Fibroblast radiosensitivity measured using the comet DNA-damage assay correlates with clonogenic survival parameters

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Summary A study was made of the neutral comet assay as a potential method for measuring normal cell radiosensitivity. Eleven fibroblast strains were studied comprising nine derived from vaginal biopsies from pretreatment cervical cancer patients and two strains from radiosensitive individuals. DNA double strand break (dsbs) dose–response curves for both initial and residual (20-h repair time) damage were obtained over the dose range 0–240 Gy, with slopes varying 3.2 and 8-fold respectively. Clonogenic cell survival parameters were available for all the cell strains following both high- and low-dose rate irradiation. There were no correlations between the dose–response slope of the initial level of DNA dsbs and parameters that mainly describe the initial portion of clonogenic radiation survival curves (SF2, α, D). A significant correlation (r = –0.63, P = 0.04) was found between the extent of residual DNA dsbs and clonogenicity for all 11 fibroblast strains. The parameter showing the highest correlation with fibroblast cell killing (D) for the nine normal fibroblasts alone was the ratio of initial/residual DNA dsb dose–response slope (r = 0.80, P = < 0.01). A significant correlation (r = –0.67, P = 0.03) with clonogenic radiosensitivity was also found for all 11 cell strains when using the ratio of initial/residual DNA dsb damage at a single dose of 180 Gy. This study shows that fibroblast radiosensitivity measured using the neutral comet assay correlates with clonogenic radiation survival parameters, and therefore may have potential value in predictive testing of normal tissue radiosensitivity.

Keywords: DNA damage; fibroblasts; comet assay; intrinsic radiosensitivity; predictive assays

Intrinsic cellular radiosensitivity has been shown to be involved in determining the severity of normal tissue response to radiotherapy (Burnet et al, 1994). This has led to interest in the potential of measuring cellular radiosensitivity in patients prior to the commencement of radiotherapy, using the results to individualize treatment and either reduce morbidity or increase local control (MacKay et al, 1998). Recently, there have been several promising correlations reported between clonogenic fibroblast radiosensitivity and the severity of late normal tissue complications following radiotherapy (Brock et al, 1995; Burnet et al, 1996; Johansen et al, 1996). However, the clonogenic assays used in the latter studies are probably too slow to be of routine clinical use because they can sometimes require several weeks to obtain a result. There is, therefore, interest in studying other potentially more rapid assays of normal cell radiosensitivity.

The precise process(es) that define intrinsic radiosensitivity are unknown. However, it is generally accepted that DNA double strand breaks (dsbs) which remain unrepaired, or are misrepaired, are important lesions (Ward, 1994). Consequently, attention has focused on assays that measure radiation-induced DNA dsb damage as potential rapid predictive tests of normal cell radiosensitivity. There is evidence for a relationship, in fibroblasts, between residual radiation-induced DNA dsbs scored either by pulsed field (PFGE) or graded voltage (GVGE) gel electrophoresis and measures of radiosensitivity derived from clonogenic survival experiments (Wurm et al, 1994; Kiltie et al, 1997; Zhou et al, 1998).

Although successful, these cell population-based electrophoresis methods are not as suited to measuring radiation-induced DNA dsbs in clinical material as is the neutral comet assay (Olive et al, 1991). In particular, the neutral comet assay requires no pretreatment radioactive precursor to label the DNA, it can be performed with a small number of cells (in theory, only a few hundred) and a measure of the heterogeneity of DNA damage within the cell population is obtained as damage is assessed on a cell by cell basis. We have recently demonstrated the potential of the neutral comet assay technique as a surrogate assay for radiosensitivity in a large series of cervical carcinoma cell lines showing a correlation between the level of unrepaired DNA dsbs and clonogenic survival measured after 2 Gy (SF2) (Marples et al, 1998). However, to our knowledge, no study has evaluated the potential of the neutral comet assay to measure DNA dsbs in a series of fibroblast strains and compared the results with clonogenic measures of radiosensitivity. Therefore, in this study, DNA dsbs were measured immediately after irradiation treatment and 20 h later in 11 fibroblast strains. The initial and residual DNA dsb dose–response curves were then compared with radiosensitivity measured using a clonogenic assay.
**MATERIALS AND METHODS**

**Cell culture**

The normal fibroblast strains were derived from vaginal biopsies obtained from patients with carcinoma of the cervix at examination under anaesthetic before radical radiotherapy (Kiltie et al., 1997). Two cell strains known to be highly sensitive to the lethal effects of ionizing radiation were used as positive controls. An ataxia telangiectasia cell strain (AT1) was obtained from Dr C Arlett (University of Sussex, Brighton, UK) and a T(–) B(–) SCID cell strain (PH1) was kindly provided by Dr JP DeVillartay (Hopital Necker-Enfants Malades, Paris, France). The fibroblasts were grown as monolayers in minimum essential medium (MEM) supplemented with 15% fetal calf serum (Biowhittaker, Wokingham, UK), 2 mM glutamine, 100 U ml−1 penicillin and 100 µg ml−1 streptomycin. All media and supplements were obtained from Gibco BRL (Paisley, UK). Cultures were maintained at 37°C in a 5% carbon dioxide humidified incubator. Plastic tissue culture flasks (25 cm² Falcon) were seeded with 10⁵ cells for initial damage experiments or with 2 × 10⁴ cells in 12.5 cm² flasks for residual damage experiments, and used in plateau phase after 7 days growth. A parallel study with these 11 cell strains indicated that this culture regime ensured the cell population was in plateau phase with a low S-phase content, as assessed by flow cytometry analysis (<7%) (Kiltie et al., 1997).

**Clonogenic assay**

Cells were irradiated using a ³²Co γ-ray source with a dose rate of either 1.08 Gy min⁻¹ at room temperature (high-dose rate) or 0.011 Gy min⁻¹ at 37°C (low-dose rate). The procedures for carrying out the clonogenic assay experiments have been described in detail elsewhere (Kiltie et al., 1997). Either two (vaginal strains) or three (AT1 and PH1) replicate experiments were performed on each cell strain. Radiation survival curves were fitted using a linear quadratic equation [surviving fraction = \exp(-\alpha D - \beta D²)] to obtain the measures \(\alpha\) (initial slope) and \(\beta\) (mean inactivation dose, the integral of fitted curves in linear co-ordinates).

**Comet assay**

Cells were irradiated using a ¹³⁷Cs γ-ray source at a dose rate of 3.1 Gy min⁻¹. For initial DNA damage experiments, plateau phase cells were trypsinized, counted and 0.5 ml of an ice-cold suspension at 4 × 10⁴ cells ml⁻¹ was irradiated with 60, 120, 180 or 240 Gy. The cells were then returned immediately on ice to prevent repair. An unirradiated control flask was included in all experiments. For measurements of residual DNA damage, the cells were irradiated as a monolayer, to the same doses, followed by reincubation at 37°C for 20 ± 2 h to allow for repair, and then processed identically to the samples used for initial dsb DNA damage. At the time of sampling, 0.5 ml of cells (4 × 10⁴ ml⁻¹) was mixed with 2.5 ml of 1% low-gelling temperature agarose (Type VII, Sigma Chemical, St. Louis, USA) and 500 µl pipetted onto a slide precoated previously with 200 µl of agarose. Once the agarose had solidified, the slides were carefully placed in lysis buffer (0.5% SDS, 30 mM EDTA, pH 8.9) for 4 h at 50°C (Olive et al., 1991) and then removed, rinsed in 400 ml 0.5 × TBE (45 mM Tris, 1 mM EDTA, 45 mM boric acid, pH 8.5) to remove excess lysis buffer and placed in fresh 0.5 × TBE overnight. Slides were then electrophoresed for 25 min in 1150 ml fresh 0.5 × TBE at 0.6 V cm⁻¹ with an average current of 5.1 mA, rinsed in double distilled water (DDW) and stained with propidium iodide (PI; 2.5 µg ml⁻¹ in 0.1 M sodium chloride) for 0.5 h. Unbound stain was removed by washing with DDW. Alternatively, slides were dried without staining and stored until required. Rehydration of the slides was by addition of 1 ml DDW for 0.5 h, followed by 1 ml PI for 0.5 h.

**Comet analysis**

Comets were analysed with a Leitz Diaplan fluorescent microscope at 200 × magnification using a Kinetic Imaging Komet system (Liverpool, UK) (Ashby et al., 1995). Comet images were selected randomly from the microscope field of view using a defined sequence of searching to ensure the same image was only scored once. The parameter used as an index of DNA damage was tail moment, 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). The mean tail moment value was calculated by the imaging software from 50 comets measured for each dose point per individual experiment. For each cell line, three independent experiments (initial and residual damage) were performed. To obtain an overall mean tail moment value for the three separate experiments, the values obtained from the individual experiments were normalized against an internal control and then averaged. This mean value and the standard error of the mean were plotted, fitted by linear regression and the slopes of the dose–response curves calculated. For statistical analysis, correlations between measures of DNA dsb damage and repair and clonogenicity were obtained using the Pearson correlation.

**RESULTS**

Dose–response curves for initial and residual radiation-induced DNA dsbs were obtained for all 11 fibroblast strains in three replicate experiments. Figure 1 illustrates typical tail moment data for one strain (SV357) and shows the magnitude of experimental
variation seen between the individual experiments. To compare directly the 11 cell strains, the data for each cell strain were normalized using the tail moment value obtained for unirradiated cells in each experiment. Figure 2 shows the results of normalization in two of the cell strains (SV350 and SV337). A clear difference is evident between the slopes of the initial and residual response curves, indicating repair of the induced DNA dsbs during the 20 h period that cells were held at 37°C after treatment.

The slopes of the DNA dsb dose–response curves from the combined data for each of the 11 fibroblast strains were calculated using linear regression, and these are shown in Table 1. These slope values ranged from 0.012 to 0.039 (3.2-fold) for initial DNA dsbs and from 0.001 to 0.008 (8-fold) for residual DNA dsbs. Statistical comparison (non-parametric one way analysis of variance) between the initial ($P = 0.16$) and residual ($P = 0.06$) slope values obtained from the individual experiments indicate no significant difference between the 11 cell strains. However, the slopes of the individual residual damage dose–response curves could be used to discriminate between the two radiosensitive (AT and PH1) and nine normal cell strains (Mann–Whitney $U$-test: $P = 0.01$). No discrimination was found for initial damage ($P = 0.11$).

For each cell line, the ratio of the dose–response slopes was also calculated by dividing values for the initial slopes by those for the residual slopes. The ratio parameter is an index of DNA damage repair capacity and the values ranged from 2 to 19 (i.e. a 9.5-fold variation between all 11 fibroblasts with a 4-fold variation between the nine normal fibroblast strains) (Table 1).

Clonogenic radiation survival curves and parameters have been published elsewhere for the two radiosensitive strains (Sproston et al, 1997) and the nine normal strains (Kiltie et al, 1997). A brief description of these curves is provided in Figure 2.
Correlations of survival parameters with repair capacity using the Pearson correlation

| LDR SF<sub>2</sub> | HDR SF<sub>2</sub> | HDR | LDR | HDR | LDR |
|------------------|------------------|-----|-----|-----|-----|
| AT1 | 0.03 ± 0.002 | 0.06 ± 0.05 | 1.19 ± 0.59 | 0.90 ± 0.23 | 0.71 | 0.99 |
| PH1 | 0.03 ± 0.004 | 0.03<sup>a</sup> | 1.68 ± 0.04 | 1.71 ± 0.10 | 0.60 | 0.58 |
| SV269 | 0.21 ± 0.01 | 0.29 ± 0.03 | 0.52 ± 0.07 | 0.21 ± 0.07 | 1.62 | 2.46 |
| SV282 | 0.18 ± 0.01 | 0.23 ± 0.01 | 0.84 ± 0.05 | 0.80 ± 0.04 | 1.20 | 1.43 |
| SV337 | 0.32 ± 0.02 | 0.30 ± 0.01 | 0.30 ± 0.07 | 0.33 ± 0.07 | 1.80 | 1.88 |
| SV350 | 0.15 ± 0.01 | 0.35 ± 0.06 | 0.96 ± 0.06 | 0.56 ± 0.03 | 1.13 | 1.90 |
| SV351 | 0.19 ± 0.01 | 0.31 ± 0.01 | 0.71 ± 0.06 | 0.33 ± 0.05 | 1.25 | 2.19 |
| SV357 | 0.18 ± 0.01 | 0.33 ± 0.03 | 0.75 ± 0.04 | 0.54 ± 0.04 | 1.32 | 1.85 |
| SV368 | 0.18 ± 0.01 | 0.31 ± 0.02 | 0.83 ± 0.08 | 0.51 ± 0.04 | 1.14 | 1.72 |
| SV371 | 0.21 ± 0.01 | 0.24 ± 0.02 | 0.75 ± 0.05 | 0.59 ± 0.05 | 1.26 | 1.62 |
| SV372 | 0.29 ± 0.02 | 0.33 ± 0.02 | 0.64 ± 0.04 | 0.48 ± 0.04 | 1.42 | 1.88 |

* Only one experiment performed for PH1. Values are means and standard errors of two or three independent experiments.

Table 3 Correlations of survival parameters with repair capacity using the Pearson correlation

| Initial slopes (all strains) | Residual slopes (all strains) | Ratio of slopes (all strains) | Ratio of slopes (SV strains only) | Ratio at 180 Gy (all strains) | Ratio at 180 Gy (SV strains only) |
|-----------------------------|-----------------------------|-----------------------------|---------------------------------|-------------------------------|---------------------------------|
| HDR SF<sub>2</sub> | r | P | r | P | r | P | r | P | r | P |
| 0.25 | 0.46 | -0.42 | 0.20 | 0.58<sup>a</sup> | 0.06 | 0.38 | 0.31 | 0.35 | 0.29 | -0.13 | 0.74 |
| LDR SF<sub>2</sub> | 0.13 | 0.71 | -0.55<sup>a</sup> | 0.08 | 0.41 | 0.21 | -0.14 | 0.72 | 0.45 | 0.17 | -0.15 | 0.69 |
| HDR | r | P | r | P | r | P | r | P | r | P |
| -0.39 | 0.24 | 0.53<sup>a</sup> | 0.10 | -0.74<sup>a</sup> | 0.01 | -0.76<sup>a</sup> | 0.02 | -0.58<sup>a</sup> | 0.06 | -0.28 | 0.47 |
| LDR | r | P | r | P | r | P | r | P | r | P |
| -0.40 | 0.22 | 0.52<sup>a</sup> | 0.10 | -0.64<sup>a</sup> | 0.03 | -0.68<sup>a</sup> | 0.04 | -0.62<sup>a</sup> | 0.04 | -0.40 | 0.29 |
| HDR D | 0.24 | 0.43 | -0.6<sup>a</sup> | 0.05 | 0.80<sup>a</sup> | 0.003 | 0.80<sup>a</sup> | 0.009 | 0.59<sup>a</sup> | 0.06 | 0.34 | 0.37 |
| LDR D | 0.30 | 0.38 | -0.63<sup>a</sup> | 0.04 | 0.68<sup>a</sup> | 0.02 | 0.60<sup>a</sup> | 0.09 | 0.67<sup>a</sup> | 0.03 | 0.48 | 0.19 |
| PFGE | -0.37 | 0.27 | 0.54<sup>a</sup> | 0.08 | -0.63<sup>a</sup> | 0.02 | -0.62<sup>a</sup> | 0.08 | -0.62<sup>a</sup> | 0.04 | -0.41 | 0.28 |

<sup>a</sup> Correlation is significant at the 0.10 level (two-tailed). <sup>b</sup> Correlation is significant at the 0.05 level (two-tailed). <sup>c</sup> Correlation is significant at the 0.01 level (two-tailed).

Correlations were then examined using the ratio of the absolute level of initial and residual DNA dsbs at a single dose point (180 Gy). No significant correlations were evident when the radiosensitive syndromic cell strains (AT1 and PH1) were excluded. However, when all 11 fibroblast strains were included, correlations of borderline significance were found (Table 3). The strongest correlation found was between the ratio of DNA dsb damage at 180 Gy and radiosensitivity (low-dose rate D) (r = 0.67 P = 0.03) is shown in Figure 5.

**DISCUSSION**

In this study, DNA dsb induction and repair were measured in nine normal and two radiosensitive fibroblast cell strains. To compare the data from all the 11 fibroblast cell strains, comet tail moment values for the individual cell strains required normalization against the unirradiated controls from within each experiment. This normalization procedure was necessary to accommodate the variation in comet-head size of unirradiated cells between the different fibroblast cell strains. The reason for this variation is unknown, but the rigid experimental protocols used for growing the cells and

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measuring DNA breaks would suggest it does not reflect experimental variability between the individual experiments (e.g. cell cycle difference, lysis conditions, PI staining intensity). Rather, it appears to be a trait of untransformed fibroblasts and may reflect the differentiation status (Rodemann and Bamberg, 1995) of each strain (e.g. differential sensitivity to the lysis conditions) because tail moment normalization for the above reason was not found to be necessary in a previous study in our laboratory measuring DNA damage in a range of cervical tumour cell lines (Marples et al., 1998).

The dose range used here was chosen after preliminary experiments (data not shown) had indicated that the tail moment values reached a plateau at doses > 240 Gy. This is not unexpected because the comet tail consists of broken strands of DNA attached to the nuclear matrix plus damaged stretched loops of DNA, and it is therefore finite in length (Fairbairn et al., 1995). Consequently, over the dose range used in this study, linear initial and residual DNA dsb dose–response curves were seen for all the cell strains, which is in agreement with other neutral comet assay studies in human cells (Olive et al., 1994; Marples et al., 1998). Studies measuring radiation-induced DNA damage using PFGE have reported non-linear initial (Wurm et al., 1994) and residual (Kiltie et al., 1997) dose responses in fibroblast strains. Analyses of data in the present study show that the majority of the repairable radiation-induced DNA dsb damage was indeed repaired during the 20 h incubation period.

The slopes of the residual DNA dsb dose–response curves for the nine normal fibroblasts varied by a factor of 8, four times that of the initial damage slopes (Table 1). A difference of borderline significance was seen in the level (slopes) of residual DNA dsbs between the 11 cell strains (P = 0.06). Moreover, discrimination was seen between the level of residual damage between the radiosensitive strains and normal strains (P = 0.01).

A significant correlation was seen between the extent of residual DNA dsbs measured after 20 h using the neutral comet assay and clonogenic radiosensitivity for all 11 fibroblast strains (r = –0.63, P = 0.04) (Figure 3). This is in agreement with studies that have shown correlations between cell kill and the extent of DNA dsb repair as assessed by PFGE in fibroblast strains at 4 and 24 h after
irradiation (Zhou et al, 1998; Wurm et al, 1994; Kiltie et al, 1997). In this comet study, stronger relationships were seen when the ratio of initial and residual DNA damage over 0–240 Gy dose range, reflecting the proportion of DNA dsbs unrepaird, was compared with a measure of clonogenic radiosensitivity for the nine normal and all 11 fibroblasts (Table 3, Figure 4). Clearly, these data indicate the potential of residual DNA dsbs and/or the ratio of initial/residual breaks, as measured by the neutral comet assay, as a method of predicting the radiosensitivity of fibroblasts. However, for a predictive assay of radiosensitivity to be adopted clinically, it must also be rapid and simple. We, therefore, compared the absolute level of DNA dsbs assessed at a single dose of 180 Gy with clonogenic radiosensitivity. However, significant correlations were found only when the data from all 11 fibroblasts were compared with clonogenicity, and non-significant trends were seen for the nine normal fibroblasts (Table 3, Figure 5).

For a novel rapid assay to be beneficial for predictive testing, a clear discrimination must be detected between radiosensitive (e.g. AT1 and PH1) and normal cell strains because clear differences are evident in the clonogenic data (Sproston et al, 1997; Kiltie et al, 1997). Statistically significant discrimination was detected only between the two radiosensitive strains and normal fibroblasts in this study when comparing the ratio of initial and residual damage slopes with \( D (P = 0.04) \), supporting the use of this ratio parameter in predictive testing of clonogenicity using the neutral comet assay.

A comparable study on the same fibroblast cell strains as used here, but assessing DNA dsbs using the PFGE assay, also found correlations between residual damage at 24 h and clonogenicity data (Kiltie et al, 1997). The PFGE assay correlations were stronger than those reported here, possibly indicating that the PFGE results give a better prediction of radiosensitivity than those from the neutral comet assay. In addition, strong significant correlation was also reported after a single dose of 150 Gy in the PFGE study, in contrast to the comet data reported here. In summary, this study has shown that there is a relationship between neutral comet assay results and clonogenic measurements of normal cell radiosensitivity. The ratio of initial and residual DNA dsb damage, which probably represents the fraction of DNA damage remaining after a period of repair, was found to be the best measure of radiation-induced DNA damage relating to cellular radiosensitivity.

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