the D283MED and HX142 cell lines. The two culture techniques gave the same cell survival results. The radiosensitivity of the tumour cell lines investigated in this study is shown in Figure 1. AT5BIVA has a similar sensitivity to HX142.

Cell culture

HX142 and D283MED were grown in Ham's F12 medium, supplemented with 10% fetal bovine serum. SKN.SH, AT5BIVA and cell fusions were grown in Eagle's minimal essential medium, supplemented with 10% fetal bovine serum. All cell cultures were incubated in 5% CO₂, 3% O₂ and 92% N₂. All media contained penicillin (10⁵ units per litre) and streptomycin (100 mg per litre). Freedom from mycoplasma contamination was checked regularly by testing with Hoescht 33528. All cells were grown in monolayer culture and were detached when required using trypsin (0.05%) and versene (0.02%). Cell survival following

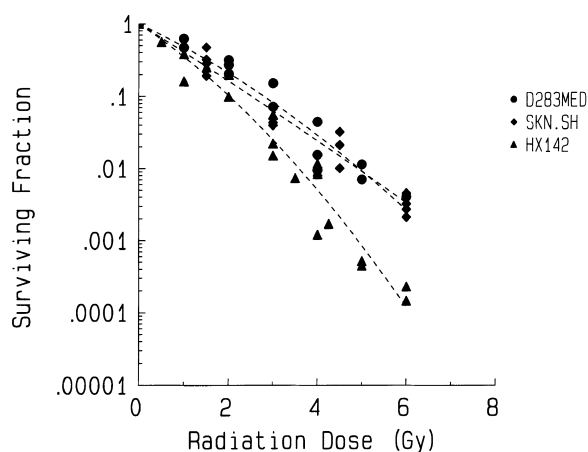


Figure 1. The high dose rate, single dose, cobalt-60 radiation cell-survival curves for HX142 ([13], (solid triangles, continuous line) SKN.SH ([JH Peacock, personal communication, 1995], solid diamonds, dashed line) and D283MED ([11], open squares, dotted line).

ionizing radiation has been reported previously [4]. SF₂ was calculated from the linear quadratic fit of the whole survival curve. The SF₂ values were 0.13 for HX142 and AT5BIVA, 0.22 for SKN.SH and 0.24 for D283MED.

Repair fidelity

Repair fidelity of radiosensitive tumour cells was measured using the plasmid transfection assay reported previously [10] and is summarised in Figure 2. A plasmid, pPMH16, with two selectable bacterial genes, was used [18]. The first gene (*neo*) confers resistance to the antibiotic G418, a derivative of neomycin which crosses the mammalian cell membrane. The second gene (*gpt*) confers resistance to the medium XHATM (xanthine, hypoxanthine, aminopterin, thymidine and mycophenolic acid) by utilizing xanthine to make guanine whose production is otherwise inhibited [19]. No function of these genes occurs naturally in mammalian cells: when present they must be derived from transfected DNA. Both genes have mammalian cell promoters immediately upstream and mammalian signalling sequences downstream to ensure expression following transfection.

The principle of the assay is to use the *neo* gene as a marker of transfection, and to damage the *gpt* gene using a restriction endonuclease, which causes a double-strand break in the plasmid. The ability of the transfected cells to reconstitute the damaged *gpt* gene and to restore its function is tested.

Plasmid digestion by restriction enzyme

Restriction enzymes, *Kpn*I and *Eco*RV, cleaved pPMH16 once within the *gpt* coding region to produce a linear plasmid with staggered and blunt ends respectively. Plasmid was digested prior to transfection with 2 units of enzyme per µg plasmid for a minimum of 3 h. The linearity of the plasmid was confirmed using gel electrophoresis (0.8% agarose in Tris-Borate-EDTA).

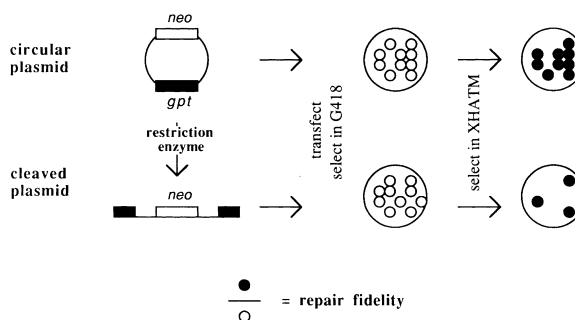


Figure 2. Schematic outline of the plasmid transfection assay of repair fidelity.

Plasmid transfection

The transfection procedure and plasmid details were as reported previously [10, 20, 21] utilizing 40 µg of DNA in a CaPO₄ co-precipitation, with the following minor modifications. The cells were washed after exposure to CaPO₄ with medium without serum, exposed to 15% glycerol in 1.5 ml of HBS for a time less than 3 min, and washed again before replacing complete medium. The cells were sensitive to the effects of prolonged exposure to glycerol, but a benefit in improved transfection frequency was obtained by using glycerol. The cells were left for 48 h (expression time) before harvesting the cells and re-seeding at 5×10^5 or 10^6 per 80 cm² flask (2–3 per experiment). Medium containing 0.2 mg ml⁻¹ G418 was added up to 48 h later to allow the seeded cells to attach prior to adding selection medium. Cell viability following the transfection procedure was 70–90% of normal plating efficiency.

After 10–14 days growth in G418 visible viable colonies could be marked. Selection for *gpt* gene function was then applied with mycophenolic acid (10 µg ml⁻¹). The proportion of marked colonies which remained viable in XHATM after 7 days was recorded as the repair fidelity. Transfection frequency was calculated as the number of drug resistant colonies obtained divided by the number of cells seeded and corrected for cell viability, and was approximately one in 10⁵.

Processing of integrated plasmid

In parallel with colony scoring in selective media, one flask of G418 positive colonies was harvested, amplified and used for genomic DNA isolation (using detergent lysis and phenol then chloroform extractions). Restriction fragments of genomic DNA were analysed by 0.8% agarose gel electrophoresis and capillary blotting (Southern analysis). 10 µg of DNA were digested for each lane, and equal lane loading was checked using ethidium bromide staining of the agarose gel. Radiolabelled probes for hybridization were prepared by random priming. The probes were made from the 2.3 kb *EcoRI* fragment of pPMH16 containing the *neo* gene, and a 1.1 kb *HindIII*–*BamHI* fragment of pL10 containing the *gpt* gene without SV40 sequences. Gels were run with lanes containing plasmid solutions equivalent to one, two and five copies per diploid cell (13.3 pg = one copy in 10 µg) and the relative and absolute copy numbers were estimated. The number of intact copies of the *neo* and *gpt* genes were recorded.

Cell fusion

Cell fusion was carried out using PEG 170 [22]. HX142 contained the *neo* gene marker and

AT5BIVA contained the *gpt* gene marker, and confirmation of fusion required G418 and mycophenolic acid resistance. Cell survival following exposure to ionizing radiation was carried out with selection maintained.

Results

Repair fidelity

The repair fidelity of HX142, measured by plasmid reconstitution, is shown in Table 1. Overall repair fidelity was 18.1% for *KpnI*-cleaved and 6% for *EcoRV*-cleaved plasmid. The transfection of circular plasmid, selecting first in G418, led to a functioning *gpt* gene in 47.3% of colonies evaluated.

The repair fidelity measured for SKN.SH is shown in Table 1. Overall repair fidelity was 19.4% for *KpnI*-cleaved and 14.3% for *EcoRV*-cleaved plasmid. The transfection of circular plasmid led to a functioning *gpt* gene, after G418 selection, in 51.2% of colonies. The repair fidelity of D283MED was 16.3% for *KpnI*-cleaved plasmid while the transfection of circular plasmid led to a functioning *gpt* gene, after G418 selection in 43.4% of colonies (Table 1).

Cell-mediated plasmid processing

The processing of plasmid was evaluated by harvesting the DNA from more than 30 colonies which had been amplified to 5×10^5 cells. For HX142, the mean copy number of *neo* genes integrated after transfection of circular plasmid was eight. An estimated five copies of normal sized *neo* bands is matched by the same number of normal sized *gpt* genes. The abnormal *gpt* genes show loss or gain of sequence, and some *gpt* sequences are completely deleted (Figure 3).

Transfection of plasmid cleaved by *KpnI* leads to no detectable cell-mediated correct repair of the *gpt* gene: cleavage of the *gpt* gene fragment by *KpnI* was not seen. Control digestions of the plasmid were satisfactory (marker lanes = first two lanes from left). Misrepair events were predominantly small insertions (2.3 kb *gpt* band, with normal sized *neo* band) and possibly deletions (reduced copy number of *gpt* relative to *neo*). The detection of discrete bands from the DNA of pooled colonies implies the same misrepair is occurring in multiple colonies.

Plasmid cleaved by *EcoRV* led to complete deletion of the *gpt* gene. The size of the *neo* gene band implied loss of the *BamHI* site or the *HindIII* site as part of the deletion in some copies. Bands larger than 8 kb imply a more complex mis-repair. As a further comparison, *PvuI*-cleaved plasmid was transfected, which revealed deletions around the cleavage site, not affecting the *neo* gene or the *gpt*

Table 1. Repair fidelity in radiosensitive human tumour cell lines

	Colonies resistant to G418+XHATM per transfected cell (G418 ^R)			
	Expt. no.	Circular	<i>KpnI</i> cut	<i>EcoRV</i> cut
HX142	1	67/134	12/73	2/22
	2	46/90	27/64	
	3	2/9	3/56	
	4	3/11	0/10	
	5	4/11	0/26	1/22
	6	3/9	0/3	0/6
	Total	125/264 (47.3%)	42/232 (18.1%)	3/50 (6%)
SKN.SH	1	53/94	36/77	
	2	44/53	15/80	
	3	40/95	0/84	
	4	31/40	32/117	
	5	22/86	18/160	30/189
	6	20/42	16/160	10/91
	Total	210/410 (51.2%)	117/603 (19.4%)	40/280 (14.3%)
D283MED	1	18/40	10/40	
	2	15/36	5/52	
	Total	33/76 (43.4%)	18/92 (16.3%)	

The repair fidelity was measured in independent transfections as outlined in Figure 2. The measure of repair fidelity reflects the number of colonies with mycophenolic acid resistance out of the total number showing G418 resistance. For cleaved plasmid transfection, this ratio reflects the proportion of colonies showing accurate repair per transfected cell.

gene. No insertions were detected. Overall, the copy number integrated for circular plasmid (8) was higher compared with *KpnI*-cleaved plasmid (3) and *EcoRV*-cleaved plasmid (1).

The processing of cleaved plasmid by SKN.SH showed differences from HX142 in that correct sized bands containing the *gpt* gene were found, and they were cleaved by *KpnI* (Figure 4). Rearranged bands greater than 1.9 kb (lower panel, *gpt* gene probe) were found, which did not cleave with *KpnI*, suggesting misrepair by insertion. Distinguishing whether the misrepair is insertion of a plasmid fragment lacking the *BamHI* and *HindIII* sites, or insertion of genomic DNA between plasmid copies at the *KpnI* site was not determined. Observation of *neo* containing bands less than 6.1 kb (top panel) suggests deletions, but whether the deletions and insertions are in separate clones or within the same clone cannot be determined from this analysis. By contrast, the processing of circular plasmid, insertion into the genome via the *gpt* gene sequence was not found, as only the correct sized *gpt* band was seen. Rearrangement of the *neo* containing band was limited to three altered bands. This suggests that the integration site is not randomly located within the plasmid, but limited to certain sequences within the plasmid. Whether this led to "hot-spots" of integration into the genome was not determined. The copy number overall was greater for linear

plasmid relative to circular plasmid, in contrast with HX142.

Fusion of HX142 and AT5BIVA

The radiation sensitivities of HX142 and AT5BIVA were almost identical. SF₂ of HX142 and AT5BIVA was 0.13, and 0.001 survival was obtained with 4.5 Gy. Fused cells had an SF₂ of 0.55 and 0.001 survival with 10 Gy (Figure 5). The sensitivity of the fused cells was in the midrange for human cells.

Discussion

It has been demonstrated that radiosensitive tumour cells have low repair fidelity using a plasmid transfection repair assay. This is in keeping with the idea that misrepair of DNA damage can be a reason for radiation sensitivity in tumour cell lines. The tumour lines investigated all showed a specific difficulty in the accurate closure of cleaved plasmid, suggesting that DNA termini were relatively unstable structures in these cells. The observation that staggered ends were closed with insertions and blunt ends were closed after losing sequence at the termini implies that excess degradation of DNA termini was not the cause of misrepair. It suggests that the termini are unstable, possibly unprotected and/or recombinogenic. The

confirms the separate genetic basis to their radiosensitivity and confirms that radiosensitivity is a recessive phenotype in the two cells tested. The analysis of plasmid processing had suggested there were differences between A-T cells and the radiosensitive tumour cells. In A-T cells, misrepair appeared to be the same with linear and circular plasmid and the error was often reproduced in multiple copies, suggesting the error was linked to the integration step [11]. In the radiosensitive tumour cells, errors were found predominantly with linear plasmid rather than circular plasmid. The integrated plasmid copy number in HX142 was higher for circular plasmid relative to linear plasmid and was the reverse for SKN.SH, also suggesting a different basis to misrepair in the two tumour cells analysed.

An increased rate of intrachromosomal recombination has been observed in tumour cells, cells lacking functional p53 [23] and A-T cells [24, 25] using an integrating DNA vector containing two mutant reporter genes. Recombination is demonstrated by rearrangement of the mutant sequences to make an intact gene. This assay is, in theory, measuring two effects: first, the ability to initiate and complete a recombination event; and second, the ability to undergo recombination with accuracy or fidelity. We are now performing experiments to distinguish recombination rate and accuracy, which occur either spontaneously or initiated via directed DNA damage, to establish whether recombination errors correlate with radiosensitivity.

Acknowledgments

This work was supported by the Cancer Research Campaign UK, the Bob Champion Cancer Trust and CA58985 from the National Cancer Institute.

References

1. Lowe SW, Ruley HE, Jacks T, Housman DB. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* 1993;74:957-67.
2. Meyn RE, Milas L. The role of apoptosis in tumor response to radiation. In: Hagen U, Harder D, Jung H, Streffer C, editors. *Radiation Research 1895-1995, Proceedings of 10th International Congress of Radiation Research*. 1995:653-6.
3. Schwartz JL, Mustafi R, Beckett MA, Czyzewski EA, Farhangi E, Grdina DJ, et al. Radiation-induced DNA double-strand break frequencies in human squamous cell carcinoma cell lines of different radiation sensitivities. *Int J Radiat Biol* 1991;59:1341-52.
4. McMillan TJ, Cassoni AM, Edwards S, Holmes A, Peacock JH. The relationship of DNA double-strand break induction to radiosensitivity in human tumour cell lines. *Int J Radiat Biol* 1990;58:427-38.
5. Whitaker SJ, Ung YC, McMillan TJ. DNA double strand break induction and rejoining as determinants of human tumour cell radiosensitivity. A pulsed field gel electrophoresis study. *Int J Radiat Biol* 1995;67:7-18.
6. Ruiz de Almodovar JM, Nunez MI, McMillan TJ, Olea N, Mort C, Villalobos M, et al. Initial radiation-induced DNA damage in human tumour cell lines: a correlation with intrinsic cellular radiosensitivity. *Br J Cancer* 1994;69:457-62.
7. Kemp LM, Sedgwick SG, Jeggo PA. X-ray sensitive mutants of Chinese hamster ovary cells defective in double-strand break rejoining. *Mutat Res* 1984; 132:189-96.
8. Wlodek D, Hittelman WN. The repair of double-strand DNA breaks correlates with radiosensitivity of L5178Y-S and L5178Y-R cells. *Radiat Res* 1987;112:146-55.
9. Schwartz JL, Rotmensch J, Giovanazzi S, Cohen MB, Weichelbaum RR. Faster repair of DNA double-strand breaks in radioresistant human tumour cells. *Int J Radiat Oncol Biol Phys* 1988;15:907-12.
10. Powell SN, McMillan TJ. The repair fidelity of restriction enzyme-induced double strand breaks in plasmid DNA correlates with radioresistance in human tumour cell lines. *Int J Radiat Oncol Biol Phys* 1994;29:1035-40.
11. Powell S, Whitaker S, Peacock J, McMillan TJ. Ataxia telangiectasia: an investigation of the repair defect in the cell line ATSBIVA by plasmid reconstitution. *Mutat Res* 1993;294:9-20.
12. Cox R, Debenham PG, Masson WK, Webb MB. Ataxia-telangiectasia: a human mutation giving high-frequency misrepair of DNA double-stranded scissions. *Mol Biol Med* 1986;3:229-44.
13. Deacon JM, Wilson P, Steel GG. Radiosensitivity of neuroblastoma. *Prog Clin Biol Res* 1985; 175:525-31.
14. Deacon JM, Wilson PA, Peckham MJ. The radiobiology of human neuroblastoma. *Radiother Oncol* 1985;3:201-9.
15. Paffenholz V, Ebener U, Koruhuber B. Uptake and release of iodine-labelled m-iodobenzylguanidine in a neuroblastoma cell culture system and its importance in neuroblastoma therapy. *J Cancer Res Clin Oncol* 1989;115:269-75.
16. Friedman HS, Burger PC, Bigner SH, Trojanowski JQ, Wikstrand CJ, Halperin EC, et al. Establishment and characterization of the human medulloblastoma cell line and transplantable xenograft D283 Med J Neuropathol Exp Neurol 1985;44:592-605.
17. Courtenay VD, Mills J. An *in vitro* colony assay for human tumours grown in immune-suppressed mice and treated *in vivo* with cytotoxic agents. *Br J Cancer* 1978;37:261-8.
18. Debenham PG, Webb MB, Stretch A, Thacker J. Examination of vectors with two dominant, selectable genes for DNA repair and mutation studies in mammalian cells. *Mutat Res* 1988; 199:145-58.
19. Mulligan RC, Berg P. Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase. *Proc Natl Acad Sci USA* 1981;78:2072-6.
20. Powell SN, Whitaker SJ, Edwards SM, McMillan TJ. A DNA repair defect in a radiation-sensitive clone of a human bladder carcinoma cell line. *Br J Cancer* 1992;65:798-802.

21. Powell SN, Steel GG, McMillan TJ. *In vitro* radiosensitivity of human medulloblastoma cell lines (letter). *J Neurooncol* 1993;15:91–2.
22. Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning: A laboratory manual* (2nd edn). New York: Cold Spring Harbor Laboratory Press, 1989.
23. Mekeel KL, Tang W, Kachnic LA, Luo C-M, DeFrank JS, Powell SN. Inactivation of p53 results in high rates of homologous recombination. *Oncogene* 1997;14:1847–57.
24. Meyn MS. High spontaneous intrachromosomal recombination rates in ataxia–telangiectasia. *Science* 1993;260:1327–30.
25. Luo C-M, Tang W, Mekeel KL, DeFrank JS, Anne PR, Powell SN. High frequency error-prone DNA recombination in ataxia-telangiectasia cell lines. *J Biol Chem* 1996;14:4497–503.