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Acknowledgements—The study was supported by a grant (Di-245/3-5) from the Deutsche Forschungsgemeinschaft.

Eur J Cancer, Vol. 28A, No. 10, pp. 1610–1614, 1992.
Printed in Great Britain

0964–1947/92 $5.00 + 0.00

Differences in the Level of DNA Double-strand Breaks in Human Tumour Cell Lines Following Low Dose-rate Irradiation

Anna M. Cassoni, Trevor J. McMillan, John H. Peacock and G. Gordon Steel

It is now well accepted that differences exist in the intrinsic radiosensitivity of human tumour cells although the molecular basis of this is still unclear. Current evidence suggests that of the lesions induced in DNA by ionising radiation, double-strand breaks (DSB) are the most closely linked to cell death. In this study, levels of DSB were measured by neutral filter elution under conditions of both repair inhibition and maximum recovery and compared with clonogenic survival curves for high (HDR) and low dose-rate (LDR) irradiation in human carcinoma lines of differing radiosensitivity. Four human lung carcinoma lines were used, two small-cell (SCLC; HC12 and HX149) and two non-small cell lines (NSCLC; HX147A7 and HX148G7). Cell survival was measured by soft agar and monolayer colony-forming assays as appropriate and a large variation in sensitivity of the cell lines was seen (α values of 0.06 to 0.56 Gy^{-1}). We have previously reported that the damage induced at high dose rate does vary in these cell lines but not in a way which correlates with their cell survival response [5]. Following irradiation to 15 Gy at low dose rate essentially no DSBs were detected in any of the four lines but at 70 Gy the more sensitive SCLC showed more residual damage than in the more radioresistant NSCLC lines. The prime determinant of the difference between the LDR and HDR damage curves is likely to be repair occurring during irradiation. These data suggest that whatever the determinant, whether the degree of damage induction or repair, the level of DSB after LDR correlates well with cellular sensitivity in these four cell lines. Thus, DNA damage studies after low dose-rate irradiation may not only enable the examination of irrepairable lesions which are important in cell killing but they may also provide a useful predictive test of cellular radiosensitivity.
Eur J Cancer, Vol. 28A, No. 10, pp. 1610–1614, 1992.

INTRODUCTION

THE ANALYSES of Fertil and Malaise [1] and Deacon *et al.* [2] have shown that if the initial portion of survival curves are examined, there is considerable variation in radiosensitivity of human tumour cell lines and this can be related to clinical curability. The factors underlying these differences in radiosensitivity have been considered in terms of recovery [3] and, by implication, repair ability and more recently in terms of damage induction [4, 5].

Of the many lesions induced in DNA, that which appears most closely linked to cell damage is the double-strand break (DSB). In yeast it has been shown that one unrepaired double-strand break is lethal [6]. Unfortunately, methods available for measuring DSB in human DNA are not sufficiently sensitive to be able to relate one unrepaired break directly to a lethal lesion. However, Radford [4, 7] has shown different levels of sensitivity to DSB induction in various cell lines. His finding of a direct and constant relationship between initial DSB and cell lethality

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Revised 1 Apr. 1992; accepted 2 Apr. 1992.

Table 1. Biological properties of the cell lines

Cell line	Type	*In vitro* doubling time (h)	P.E.* (Range) (%)	DNA† content
HC12	SCLC	51	19.6 (13–30)	1.52
HX149	SCLC	71	17.5 (5–35)	1.46
HX147A7	large cell	60	37 (26–43)	1.68
HX148G7	adeno-carcinoma	61	5 (3–7)	2.3

*Plating efficiency.
†DNA content: ratio of positions of G1 peaks on FACS II analysis, tumour cells vs. human lymphocytes.

suggests that differences in the level of enzymatic repair of DSB between cell lines are not important. This has been challenged by other authors [8] who have failed to find this constant relationship.

Where no difference in induction is found, differences in DNA repair is an obvious possible cause of variation in radiosensitivity. A relationship between rejoining of DNA DSB and radiosensitivity has been suggested in CHO mutants [9], radiosensitive L5178Y-S murine lymphoblast cell lines [10] and an ataxia telangiectasia (AT) cell line [11]. However, this relationship has not been demonstrated in CHO radiosensitive lines EM9 and NM2 [12] or four other AT lines [13]. Schwartz *et al.* [14] while showing no differences in ultimate completeness of rejoining of DSB showed a correlation between rate of rejoining and sensitivity in 12 human tumour lines.

The relative insensitivity of available methods of measuring DSBs in human DNA and the fact that the majority are rejoined [15] complicates the investigation of the relationship between DSB and lethality. By examining DSB after low dose-rate irradiation (LDR), where cellular recovery and presumably DNA repair can take place during treatment, we have attempted to measure those DSB that are more closely related to cell lethality.

MATERIALS AND METHODS

Cell lines and culture conditions

Four human lung carcinoma lines were used (Table 1). HC12 and HX149, small cell lung carcinoma (SCLC) lines have been previously desribed [16]. Two non-small cell lung carcinoma (NSCLC) lines HX147A7 and HX148G7 were cloned from previously described lines [16] by serial dilution. They grew in Hams F12 medium supplemented by 16% aseptic calf serum (Gibco), with penicillin (10^5 U/l) and streptomycin (100 mg/l). Cells were incubated in 5% CO_2, 5% O_2 and 90% N_2. Freedom from mycoplasma contamination was checked by testing with Hoechst 33528.

Survival assay

Clonogenic cell survival in the SCLC was assayed with the Courtenay–Mills soft agar method using August rat blood and heavily irradiated tumour feeder cells [17]. Survival of the NSCLC lines was assessed using a monolayer method on a feeder layer of heavily irradiated Swiss mouse 3T3 cells. Colonies of more than 50 cells were counted 2–4 weeks after plating, depending on the cell line. Triplicate plates were counted per dose point in each experiment in a minimum of three separate experiments performed on each cell line. Survival data were fitted by the linear quadratic equation $S = \exp(-\alpha D - \beta D^2)$ [18] using nonlinear regression analysis.

Double-strand break assay

The neutral filter elution technique of Bradley and Kohn [19] was used with the addition of 3T3 cells irradiated to 100 Gy as internal reference cells. The cells were labelled as follows: test cells were labelled with [^{14}C] thymidine (specific activity 2 GBq/mol, Amersham), 3.7 kBq/ml for 1.5–2 doubling times. 3T3 cells were labelled for 48 h with [^{3}H] thymidine 20 kBq/ml (1 GBq/mol) plus 10^{-5} mol/l unlabelled thymidine. Fifteen hours before assay the medium was replaced by fresh non-radioactive medium.

Single-cell suspensions were produced by mechanical disaggregation for HC12 and HX149 and by treatment with trypsin (0.005%) and versene (0.02%) for 3 min for the remainder. Previous comparison of trypsination and mechanical disaggregation of monolayers have shown no difference in subsequent DSB induction [20]. All cells were kept at ice bath temperature for 15 min before irradiation and until assay immediately afterwards. Following irradiation, aliquots containing 1–2 $\times$ 10^5 test cells mixed with 10^5 internal reference cells in 10 volumes of ice-cold phosphate-buffered saline (PBS) were sucked gently onto prewetted, ice-cold 2 μm pore polycarbonate filters (Nucleopore) on Swinnex filter units (Millipore) attached to 50 ml syringe barrels. 3 ml of elution buffer (Tris 0.05 mol/l, glycine 0.05 mol/l, Na_2 EDTA 9.025 mol/l and 2 g/l sodium lauryl sulphate at pH 9.6) containing 0.5% freshly made proteinase K (Sigma) were added gently, avoiding the formation of bubbles and cells were lysed for 15 min before elution was started using a peristaltic pump to give a flow of 2 ml/h. One hour later 30 ml of elution buffer were added and elution continued for 16 h. Samples were collected 2-hourly. At the end of elution, the filters were hydrolysed in 0.8 ml of 1 mol/l HCl at 60°C for 1 h. 5 ml of 0.4 mol/l NaOHwas then added and 1 h later 14 ml scintillant (Picofluor-40, Canberra Packard) was added to all samples. Counting was performed in a Canberra Packard Tricarb 2000 CA with correction for luminescence and 'spill over' into the ^{3}H channel.

Double-strand break data are presented as relative elution (R.E.): that is the fraction of DNA eluted in irradiated cells minus the fraction eluted from unirradiated cells at a time when 40% of the 3T3 DNA had eluted. R.E. values from three elution columns were averaged to produce a single value for each dose in each experiment.

Irradiations

Radiation treatments were performed using 33 and 2 TBq ^{60}Co sources. Dosimetry was checked with an Ionex 2500/3. Acute survival experiments were carried out at 1 Gy/min at 37°C. Acute DSB induction experiments were performed at ice bath temperature and at 3 Gy/min. All low dose-rate experiments were performed at 37°C at 0.02 Gy/min in full culture medium with the immediate addition of ice-cold medium at the end of irradiation for the DSB assay.

RESULTS

Clonogenic cell survival

The four cell lines showed a range of radiosensitivity at high dose rate (Fig. 1 and Table 2), the SCLC being more sensitive with α values of 0.56 and 0.35 Gy^{-1}, compared with 0.17 and 0.06 Gy^{-1} for the NSCLC. The results for the SCLC lines are similar to those previously published [5]. The cloned cell lines of the NSCLC cell lines are more radioresistant than previously found for their parent lines: HX147A7 had an α of 0.17 Gy^{-1} compared with 0.23 Gy^{-1} in its parent line while HX148G7 has

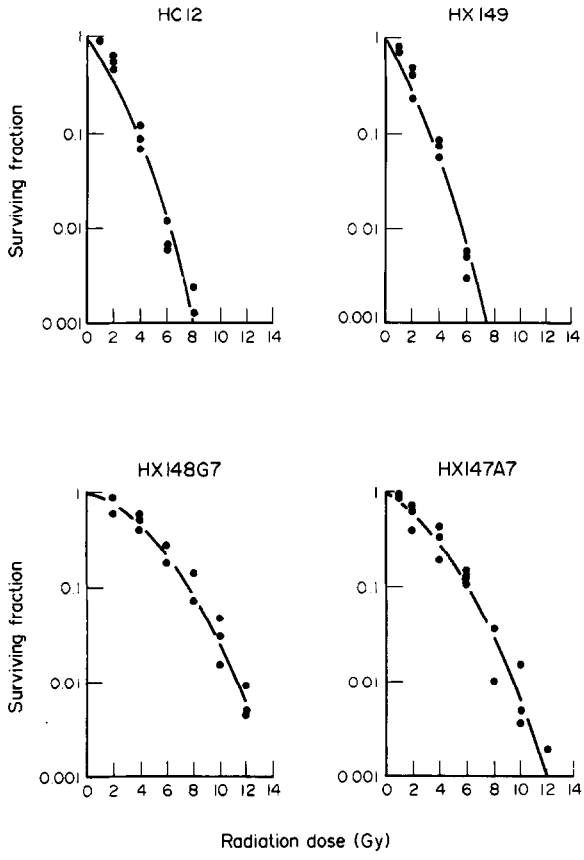

Fig. 1. Clonogenic survival curves at 100 cGy/min. Data points from at least three independent experiments. Curve calculated by linear quadratic equation fitted to the data.

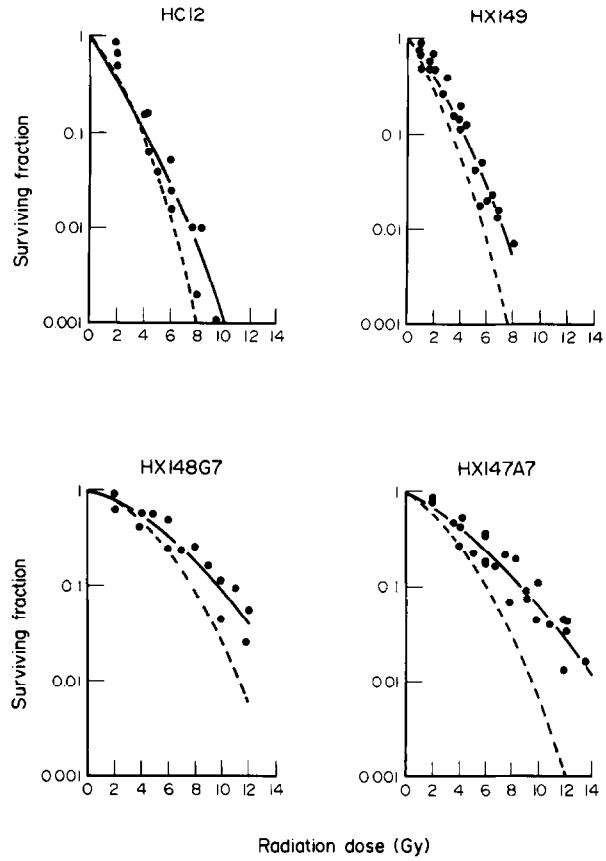

Fig. 2. Clonogenic survival curves. Data points and solid line at 0.02 Gy/min. Dashed line curve is from acute dose rate.

α value of 0.06 Gy^{-1} compared with 0.137 Gy^{-1} in the parent [16].

At 0.02 Gy/min all lines showed increased survival although in none was the β component eliminated, suggesting that recovery even at this low dose rate remained incomplete in these lines (Fig. 2 and Table 2). There was no difference in low dose-rate sparing at this dose rate between SCLC and NSCLC cell lines, as assessed by the dose reduction factor (the ratio of dose required to reduce survival to 0.01 at low and high dose rate).

Table 2. Survival parameters of cell lines at high and low dose-rate

Cell line	High dose-rate (1Gy/min) α	β	Low dose-rate (0.02 Gy/min) α	β	DRF*
HC12	0.35 (0.003)	0.06 (0.003)	0.47 (0.084)	0.02 (0.009)	1.19
HX149	0.56 (0.213)	0.05 (0.002)	0.38 (0.025)	0.03 (0.0035)	1.37
HX147A7	0.17 (0.006)	0.03 (0.016)	0.20 (0.006)	0.008 (0.0001)	1.57
HX148G7	0.06 (0.004)	0.03 (0.01)	0.07 (0.023)	0.02 (0.0025)	1.3

*Dose reduction factor = ratio of doses to reduce survival to 0.01 at low and high dose rate. Units: α/Gy^{-1}, β/Gy^2.

Levels of double-strand breaks

The relationship between survival and DSB at acute dose rates has been reported elsewhere [5]. The data points were well fitted by a linear regression line constrained through the origin (Fig. 3). At acute dose rates DSB could not be assessed accurately beyond 30 Gy. The sensitivity of the cell lines to damage induction differed by a factor of up to 1.5 (ratio of the slopes of the damage induction curves) but did not correlate with survival. The most resistant HX148G7 had a steeper DSB induction curve than the most sensitive, HX149.

After LDR irradiation the level of damage detected was markedly less than was seen at acute dose rates, with essentially no damage detected in any line at 15 Gy, a dose which produced easily detectable levels in all lines at the acute dose rate. This suggests that all four cell lines efficiently rejoined DSB. However, when higher doses were examined, residual damage was detected in the SCLC lines with mean relative elution values of 0.2 and 0.27 at 70 Gy for HC12 and HX149, respectively. At this dose relative elution was only 0.015 in HX148G7 and 0.055 in HX147A7.

Comparison of damage measured at LDR to 70 Gy and that extrapolated to the 70 Gy level from acute data shows that the R.E. values were reduced by 71, 51, 98 and 89% in HC12, HX149, HX148G7 and HX147A7, respectively. This suggests that a higher proportion of the DSBs inflicted may be repaired in the NSCLC cell lines.

The comparison of neutral elution data for cells irradiated at acute and low dose rate assumes that the total number and nature of the lesions inflicted are the same under both conditions. Double-strand breaks were also assessed following LDR

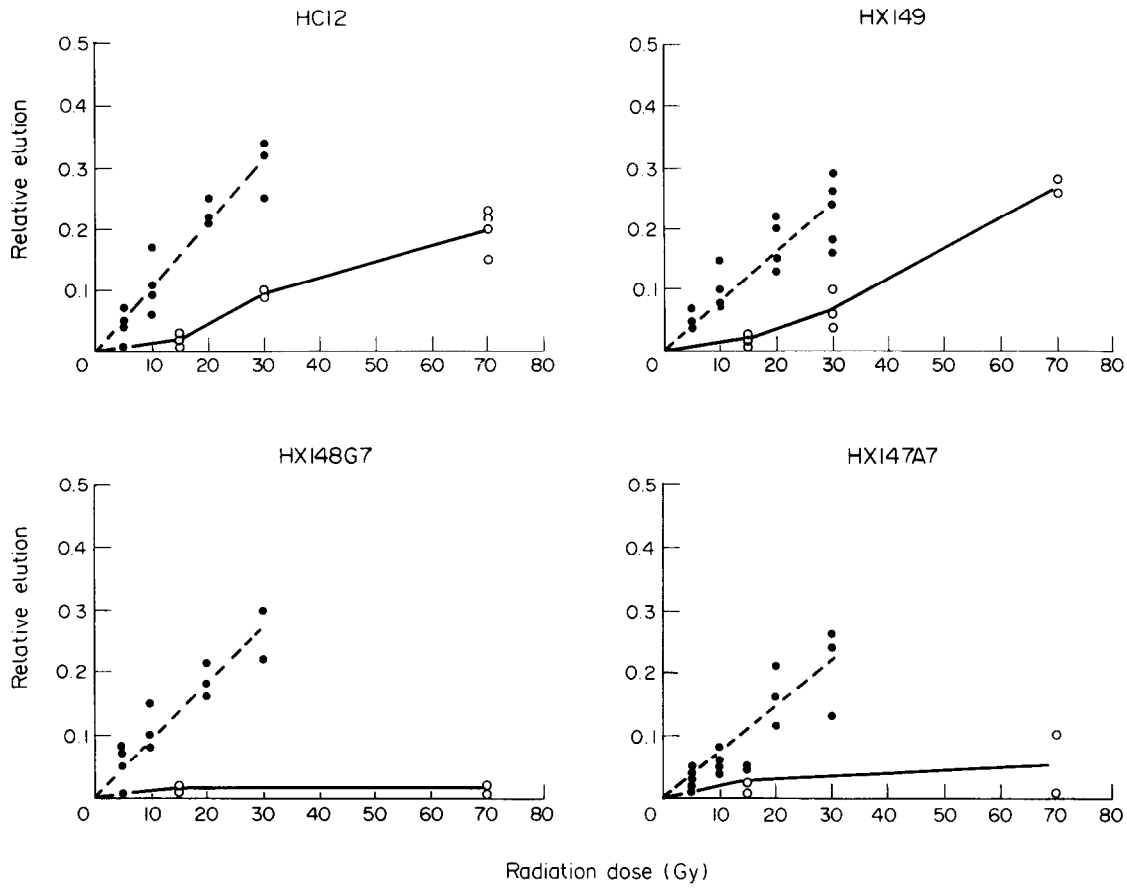

Fig. 3. Relationship between relative elution and dose for acute irradiation (●) and low dose-rate irradiation (○). Individual points from all experiments are shown.

irradiation to 15 Gy at ice bath temperature on each cell line. Assessment at higher dose and therefore longer time at low temperature was unreliable because of increasing DNA loss in unirradiated controls. Two separate experiments (minimum three channels per dose point) were performed for HX149 and one for each of the other lines producing relative elution within the range seen for 15 Gy at acute dose rate and were as follows: HC12, 0.2; HX149, 0.1 and 0.06; HX148G7, 0.11; HX147A7, 0.08. Despite the limited number of experiments we feel this offers support for the assumption that the total number of lesions inflicted is not markedly different at high and low dose rate.

DISCUSSION

Four human lung carcinoma cell lines have been used in this study of cell killing and DNA damage following irradiation at high and low dose rate. In terms of cellular response they are representative of the range of radiosensitivities generally seen in human tumour cell lines [21].

The dose reduction factors (DRF) for cell survival in cell lines examined here vary from 1.19 to 1.57 with no clear difference between the sensitive and resistant lines (Table 2). Similar results have been found by other authors [21]. However, while Steel *et al.* [21] found linear or near-linear curves at 0.016 Gy/min, in none of the lines in the present study was the β component eliminated. This suggests that recovery of potentially lethal damage was not complete even at 0.02 Gy/min.

Lowering the radiation dose rate causes considerable sparing of cell kill; this is thought to be due to repair of potentially lethal DNA lesions under these conditions. As the majority of induced double-strand breaks are repaired [15] examination of DNA damage following conditions allowing maximum repair should focus attention on that damage which persists and allow more relevant comparison between cell lines. This current study shows a clear difference in the level of residual DSBs following low dose-rate irradition in cells that are judged radiosensitive or radioresistant on the basis of clonogenic survival. There are two components which determine this level of residual damage. Firstly, the level of damage present immediately after HDR irradiation has been found to vary in these lines, but not in a manner which correlates with cellular sensitivity. Secondly, the extent of DNA repair plays a role. With the current approach it is difficult to quantify this as we cannot accurately define the shape of the LDR elution curves. However, a comparison between LDR at 70 Gy and the HDR curves extrapolated to 70 Gy did suggest that a higher proportion of damage was repaired in the two radioresistant NSCLC cell lines.

In this analysis we have assumed that the prime determinant of the difference between the HDR and LDR elution curves is DNA repair and that repair is complete immediately after irradiation at LDR. This second assumption is not strictly true as lesions inflicted towards the end of the treatment time will not have been repaired. However, with an overall irradiation time of 58 h for 70 Gy and recognised DSB repair half-times of 10–40 min using neutral filter elution [19] the proportion with an insufficient repair time is likely to be very small. In other studies we have demonstrated this to be the case using pulsed field gel electrophoresis (unpublished results).

Studies of the role of DSB rejoining in the determination of

radiosensitivity have come to mixed conclusions. Differences in residual levels of DSB following repair after acute irradiation have been demonstrated in radiosensitive CHO mutants where 57–90% of induced double-strand breaks remained unrepaired compared with 28% in the more resistant parent line [9]. A dose of 90 Gy was used and no further rejoining was noted after 2 h. Within the mutants, however, the level of DSB rejoining could not, within the sensitivity of the method, be related directly to radiosensitivity. The two most sensitive, XRS-5 and XRS-6, which showed two decades of kill at 2–2.5 Gy (compared with 5–6 Gy in HC12 and HX147) had residual DSBs of 54–78%, while XRS-2 and XRS-4 which showed two decades of kill at 4 Gy had residual DSB of 50–57%. Kelland *et al.* [20] using a dose of 30 Gy and analysing repair for 1 h showed residual DSBs of 50–53% in his two more sensitive lines and 1–22% in his more radioresistant lines. Wlodek and Hittelman [10] after a dose of 30 Gy found that 20% of the initial double-strand breaks remained unrejoined in the resistant L5178Y-R, as opposed to 45% remaining in the sensitive L5178Y-S. Other authors have failed to show differences in DSB rejoining in cells differing in sensitivity [12] in experimental systems and in only one A–T line has a rejoining defect been detected [11]. Where a lack of correlation between DSB rejoining and sensitivity exists it has been suggested that the degree of misrepair may be a critical factor in some cases [22].

In summary, the data presented suggest that differences exist in the level of damage as measured by neutral elution remaining after low dose-rate irradiation in human tumour cell lines and that these may be related to differences in radiosensitivity. It appears that damage induction and repair may be influential in determining the final level of this residual damage. Therefore, while a measure of the damage induced or repaired may not always provide a good predictive assay of radiosensitivity, the assessment of residual damage after low dose-rate irradiation may be useful in this context.

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Acknowledgements—We are grateful to Miss R. Couch and Mrs S. Stockbridge for preparing this manuscript. This study was supported by the Cancer Research Campaign.