British Journal of Cancer (1998) 77(7), 1108–1114

The ratio of initial/residual DNA damage predicts intrinsic radiosensitivity in seven cervix carcinoma cell lines

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Summary The single-cell gel electrophoresis (comet) assay was used to measure radiation-produced DNA double-strand breaks (dsbs) in a series of seven cervical tumour cell lines (ME180, HT3, C33A, C41, SiHa, MS751 and CaSki). The proportion of DNA dsbs was measured immediately after radiation treatment (initial damage) and 16 h later after incubation at 37°C (residual damage). Linear dose–response curves were seen for initial (slopes 0.23–0.66) and residual (slopes 0.16–0.87) DNA dsbs. Neither of the slopes of the linear regression analysis on the initial and on the residual DNA dsbs dose–response curves (range 0–80 Gy) correlated with SF_2 (surviving fraction at 2 Gy) measured after high- (HDR) or low-dose-rate (LDR) irradiation. An association was evident between SF_2 after HDR and LDR irradiation and the ratio of the absolute level of initial and residual damage after a single dose of 60 Gy. However, a significant correlation was found between HDR (r = –0.78, P = 0.04) and LDR (r = –0.86, P = 0.03) SF_2 values and the ratio of the slopes of the initial and residual DNA dsbs dose–response curves (range 0.47–0.99), representing the fraction of DNA damage remaining. These results indicate that the neutral comet assay can be used to predict radiosensitivity of cervical tumour cell lines by assessing the ratio of initial and residual DNA dsbs.

Keywords: predictive assay; intrinsic radiosensitivity; comet assay; cervix cancer

The variation in cellular radiosensitivity of human fibroblasts measured in vitro can be explained largely by the extent of DNA double-strand break (dsb) repair (Würm et al, 1994; Kiltie et al, 1997). However, for tumour cells, no firm consensus exists for a correlation between in vitro radiosensitivity and the magnitude of radiation-induced DNA dsb damage or repair. A number of reports indicate that tumour cell radiosensitivity is positively correlated with the levels of initial radiation-induced DNA dsbs (Kelland et al, 1988; Peacock et al, 1989; McMillan et al, 1990; Ruiz de Almodovar et al, 1994), while others have shown no correlation (Smeets et al, 1993; Olive et al, 1994; McKay and Kefford, 1995). Positive correlations between tumour cell radiosensitivity and the extent of residual DNA dsbs (Giaccia et al, 1992; Zaffaroni et al, 1994), the rate of DNA dsb repair (Schwartz et al, 1988) and the misrepair of radiation-induced DNA damage (Powell et al, 1992; Powell and McMillan, 1994) have also been reported.

The aim of this study was to measure the level of initial and residual radiation-induced DNA dsbs in seven cervical carcinoma cell lines of differing radiosensitivity, as assessed by clonogenic survival after high- (HDR) and low-dose-rate (LDR) irradiation to evaluate the potential of the neutral comet assay as a predictor of radiosensitivity. This method of measuring DNA dsbs was used as it is rapid (Olive et al, 1991; Fairbairn et al, 1995), is less technically demanding than other assays (e.g. neutral filter elution and pulse field gel electrophoresis) and does not require the use of pretreatment radiolabelling to quantify DNA dsb damage, attributes that make the assay an attractive surrogate measure for radiosensitivity for routine measurements of DNA dsb repair capacity on clinical material.

Received 4 April 1997
Revised 4 September 1997
Accepted 9 September 1997

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MATERIALS AND METHODS

Cell culture

The seven cervical carcinoma cell lines used in these studies (CaSki, SiHa, ME180, HT3, C41, C33 and MS751) were obtained originally from the American Type Culture Collection (ATCC). All cells were grown in minimum essential medium (MEM), supplemented with L-glutamine (2 mM), penicillin (100 IU ml^{-1}), streptomycin (0.1 mg ml^{-1}) and 10% fetal calf serum (Biological Industries, kibbutz beth haemek, Israel; batch 501104 and 785225). All media and supplements were obtained from Gibco, Paisley, UK. Cells were grown in sealed T25 tissue culture flasks containing 5 ml of medium and gassed with a mixture of 95% air–5% carbon dioxide when reseeded. Subculturing occurred weekly (seeded at 5×10^5 cells ml^{-1}) with twice-weekly feeding with complete fresh culture medium.

Survival after ionizing radiation

Cell survival was assessed using a conventional clonogenic survival assay after high- (3.1 Gy min^{-1}) or low- (0.112 Gy min^{-1}) dose-rate irradiation using a ^{60}Co-γ source (Wilks et al, 1996). Briefly, cells were irradiated either as single-cell suspensions at room temperature (high dose rate) or cell monolayers in tissue culture flasks at 37°C (low dose rate). In all cases, control (unirradiated) cells were sham irradiated and treated identically to the irradiated cells. After radiation treatment, the cells were

trypsinized (low dose rate only), and cell suspensions were diluted in complete tissue culture medium to an appropriate density and plated to ensure 100 surviving colonies per 50-mm Petri dish, irrespective of the radiation dose given. Separate plates were set up at two or three cell densities for unirradiated cells to obtain a precise estimate of plating efficiency. The dishes were incubated at 37°C in an atmosphere of 95% air–5% carbon dioxide for 3 weeks. Plates were then stained with Gentian violet, and colonies assessed as containing more than 50 cells were scored as survivors.

Survival curve analysis

Surviving fractions were calculated as described previously (Marples and Joiner, 1993). Briefly, for each individual experiment, the plating efficiencies (PE) of the replicate control (unirradiated) plates were determined and a mean value calculated, this was then used to determine the surviving fraction of irradiated cells in that experiment. The plating efficiencies of the seven tumour cell lines ranged from a mean value of 15% for C41 cells to 67% for SiHa cells. For each individual experiment, surviving fraction was calculated using a minimum of three replicate Petri dishes per dose point. The values calculated from the independent experiments (n = 3–12) were averaged to give an overall mean value of survival for the complete data for each cell line. These data were plotted and fitted using a linear quadratic model [surviving fraction = $\exp(\alpha d + \beta d^2)$]; a mathematically derived estimate of the surviving fraction at 2 Gy (SF_2) was then calculated from the fitted survival curves.

Treatment of samples for comet analysis

Two treatment regimens were used to measure initial and residual DNA dsbs. For assessment of initial damage, a confluent monolayer of cells was trypsinized and resuspended in bijou tubes at 8×10^4 cells ml^{-1} in 2 ml of ice-cold complete medium and placed on ice. For each experiment, a cell cycle profile of the cell population was measured using flow cytometry (see below) (Ormerod, 1990). The ice-cold cell suspension (8×10^4 cells ml^{-1}) was irradiated at room temperature and returned immediately to ice to minimize enzymatic DNA dsb repair. A 1-ml aliquot of the ice-cold treated cell suspension was mixed with 2.5 ml of pre-warmed (45°C) 1% agarose (low-gelling-point type VII, Sigma Chemical), then 1 ml of the cell–agarose mixture was rapidly applied to a standard microscope slide, precoated 24 h previously with 400 µl of 1% agarose. The slides were placed immediately onto an ice-cold metal surface to hasten the solidification of the agarose and reduce the time for DNA repair to occur. Once the agarose had set, the slides were carefully submerged in 500 ml of a freshly prepared 50°C lysis solution of 0.5% sodium dodecyl sulphate (SDS) and 30 mM EDTA, pH 8, for 4 h. Subsequently, the slides were rinsed in a solution of TBE buffer (45 mM Tris base, 45 mM boric acid, 2 mM EDTA, pH 8.2) overnight followed by four 15-min washes in fresh TBE buffer. The slides were then transferred to an electrophoresis tank containing 1150 ml of TBE buffer at room temperature. The electrophoresis and washing tanks are made of black perspex, ensuring all light was excluded. Electrophoresis was carried out at room temperature for 25 min at 20 V (~ 0.6 V cm^{-1}). Subsequently, the slides were rinsed by submerging in double-distilled water and stained with 2.5 µg ml^{-1} of PI in 0.1 M sodium chloride for 60 min followed by a 30-min rinse in double-distilled water to remove unbound propidium iodide. Slides were dried at room temperature for storage and rehydrated by placing in double-distilled water for 45 min to score. In order to measure residual damage, monolayers of cells in T25 flasks containing 5 ml of culture medium were irradiated at room temperature and immediately returned to a 37°C incubator. After 16 h, the cells were trypsinized, suspended at 8×10^4 cells ml^{-1} and processed for comet analysis as described above. Experiments measuring the kinetics of DNA dsb rejoining using the most radiosensitive (ME180) and radioresistant cell line (MS751) indicated that small differences were evident in the rate of break rejoining at times < 4 h, however no differences were detected after a 12-h repair period. Consequently, to ensure sufficient time for all the 'rejoinable' breaks to rejoin, a 16-h repair period was used in experiments measuring the absolute proportion of residual DNA dsbs. In addition, 16 h is experimentally appropriate for the development of a routine predictive radiosensitivity test using the neutral comet assay, as it represents an overnight time interval.

Analysis of initial and residual damage

A minimum of three independent experiments were carried out per cell line. Comets were analysed using a Leitz Diaplan fluorescent microscope at 200× magnification using a Kinetic Imaging Komet system (Liverpool, UK) (Ashby et al, 1995). Tail moment was used as an index of DNA damage, which combines a measure of the length of the comet tail and the proportion of DNA to migrate into the tail (Olive and Banáth, 1993). Before comet scoring, the control sample slide (unirradiated) was examined manually and the brightest comet used to calibrate the CCD camera by ensuring the fluorescence of the comet head did not exceed 255 grey scales. This procedure ensured that subsequent measurements of other comet images did not saturate the CCD camera preventing incorrect head to tail ratios being scored. Comets were measured by randomly selecting images from the microscope field of view and a defined sequence of searching guaranteed that the same image was not scored twice. The mean tail moment value was calculated from 100 comets on two replicate slides per dose point in each of the three individual experiments using the Kinetic Imaging analysis software. To obtain an overall mean tail moment value from the repeat experiments, the mean values from the individual experiments were averaged and standard error on the mean calculated and plotted.

Flow cytometry

Immediately before each experiment an aliquot of the cell population was washed twice in phosphate-buffered saline (PBS) and resuspended in 0.5 ml of PBS and fixed with 4.5 ml of ice-cold 70% ethanol (in PBS) for a minimum of 24 h at 4°C. Subsequently, the cells were washed twice in PBS, pelleted and resuspended in 800 µl of PBS, 100 µl of RNAase (1 mg ml^{-1}) and 100 µl of propidium iodide (1 mg ml^{-1}) for 30 min at 37°C (Ormerod, 1990). Analysis of cell cycle phase distributions were carried out on a Becton Dickinson FACScan flow cytometer at 488 nm using a long-pass filter.

RESULTS

The radiation survival curve parameters after high- (HDR) and low-dose-rate (LDR) irradiation for the seven cell lines used are given in Table 1, and the data are illustrated in Figure 1. The data

Table 1 Parameters from the linear quadratic model fitted to the survival data in Figure 1 and the mathematically derived SF_2 values

	High-dose-rate survival parameters			Low-dose-rate survival parameters		
Cell line	**SF_2 ± (95% CL)**	**α ± (95% CL)**	**β ± (95% CL)**	**SF_2 ± (95% CL)**	**α ± (95% CL)**	**β ± (95% CL)**
MS751	0.78 ± 0.09	0.07 ± 0.03	0.03 ± 0.01	0.87 ± 0.18	0.06 ± 0.05	0.01 ± 0.01
ME180	0.31 ± 0.06	0.56 ± 0.05	0.01 ± 0.02	0.38 ± 0.07	0.42 ± 0.01	0.03 ± 0.01
C33A	0.55 ± 0.16	0.25 ± 0.05	0.03 ± 0.01	ND	ND	ND
C41	0.61 ± 0.02	0.15 ± 0.03	0.05 ± 0.01	0.74 ± 0.03	0.12 ± 0.08	0.04 ± 0.02
HT3	0.38 ± 0.10	0.41 ± 0.04	0.03 ± 0.01	0.72 ± 0.23	0.12 ± 0.03	0.03 ± 0.01
SiHa	0.75 ± 0.05	0.13 ± 0.12	0.02 ± 0.05	0.60 ± 0.14	0.25 ± 0.11	0.01 ± 0.02
CaSki	0.87 ± 0.06	0.09 ± 0.06	0.08 ± 0.02	0.73 ± 0.08	0.08 ± 0.02	0.04 ± 0.01

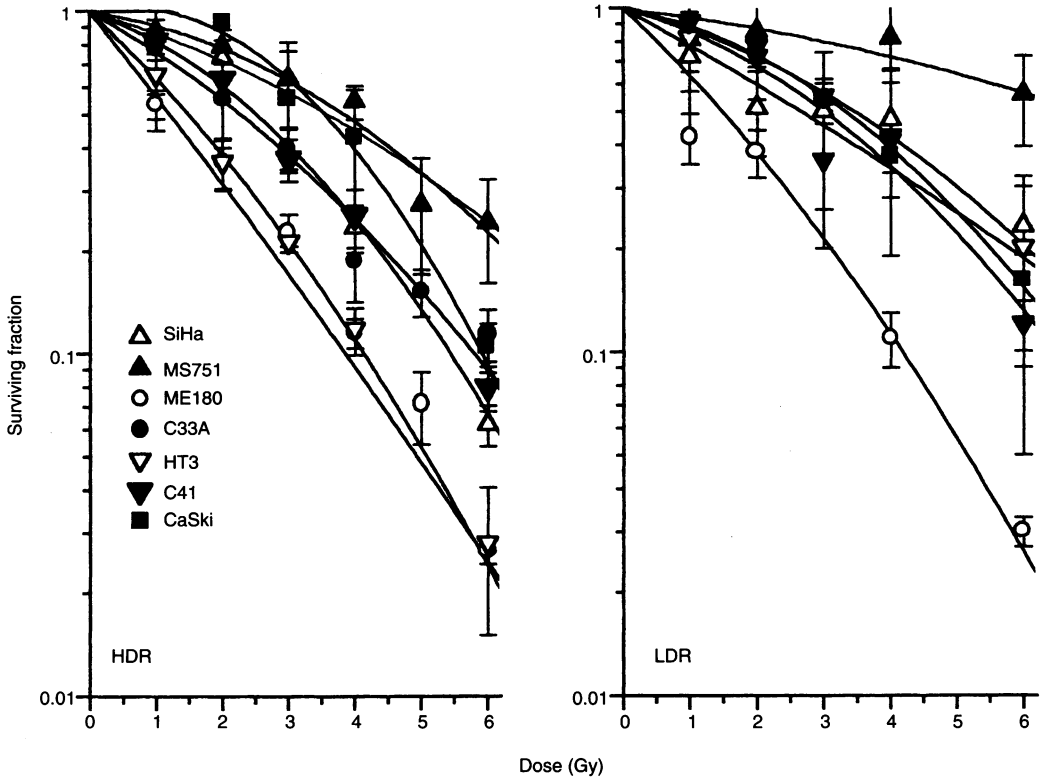

Figure 1 Radiation survival curves for the cervical tumour cell lines after HDR (right panel) and LDR (left panel) irradiation. The lines are a linear quadratic fit to the data

points for HDR irradiation represent the mean survival (± standard deviation) calculated from a minimum of three independent experiments (n = 3–12) with three to six replicate dose measurements; survival after LDR irradiation was calculated from two or three independent experiments. A range of cellular radiosensitivity was seen, with the SF_2 values differing by a factor of 2.8 (range 0.31–0.87) after HDR irradiation and by a factor of 2.3 (range 0.38–0.87) after LDR radiation treatment.

Figure 2 shows the initial and residual DNA dsb dose–response curves obtained from the three individual experiments for two of the tumour cell lines used. Linear dose–response curves were evident in each experiment for initial and residual damage. A linear relationship between dose and initial and residual DNA dsbs was also seen in the majority of the individual experiments for the other five cell lines, albeit with greater variability (correlation coefficients ≥0.74) (data not shown). In all experiments, the influence of cell age on assay variability was minimized by using confluent cultures to reduce the proportion of cells in S-phase, as the presence of replication forks in DNA can restrict migration under electrophoresis (Olive et al, 1992; 1993). In each experiment, flow cytometry analysis indicated that the proportion of cells in the S-phase of the cell cycle was always below 18% (range 7–17%, mean 12%), irrespective of the cell line used.

The combined initial and residual DNA dsb dose–response curves compiled from the data of the individual experiments for all seven cell lines are shown in Figure 3. For each cell line, the data points represent the mean (± s.e.m.) tail moment (TM) value calculated from the average TM values from the individual experiments

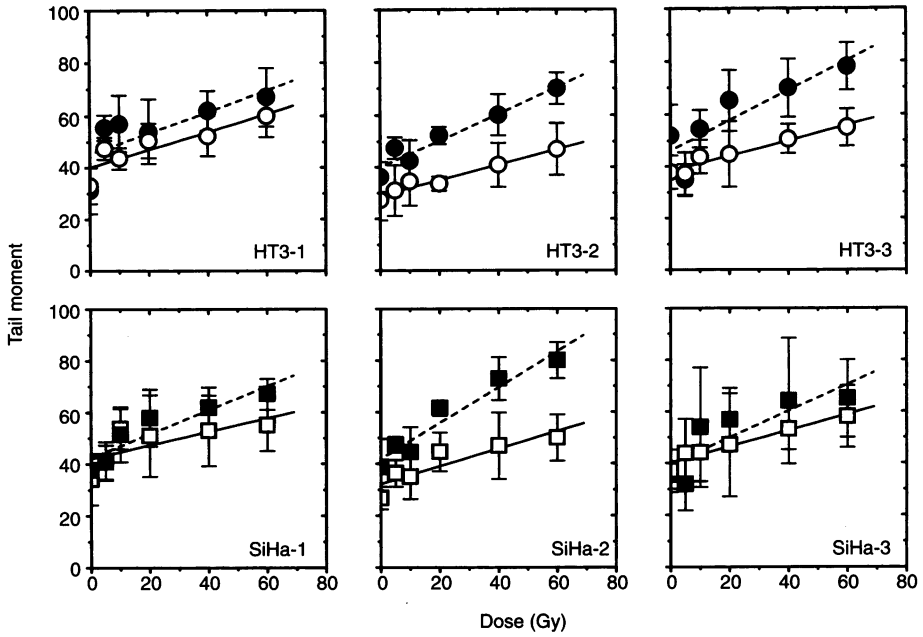

Figure 2 Dose–response curves for initial (- - -) and residual (—) DNA dsbs measured using the neutral comet assay in the three individual experiments for the HT3 (upper panels) and SiHa (lower panels) cervical tumour cell lines. Each point represents the mean (± s.d.) tail moment value calculated from two replicate slides per dose point per individual experiment

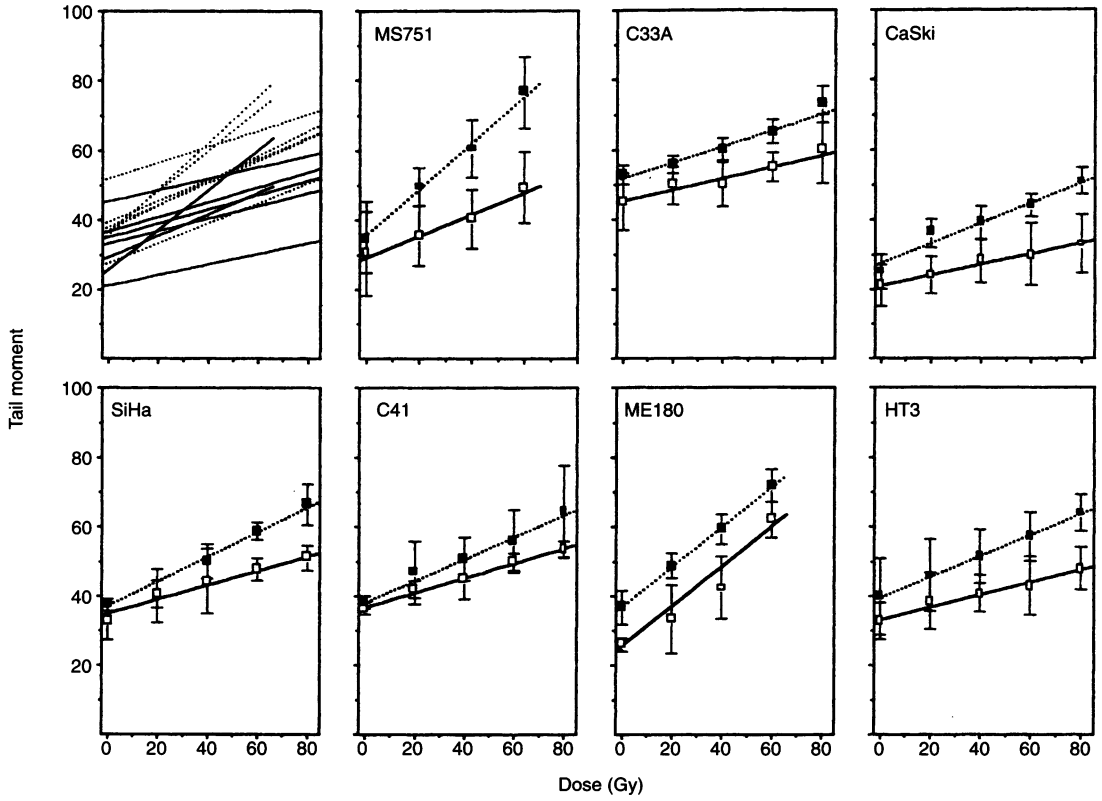

Figure 3 Dose–response curves for initial (- - -) and residual (—) DNA dsbs measured using the neutral comet assay. Each point represents the mean (± s.e.m.) calculated from replicate independent experiments. The lines are fitted by linear regression

and are fitted by linear regression. The combined data shown in Figure 3 were all well described by a linear fit, with correlation coefficients of 0.96 or higher.

The slopes of the fitted linear regression to the initial DNA dsb dose–response data varied by a factor of 2.9 (range 0.23–0.66) and were steeper than those for residual damage. The shallower residual

Table 2 Slopes (± 95% CL) from linear regression analysis of the initial and residual dose–response curves in Figure 3. Ratio of damage is calculated as residual damage/initial damage

Cell line	Initial damage	Residual damage	Ratio
MS751	0.66 ± 0.04	0.31 ± 0.04	0.47
ME180	0.58 ± 0.02	0.58 ± 0.05	0.99
C33A	0.23 ± 0.02	0.16 ± 0.03	0.72
C41	0.32 ± 0.02	0.22 ± 0.01	0.68
HT3	0.30 ± 0.01	0.18 ± 0.01	0.61
SiHa	0.36 ± 0.02	0.21 ± 0.02	0.58
CaSki	0.29 ± 0.03	0.87 ± 0.01	0.53

damage slopes indicate that there were fewer DNA dsbs after a 16-h incubation at 37°C (Table 2). The slopes of the fitted linear regression to the residual DNA dsb dose–response curves varied by a factor of 3.6 (range 0.16–0.58). The ratio of the slopes of the initial and the residual dose–response curves, representing the fraction of DNA damage unrepaired, varied by a factor of 2.1 (range 0.47–0.99).

A comparison was made of the relationship between clonogenic survival after HDR and LDR irradiation and the level of radiation-induced DNA dsbs measured using the neutral comet assay. No relationship was found between HDR SF_2 and the slopes of initial (P = 0.91) or residual (P = 0.23) DNA dsb dose–response curves fitted by linear regression. In contrast, an association was evident between HDR SF_2 and the ratio of the number of initial and residual DNA dsbs (i.e. the fraction of DNA damage unrepaired) after a 60-Gy dose of radiation (r = 0.58; P = 0.17) (Figure 4B). Moreover, a statistically significant correlation was found between HDR SF_2 and the ratio of initial and residual DNA dsbs over the complete dose range examined, as represented by the slope parameters calculated from Figure 3 (r = –0.78; P = 0.04) (Figure 4A). Stronger correlations were found between SF_2 after low-dose-rate irradiation and the fraction of DNA damage unrepaired (ratio of initial and residual damage) at 60 Gy (r = 0.78; P = 0.09) and over the complete dose range studied (r = –0.86; P = 0.03) (Figure 4C and D).

DISCUSSION

Evidence that the radiosensitivity of a tumour is an important factor involved in determining patient response to radiotherapy has been gained from work carried out using clonogenic assays (West, 1995; West et al, 1997). These assays are however too laborious for routine clinical application, and this has stimulated an interest in the development of more rapid measures of radiosensitivity. The comet assay is attractive as a potential clinical test of tumour radiosensitivity as it requires few cells and generates results rapidly compared with standard clonogenic and other electrophoresis assays.

The linear relationship between the production of radiation-induced DNA dsbs and dose shown in Figures 2 and 3 is in good agreement with previous studies using a variety of DNA dsb assays (reviewed by Núñez, 1996) and the neutral comet assay (Olive et al, 1994). In the present study, a comparison of the slopes obtained from the linear regression analysis of the initial DNA dsb dose–response curves for the seven tumour lines showed significant variation, indicating different levels of initial damage between

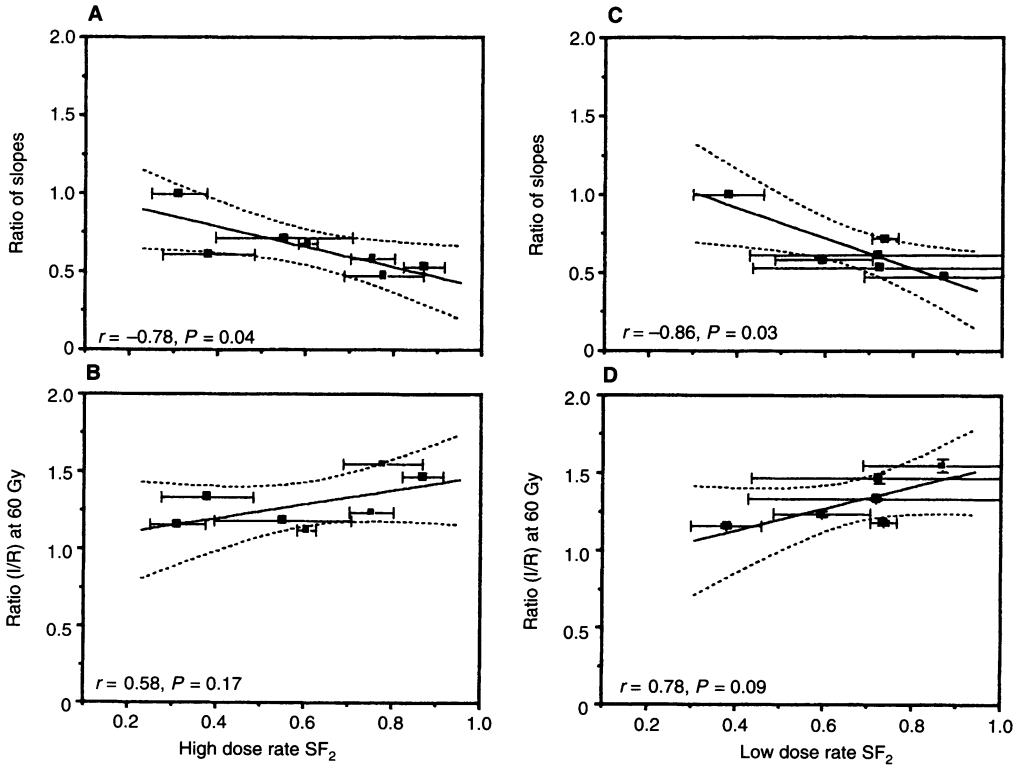

Figure 4 Relationship between ratio of damage (initial/residual) at 60 Gy (**B** and **D**) or ratio of slopes of the initial and residual DNA dsb dose–response curves and radiosensitivity expressed as SF_2 for HDR (**A** and **B**) and LDR (**C** and **D**) irradiation for the series of cervical tumour cell lines. The solid lines represent the regression fit to the data and the dashed lines the 95% confidence on the fit

Table 3 Correlation coefficients and *P*-values calculated from either HDR or LDR SF_2 plotted against the slopes of the initial and residual damage dose–response curves, the ratio of slopes of residual and initial damage dose–response curves or the ratio of the absolute level of initial residual level of DNA dsbs at 60 Gy (as determined from Figure 3)

	Initial slope		Residual slope		Ratio of slopes		Ratio at 60 Gy	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
HDR SF_2	–0.05	0.91	–0.51	0.23	–0.78	0.04*	0.58	0.17
LDR SF_2	–0.07	0.89	–0.59	0.21	–0.86	0.03*	0.78	0.09**

*Correlation is significant at the level 0.05. **Correlation is significant at the level 0.10.

the different cell lines (Figure 3 and Table 2). The present finding agrees with other studies that have shown significant differences in DNA dsb induction between human tumour cells lines using neutral elution (Kelland et al, 1988) or pulsed-field gel electrophoresis (Whitaker et al, 1995). However, the results from Figure 3 contrast with the data of Olive et al (1994) using the neutral comet assay which showed no significant differences in radiation-induced DNA dsb in a range of six histologically distinct human tumour cell lines (melanoma, prostate carcinoma, glioma, colon adenocarcinoma and cervical carcinoma).

In order to have confidence in the potential use of a rapid assay of tumour radiosensitivity, correlations must be shown with clonogenic measures of radiosensitivity. A significant correlation has been reported previously between clonogenic radiosensitivity and the levels of initial radiation-induced damage in human tumour cells (Kelland et al, 1988; Núñez et al, 1996). It was postulated that this might reflect differences in the number of lesions incurred by the radiosensitive cells compared with the resistant cells, as shown by Peacock et al (1989), or possibly differences in chromatin conformation that affect the accessibility of scavenging molecules to damaged DNA or radical attack (Oleinick et al, 1984; Olive et al, 1992) or the 'presentation' of DNA damage sites (Woudstra et al, 1996). However, the results from this study indicate that in the cervical tumour lines studied here the level of initial DNA damage does not correspond with clonogenic radiosensitivity after HDR and LDR irradiation (Table 3).

In contrast, Ward (1990) argued that the yield of molecular lesions (initial damage) is independent of tumour cell type and that radiation-sensitive cells are repair deficient, resulting in different levels of residual damage. However, in our work, there were no significant differences in the slopes of the residual DNA dsb dose–response curves fitted by linear regression and SF_2 after HDR and LDR irradiation. Nevertheless other human tumour studies measuring DNA dsbs by asymmetric field-inversion gel electrophoresis (Giaccia et al, 1992) and neutral filter elution (Zaffaroni et al, 1994) have indeed shown significant correlations between the level of residual dsbs and radiosensitivity.

A new finding in the present studies was the significant correlation with the extent of dsb repair (ratio of initial and residual slopes) after 16 h of post-irradiation incubation and radiosensitivity after HDR ($r = -0.78$, $P = 0.04$) or LDR ($r = -0.86$, $P = 0.03$) irradiation (Figure 4). DNA dsb repair measured using the neutral comet assay and cellular radiosensitivity were found not to correlate in a study by Olive et al (1992), however one possible explanation for the difference between the present study and that of Olive et al (1992) may reflect the time interval allowed for repair: 4 h in the previous study and 16 h in the present study. It has been noted that measuring residual DNA dsbs using the neutral comet assay at 4 h may underestimate the level of DNA damage as a result of the increase in the percentage of S-phase cells after irradiation, as S-phase DNA migrates inefficiently. This would not be expected in the present study using a 16 h period of repair (Olive et al, 1994).

In support of the correlation between SF_2 and repair capacity reported here, a non-significant trend was evident between LDR SF_2 and the absolute difference in residual and initial DNA dsbs assessed from a single dose of 60 Gy ($P = 0.09$) and a significant trend over the complete dose range studied ($P = 0.03$) (Figure 4B and D). The stronger relationship between the level of DNA dsbs and SF_2 measured after LDR irradiation is not unexpected and reflects the potential of each cell line to repair radiation-induced DNA dsbs during exposure. A correlation between radiosensitivity and level of dsbs in four histologically related tumour cell lines has been reported previously after low-dose-rate but not at high-dose-rate irradiation using PGFE (Cassoni et al, 1992).

The approach of examining the unrepaired DNA dsb fraction by taking a ratio of dose–response curve slopes (thereby assessing damage from a range of multiple dose points) minimizes variability between experiments on the different cell lines and suggests that it is only by doing so that a significant correlation can be seen between HDR SF_2 and DNA dsb repair capacity as measured by the neutral comet assay. Recently, we have also found a strong correlation between the ratio of slopes of initial/residual DNA dsb dose–response curves and radiosensitivity after HDR irradiation for nine normal fibroblast strains derived from vaginal biopsies from pretreatment cancer patients ($r = 0.80$, $P = 0.01$) (Marples et al, 1997). It is not clear yet whether the importance of the unrepaired fraction of DNA dsbs is related to, for example, DNA ploidy, possible influence of chromatin structure/DNA organization on the translation of residual DNA damage into potentially lethal events or on the tolerance of cells to genetic injury. Alternatively, assessing repair capacity by the ratio of damage (initial/residual) may relate to misrepair of radiation-induced DNA dsbs. Such possibilities will require further investigation.

A review of the literature (see Introduction) illustrates the disparate findings over the possible relationship between DNA dsb damage and clonogenic measures of radiosensitivity. There is clearly some relationship but the question of interest is which parameter is the most important component (initial or residual damage, ratio of damage, rate of repair, time at which repair is assayed, etc.). The balance of evidence suggests that residual damage is likely to be the most critical aspect but, in view of the published data, the significance of initial damage cannot yet be discounted. The divergent results may relate to differences in the assays used to score DNA dsb damage and/or experimental design (e.g. selection and number of cell lines, doses used, time allowed for repair). In an attempt to overcome some of the limitations of other studies, we have selected a large series of cell lines of the same histological origin (seven human cervical carcinoma cell lines) and have assessed both initial and residual DNA damage over a range of radiation doses. In doing so, we have demonstrated a significant relationship between the ratio of initial and residual radiation-induced DNA dsbs, measured using the neutral comet assay, and cellular radiosensitivity measured using a clonogenic assay. It is likely that the continued improvements of assay conditions may improve this further, e.g. more sensitive DNA-binding dyes, more optimal lysing conditions. The work supports the

continued use of the neutral comet assay as a potential rapid predictive test for tumour radiosensitivity in radiotherapy.

ACKNOWLEDGEMENTS

This work was supported by the Association for International Cancer Research and the Cancer Research Campaign, UK. The assistance of Mr J Barry in carrying out the flow cytometric measurements is acknowledged. We particularly thank Dr Peggy Olive (Medical Biophysics, British Colombia Cancer Research Centre, Vancouver, Canada) for discussion of the comet assay data. Also, the authors would like to thank the staff and customers at Asda (Chadderton, UK) for funds to purchase the image analysis system.

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