

Chromatin structure and cellular radiosensitivity: a comparison of two human tumour cell lines

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Abstract. The role of variation in susceptibility to DNA damage induction was studied as a determinant for cellular radiosensitivity. Comparison of the radiosensitive HX142 and radioresistant RT112 cell lines previously revealed higher susceptibility to X-ray-induced DNA damage in the sensitive cell line using non-denaturing elution, but not when using alkaline unwinding. The present data also show that no difference in the amount of initial damage is seen when pulsed-field gel electrophoresis (PFGE) or comet analysis are used for DNA damage assessment. However, using the halo assay or a modified version of PFGE in which the higher DNA architecture remained partially intact, the radiosensitive cells showed steeper dose-response curves for initial DNA damage than the radioresistant cells. Analysis of the protein composition of DNA-nucleoid structures revealed substantial differences when isolated from HX142 or RT112 cells. From our data, it is concluded that HX142 and RT112 differ in their structural organization of chromatin. As no differences in the kinetics of DNA damage rejoining were found, it is hypothesized that the same amount of lesions have a different impact in the two cell lines in that the 'presentation' of DNA damage alters the ratio of repairable to non-repairable DNA damage.

1. Introduction

Variations in radiosensitivity amongst cell lines have often been related to differences in the capacity of cells to repair DNA damage caused by ionizing radiation (Frankenberg-Schwager 1989, Olive *et al.* 1994, Zdzienicka 1995). However, these and other investigators have often observed no difference in DNA damage rejoining among various tumour cell types, despite large differences in radiosensitivity (Smeets *et al.* 1993, Olive *et al.* 1994).

Variations in the susceptibility of DNA to damage by ionizing radiation have also been proposed as a determinant of cellular radiosensitivity. Using the non-denaturing elution technique

(NDE assay) several authors showed correlations between radiosensitivity and the amount of initial DNA damage detected (Radford 1986, McMillan *et al.* 1990). One of the explanations for the observed variations between cell lines in detected DNA damage may be differences in intracellular scavenger concentration. However, this is unlikely since it would require considerable differences in scavenger concentration in the close vicinity of the DNA (Ward 1990). Alternatively, variations in chromatin structure between cell lines have been proposed as being responsible for differences in the induction of DNA damage (Olive *et al.* 1986, Jorgensen *et al.* 1990, Ward, 1990, Ljungman, 1991, Schwartz *et al.* 1993).

Differences in chromatin structure have not only been related to differences in induction of DNA damage but also directly to differences in cellular radiosensitivity (Gordon *et al.* 1990, Lynch *et al.* 1991, Malyapa *et al.* 1994, 1996). Measuring light scattering from individual nucleoids within a flow cytometer, Gordon *et al.* (1990) and Lynch *et al.* (1991) both found a decreased condensation (increased relaxation) of supercoiled DNA from radiosensitive cells compared to radioresistant cells. Also a higher resistance to detergent-induced degradation of the DNA-protein matrix was found for the resistant cells (Gordon *et al.* 1990). Using the halo assay, Malyapa *et al.* (1994, 1996) found that the degree of inhibition of loop rewinding was greater in radiosensitive cells compared to resistant cells, which inversely correlated with the clonogenic survival of these cells after exposure to ionizing radiation. The latter results, however, are inconclusive in terms of whether differences in chromatin structure have led to an altered induction of lesions in DNA or whether these have caused an altered detectability of the same number of lesions in the DNA.

To investigate in more detail the role of chromatin structure as cause for differences in cellular radiosensitivity, a radiosensitive and a

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radioresistant human tumour cell line were used that showed variations in the rate of elution measured with the NDE that were related to their differences in radiosensitivity (Peacock *et al.* 1989, McMillan *et al.* 1990). The rate of elution is presumed to be proportional to the amount of DNA double-strand breaks induced; therefore, these data suggest that the difference in sensitivity to ionizing radiation between these two cell lines is caused by a dissimilar amount of initial DNA damage. However, when alkaline unwinding (measuring strand breaks and alkali-labile sites) was used, the same induction of DNA damage was detected in both cell lines (Woudstra *et al.* 1996). The same result was observed with the pulsed field gel electrophoresis assay, measuring double-strand breaks only (Woudstra *et al.* 1996). However, another study did reveal differences between these two cell lines using the same assay (Whitaker *et al.* 1995). Since it has been suggested that the double-strand break measurements by the NDE assay are strongly influenced by features of chromatin structure (Olive *et al.* 1992, 1994), we previously proposed that 'DNA damage presentation' is different for the two cell lines, affecting the detectability of DNA lesions in some assays (Woudstra *et al.* 1996), which can also be related to cellular radiosensitivity. This hypothesis was further explored in the current study in which irradiation of intact cells was followed by differential preparation of the DNA/chromatin. These fractions were subsequently analysed in different assays for the amount of detectable DNA lesions.

2. Materials and methods

2.1. Cell culturing procedures

Two human tumour cell lines were used in this study. HX142, a neuroblastoma cell line being more sensitive to ionizing radiation than RT112, a bladder carcinoma cell line (Peacock *et al.* 1989). Surviving fraction at 2 Gy (SF_2) for HX142 = 0.09 and for RT112 SF_2 = 0.70. Cells were exponentially grown at 37°C as a monolayer in Costar plastic flasks containing Ham's F12 medium supplemented with 10% foetal calf serum (all Life Technologies). Cell-cycle distribution was measured by flow cytometry and was similar for both cell lines (the percentage of cells in G₁/S/G₂M was 55/26/19%). Cells were incubated in an atmosphere of 3% O₂, 5% CO₂, and 92% N₂. All our standard laboratory chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA) or Merck (Darmstadt, Germany).

2.2. Irradiations

Cells were trypsinized, after which the trypsin was neutralized with medium. After centrifugation (5 min at 150 g), the cells were resuspended in fresh complete medium at 10⁶ cells/ml for the halo and PFGE assay or 5 × 10⁴ cells/ml for the comet assay. Irradiations were performed on ice using a Philips-Muller MG 300 X-ray machine, operating at 200 kV and 15 mA at a dose rate of 5 Gy/min. The dosimetry was performed with an ionization chamber (Philips 37489/10) calibrated with a ⁹⁰Sr source (Philips XL 2011/00).

2.3. Alkaline comet assay

Fresh complete medium (5 × 10⁴ cells/ml) were irradiated on ice. For analysis of DNA repair, cells were allowed to repair damage after irradiation by incubation at 37°C. Following irradiation and repair, cells were resuspended in ice-cold phosphate-buffered saline (PBS; pH 7.4). Starfrost microscope slides were pretreated with a thin layer of 0.5% low melting point (LMP) agarose (Bio-Rad) in PBS at 37°C. Of LMP agarose (1% in PBS) at 37°C, 1.5 ml was added to a tube containing 0.5 ml cold cell suspension and quickly pipetted onto the pretreated microscope slide. The slides were covered with a thin layer of 0.5% LMP agarose and then positioned on an ice-cold surface for 2 min.

The slides were placed in an ice-cold high salt/alkali lysing solution (1 M NaCl, 0.03 M NaOH, 0.1% sarkosyl) for 1 h at room temperature then rinsed in an ice-cold solution of 0.03 M NaOH and 1 mM EDTA (1 h at room temperature), before transfer to a horizontal gel electrophoresis apparatus. Slides were covered with 0.03 M NaOH and 1 mM EDTA and exposed to 1 V/cm for 20 min. Following electrophoresis, slides were rinsed in distilled water (20 min) and stained at room temperature with propidium iodide (2.5 µg/ml in H₂O) for 10 min in the dark. After staining, slides were kept in a humidified box to protect the gels from shrinkage until the slides were viewed and analysed within 24 h.

Individual cells were viewed using a fluorescence microscope (Olympus IMT-2) attached to a sensitive silicon-intensified tube (SIT-66) camera (DAGE, MTI Inc.) connected to a digital signal, remote-control processor (DSP 100: DAGE) and a CUE II image analysis system (Olympus). Slides were illuminated with green light from a 100-W mercury source.

Images were selected randomly (40–100 images for each dose/repair point). Overlapping comets were excluded from analysis. Gain- and black-level settings were held constant throughout the entire measurement.

Using the protocol of Olive and coworkers (1990), we found that tails appeared in non-irradiated samples and that only a weak radiation dose-dependency for tail formation was obtained (data not shown). By adding 0.1% sarkosyl to the lysis solution, tail formation in untreated samples was reduced to a minimum, whilst there was a good dose–response. Furthermore, more reproducible data were obtained when after cell lysis the slides were rinsed in NaOH/EDTA rather than in water. Finally, optimal electrophoresis conditions for comet analysis were found at 1 V/cm for 20 min.

For quantification, we used the analysis of Kent *et al.* (1995). In this analysis, the total comet moment is defined as the product of the amount of DNA at distance n and the distance migrated, summed for 100 intervals of n (where n is the distance from the centre of mass of the head, divided by the total amount of DNA). Thus for this type of analysis, identification of separate head and tail regions is not required.

2.4. Halo assay

DNA loop-size was measured using the fluorescent halo technique of Roti Roti and Wright (1987). Cells were trypsinized, neutralized, centrifuged for 5 min at 150 g and resuspended in PBS to a concentration of 10^6 cells/ml followed by irradiation on ice. Cell suspensions were diluted 10 times in PBS to yield 10^5 cells/ml. For repair studies, 10^6 cells/ml medium were irradiated on ice (12 Gy) followed by incubation at 37°C for 15 or 30 min. After repair cells were resuspended to a concentration of 10^5 cells/ml PBS. Cells (100 μ l) were lysed on ice on poly-L-lysine-pretreated, four-well Lab-Tek slides (Nunc) by dilution into the dye-lysis solution (1:1 in 2 M NaCl, 10 mM EDTA, 2 mM TRIS pH 8.0, 0.5% TRITON X-100 plus two times the desired propidium iodide [PI] concentration). After lysis the individual nucleoids were visualized as halos by illumination with green light from a 100-W mercury source and halo areas were measured by the image analysis system described above. Images (50–100) were analysed per PI point to obtain total halo areas and core areas. From these DNA loop sizes ($2 \times \text{radius}_{\text{total halo}} - \text{radius}_{\text{core}}$) were calculated.

2.5. Pulsed-field gel electrophoresis assay

The CHEF gel electrophoresis was used described by Blöcher *et al.* (1989) using the detection method described by Rosemann *et al.* (1993). About 2×10^7 cells/ml culture medium were mixed with an equal volume of 1% LMP agarose (Bio-Rad) in PBS at 37°C. Plugs were formed by pipetting 35 μ l of this mixture into a Bio-Rad plug mould. After 15 min gelation at 4°C, the plugs were placed in Eppendorf vials containing fresh medium and put on ice for irradiation. After irradiation, the embedded cells were processed either by standard SDS-proteinase K treatment (protocol A) or using a triton X-100/2M NaCl extraction procedure similar to that used for the halo assay (protocol B). Protocol A involves lysing the cells in a solution of 2% sarkosyl, 0.5 mg/ml proteinase K (Sigma, cat. no. P-0390) and 500 mM EDTA (pH 7.6) in PBS (2 h 0°C + 16 h 37°C). Next, the plugs are washed twice with PBS and treated for 1 h with RNase A (0.2 mg/ml in PBS; Sigma, cat. no. R-5000) at 37°C. Protocol B involves high salt lysis in 2 M NaCl, 10 mM EDTA, 2 mM tris pH 8.0, 0.5% triton X-100) for 2 h at 0°C.

Next, for both protocols, the plugs were washed twice with TBE (45 mM tris–base, 45 mM boric acid, 2 mM EDTA, pH 8.2) and inserted into the wells of a 0.5% agarose gel (Bio-Rad, Chromosomal Grade).

CHEF electrophoresis was carried out at 14°C using a Bio-Rad DR-II system in TBE buffer at 40 V for 25 h using a switching interval of 75 min. After staining with ethidium bromide, the fraction of DNA migrating from the plug into the lane (% DNA extracted; % DNA_{extr}) was measured using an UV-transilluminator (312 nm) and image analysis as previously described (Rosemann *et al.* 1993).

2.6. Nucleoid protein pattern

Nucleoids were prepared as previously described (Kampinga *et al.* 1989). Ten million cells in 7.5 ml PBS were 1:1 diluted with lysis buffer (2 M NaCl, 10 mM EDTA, 2 mM TRIS, pH 8.0, 0.5% TRITON X-100). After 15 min on ice the material was spun down for 20 min at 15,000 g. The resulting pellet was resuspended in 50 μ l TNMP (10 mM tris-HCl, pH 7.4, 10 mM NaCl, 5 mM MgCl₂, 0.1 mM phenylmethylsulphonyl fluoride [PMSF]) and digested with DNase I (stock: 5 mg/ml; 12.5 μ g/10⁶ cells) for 15 min at 37°C. Samples were diluted 1:1 with SDS-gel loading buffer (2 \times) after which 15 μ l of

the samples were loaded on a 10% SDS-polyacrylamide gel. After electrophoresis at 12 mA overnight, the gels were fixed for 1 h (MeOH/acetic acid/H₂O [5:1:4 v/v]), stained with coomassie brilliant blue (0.25 g coomassie brilliant blue; 0.25 g CuSO₄; 50 ml acetic acid; 62.5 ml MeOH; 137.5 ml H₂O) for 1 h and destained in MeOH/H₂O (1:4) for several hours.

3. Results

Neutral elution studies had revealed that the radiosensitive HX142 cells showed higher elution rates after irradiation compared to the radioresistant RT112 cells (McMillan *et al.* 1990). Such differences were interpreted to suggest that HX142 cells suffered more initial DNA damage than RT112 cells for a given dose of X-rays. However using PFGE as well as alkaline unwinding we were unable to detect any differences in initial DNA damage between these two cell lines (Woudstra *et al.* 1996). To obtain a better insight in these apparent controversies we therefore pursued these studies using two other methods for DNA damage detection.

3.1. Comet studies

In the tumour cell lines used in our study, a slightly modified protocol described by Olive *et al.* (1990) was used as described in §2. Damage after doses as low as 2 Gy was detectable and a linear increase in comet moment was observed up to 15 Gy for both cell lines (Figure 1a). It is clear from Figure 1a that there are no detectable differences in the amounts of radiation-induced damage: although the curve for HX142 lies consistently above the curve for RT112, the slopes of the induction curves were not found to differ significantly (4.76 ± 0.44 for HX142 versus 4.80 ± 0.20 u/Gy for RT112).

When cells were reincubated at 37°C after 5 Gy of X-irradiation and before comet analysis, 70–80% of the damage was found to be repaired within 30 min (Figure 1b). Repair kinetics were indistinguishable for HX142 and RT112 cells. Similar data were obtained for 10 Gy of X-irradiation (data not shown). There was no evidence for radioresistant or sensitive subpopulations (data not shown).

These data are in agreement with our PFGE and alkaline unwinding data (Woudstra *et al.* 1996),

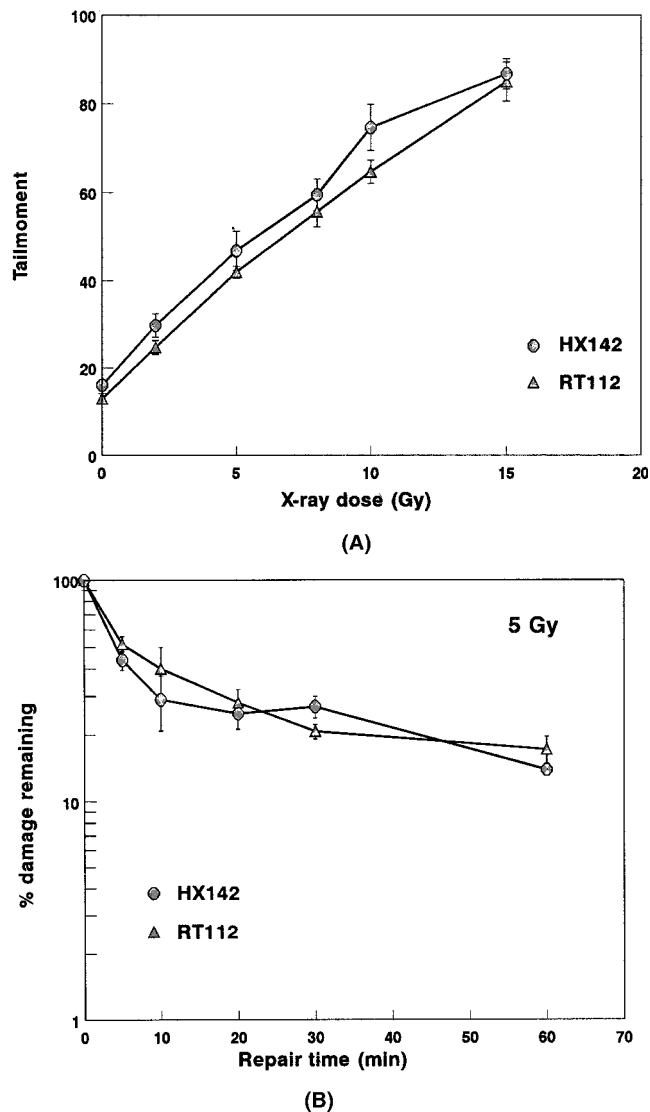


Figure 1. DNA damage induction and repair as measured with the comet assay in HX142 and RT112 cells. RT112 (triangles) and HX142 (circles) were irradiated with graded doses of X-rays and comets were prepared as described in §2. Mean \pm SEM of at least three independent experiments are given. In each experiment, 50–100 comets were analysed per dose point. (A) Images were analysed and comet moment analysis was performed immediately after irradiation. The slopes for the induction curves are: HX142 4.76 ± 0.44 ; and RT112 4.80 ± 0.20 u/Gy. (B) Images were analysed and comet moment analysis was performed after 5 Gy X-rays followed by 0–60 min at 37°C. The percentage of DNA damage remaining (as tail moment) is expressed relative to the percentage damage without repair (100%) after subtraction of the unirradiated control level and plotted versus the repair time (min).

but again apparently contrasting to observations with the non-denaturing elution technique, where more DNA damage was detected in the sensitive cell line HX142 (McMillan *et al.* 1990).

3.2. Halo studies

The halo assay, as developed by Roti Roti and Wright (1987), is based on the ability of the intercalating dye (propidium iodide) to cause dose-dependent unwinding and rewinding of DNA loops attached to a residual nuclear protein network, often referred to as the nuclear matrix. In eukaryotic cells DNA is packed via nucleosomes and solenoids in supercoiled loops attached to the nuclear protein matrix (Berezney 1991, Jackson *et al.* 1992). Using triton X-100/high salt extraction procedures and adding the intercalating dye propidium iodide, the unwound DNA supercoiled loops extruding from the nuclear matrix become visible as a fluorescent halo (Vogelstein *et al.* 1980, Roti Roti and Wright 1987). Figure 2A and B shows that increasing PI concentrations (causing the DNA loops to unwind) lead to larger halo sizes up to 5 µg PI/ml, when the nucleoids of both cell lines showed maximum loop sizes (relaxation point). Above 5 µg PI/ml the loop sizes of both cell lines decreased due to the rewinding of the DNA loops. Figure 2 reveals, moreover, that HX142 cells have larger loop sizes compared to RT112 cells.

Radiation-induced DNA strand breaks were shown to interfere with DNA rewinding in a dose-dependent manner (Roti Roti and Wright 1987, Kampinga *et al.* 1989). For each dose of radiation (3–12 Gy; Figure 2A and B) loss of rewinding was more extensive in HX142 cells than in RT112 cells. To quantify this effect, the parameter of excess halo size (calculated by adding up the halo areas of non-irradiated cells of the PI concentrations >5 µg/ml and subtracting this number from the overall halo area >5 µg PI/ml of the irradiated cells) was used (Roti Roti and Wright 1987). Plotting this parameter as a function of the radiation dose reveals that the slope of the induction curve is steeper for HX142 cells (143.2 ± 15.2 than for RT112 cells ($85.9 \pm 7.9 \mu\text{m}^2/\text{Gy}$), $p < 0.0001$) (Figure 2C).

Following repair of the DNA damage after irradiation of 12 Gy of X-rays (30-min incubation at 37°C), roughly 10% of the initial damage remained in both cell lines (Figure 2D). No differences could be observed in the repair kinetics of the two cell lines by means of this assay.

3.3. PFGE studies: effect of cell lysis conditions

Thus far three assays (PFGE, alkaline unwinding [Woudstra *et al.* 1996] and comet [this study]) do

not reveal differences between HX142 and RT112 cells in the amount of DNA damage detected immediately after ionizing radiation and two assays (non-denaturing elution [McMillan *et al.* 1990] and halo [this study]) do show a difference. Both the non-denaturing elution and the halo assay have been shown to be substantially influenced by residual chromatin structure after cell lysis (Kampinga *et al.* 1989, Olive *et al.* 1992, 1994). It can be speculated that detected differences in some assays in the induction of DNA damage between the two cell lines are due to chromatin structure differences between the cell lines.

To investigate this, we modified the cell lysis procedure for the PFGE assay and compared two lysis conditions. Cells were irradiated followed by lysis with either conventional PFGE lysis procedures (sarkosyl-proteinase K; protocol A) or with the halo lysis procedure (triton X-100, 2 M NaCl, 2 h 0°C; protocol B), leaving residual DNA-matrix structures intact (Figure 3). In contrast to the conventional PFGE lysis (insert) showing identical slopes for DNA damage induction, the high salt lysis revealed a higher percentage of DNA extracted from the radiosensitive HX142 cells compared to the RT112 cells. The data obtained with this lysis method had a large scattering in the absolute percentage of DNA extracted as only a minor fraction (<2%) of the DNA was extracted at doses as high as 100 Gy†. However, all individual experiments revealed a higher percentage of DNA extracted for HX142 compared to RT112 cells. To be able to compare the different experiments, the percentage DNA extracted after 400 Gy X-rays (HX142 cells) in every test was adjusted to 100% and the data were plotted relative to the 400 Gy data. The slopes of the induction curves are significantly different (0.24 ± 0.03 u/Gy for HX142 and 0.08 ± 0.01 u/Gy for RT112, $p < 0.01$).

These data again indicate that the cell lines might be different in their chromatin structures and that these structure differences affect the detectability of the damage.

†Based on the assumptions that under our conditions DNA-matrix-loop interactions remain completely intact and that there are indeed about 100,000 loops per cell with a mean loop size of 100 kb (as suggested in the literature), one can calculate that in order to release 1% of DNA (1000 of such loops, each loop requiring two double-strand breaks for its release) 2000 double-strand breaks are needed. As the assumed double-strand break frequency is around 20/Gy/cell, this means 100 Gy is needed to release only 1% DNA.

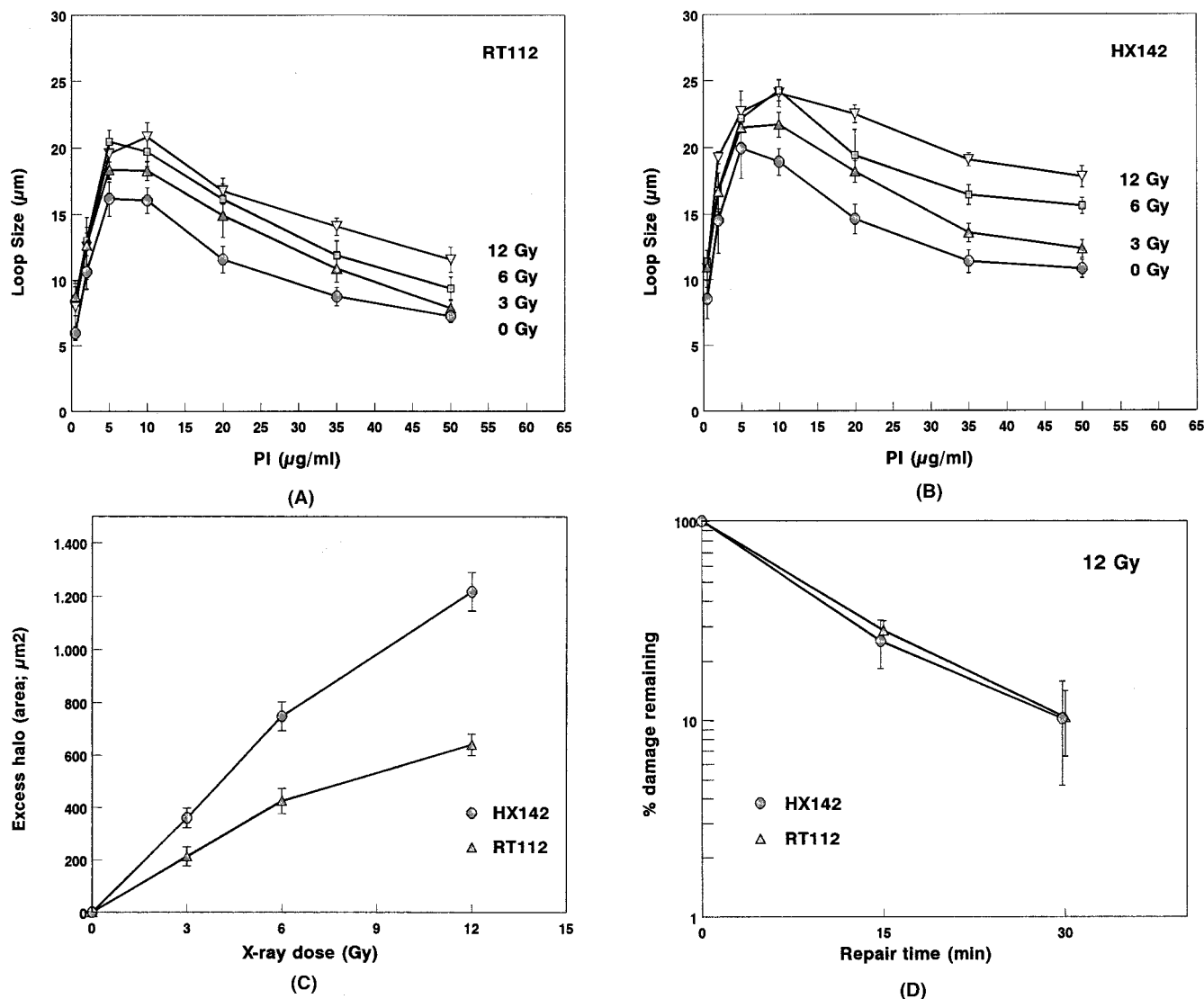


Figure 2. Full PI, DNA damage induction and DNA repair curves from HX142 and RT112 nucleoids as measured with the halo assay. RT112 (triangles) and HX142 (circles) cells were lysed in dye-lysis solution. Individual nucleoids were visualized as halos and measured by image analysis. Fifty to 100 images were analysed per PI point to obtain total halo areas and core areas. Halo sizes (halo radius) and DNA loop sizes ($2 \times (\text{radius}_{\text{total halo}} - \text{radius}_{\text{core}})$) were calculated from the halo areas. Mean \pm SEM of at least three independent experiments are given. (A) Full PI curves for RT112 cells irradiated with increasing dose (0–12 Gy) of X-rays. Halo sizes are plotted as a function of propidium iodide (PI) concentration. (B) The same as (A) for HX142 cells. (C) Excess halo area of both cell lines plotted against increasing dose (0–12 Gy) of X-rays. The initial slopes for the induction curves are: HX142 143.2 ± 15.2 ; and RT112 $85.9 \pm 7.9 \mu\text{m}^2/\text{Gy}$. (D) DNA damage remaining after HX142 and RT112 cells were irradiated with 12 Gy and allowed for 15 and 30-min repair at 37°C . The percentage of DNA damage remaining (as excess halo size) is expressed relative to the percentage damage without repair (100%) and plotted versus the repair time (min).

3.4. Nucleoid protein isolation

The DNA is bound to the nuclear protein matrix via as yet unknown proteins. One possible explanation for the differences in chromatin structures between the two cell lines could be a variation in the stability of matrix-proteins–DNA interactions. Therefore the protein compositions of the halos (nucleoids) of both human tumour cell lines were

compared using protocols previously described (Kampinga *et al.* 1989). The nucleoid proteins were isolated by lysing the cells with the same high salt lysis buffer as used in the halo assay and the ‘modified’ PFGE, which showed differences in DNA damage induction between the two cell lines.

Figure 4 shows the coomassie brilliant blue staining of the protein gel. A difference in the pattern of proteins was seen between the nucleoids

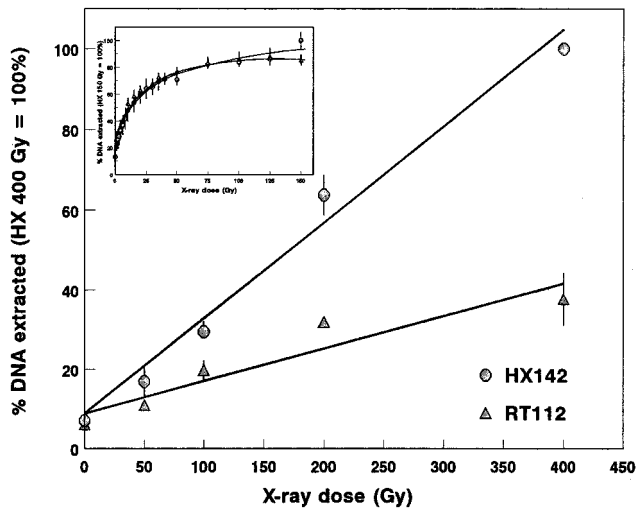


Figure 3. Effect of lysis procedures on the dose-response curve for initial radiation-induced DNA damage for HX142 and RT112 cells measured with the PFGE assay. RT112 (triangles) and HX142 (circles) cells were irradiated *in situ* (0–400 Gy). Subsequently they were processed by conventional lysis/DNA isolation procedures (insert) or treated with triton X-100, 2M NaCl for 2 h to preserve the DNA-loop-matrix structure. Hereafter, the percentage of extracted DNA was analysed as previously described (Rosemann *et al.* 1993, Woudstra *et al.* 1996). Data are the mean \pm SEM of 4–6 independent experiments. For the triton X-100/high salt method, the % extracted DNA was quite low even after 100 Gy (<1–2%) resulting in a rather large scatter between individual experiments for the absolute fraction DNA extracted. This low fraction of extracted DNA is consistent with the induction of 20 double-strand breaks/Gy in each cell containing a mean total number of \pm 100,000 loops of about 100 kb each. For reasons of comparison we plotted therefore the data relative to the percentage DNA extracted from HX142 cells after 400 Gy (= 100%). To be consistent, the same was done for the conventional method in which the data were plotted relative to the % extracted after 150 Gy in HX142 cells (data re-analysed from Woudstra *et al.* 1996).

isolated from the two cell lines. Several protein bands found in nucleoids isolated from RT112 cells were not found in those isolated from HX142 cells; these include proteins with a molecular weight of 61, 58, 52 and 38 kD. There was no change in the overall pattern of proteins of nucleoids from HX142 and RT112 cells following irradiation. No obvious differences were seen in the overall protein composition of whole cell lysates of the two cell lines (data not shown).

4. Discussion

4.1. DNA damage induction

Comparison of the current data with those

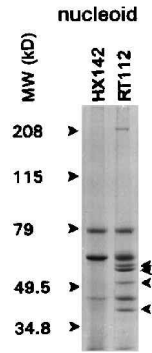


Figure 4. Protein composition of high salt-insoluble nucleoid structures isolated from HX142 and RT112. Nucleoids were isolated according to §2 and nucleoid proteins were analysed by electrophoresis through a 10% SDS-polyacrylamide gel. Arrows indicate major protein bands present in halos prepared from RT112 but absent in those isolated from HX142. The indicated numbers represent molecular weight markers.

previously published on initial DNA damage reveals that the alkaline unwinding, PFGE and comet showed no variations in dose-response between the RT112 and HX142 cells, whereas the NDE, halo and modified PFGE did show a significant difference in the dose-response curves for initial damage (Table 1).

These combined data, together with the observation that HX142 cells lack several nucleoid proteins compared to RT112 cells (Figure 4), led us to propose that not the number of double-strand breaks per Gy per dalton DNA but rather the ‘presentation’ of damage differs between the two cell lines. The following arguments further support this hypothesis. In the halo assay, DNA damage is measured in a residual DNA structure (loops attached to a protein matrix). The protein composition of this structure significantly differed between HX142 cells and RT112 cells. Work of others also has revealed that for cell lines with different DNA damage induction curves detected with the halo assay, a nucleoid protein pattern was observed in which certain proteins seemed missing in the radiosensitive cell nucleoids (Malyapa *et al.* 1994, 1996). Furthermore, when nuclear structures were preserved in another assay (PFGE) a steeper DNA damage induction curve was found for the radiosensitive HX142 cells, while the percentages DNA extracted remained the same when the conventional (more vigorous) lysis procedure was used in which chromatin structure is not preserved (Figure 3). So, the extent of cell lysis may have drastic impact on the outcome of the PFGE analysis and may (in part) explain why earlier investigations using the same two cell lines did

Table 1. Sensitivity of HX142 and RT112 to induction of DNA damage by radiation: a comparison of several assays. Indicated are the surviving fractions after 2 Gy X-rays (SF_2), the slopes of induction curves (in relative retention/Gy for NDE; % double-stranded DNA/Gy for alkaline unwinding (AU); tailmoment/Gy for comet; % DNA extracted/Gy for PFGE and PFGE (high salt) and the initial slopes of induction curves (in μm^2 excess halo area/Gy for halo, using $Y = a + bX + cX^2$).

Assay	HX142 (sensitive)	RT112 (resistant)	<i>p</i>
SF_2 ^[2]	0.1 ± 0.03	0.7 ± 0.1	<i>p</i> < 0.05
NDE ^[1]	8.5 ± 0.7	4.4 ± 0.4	<i>p</i> < 0.05
AU ^[2]	-0.058 ± 0.018	-0.061 ± 0.009	n.s.
Comet ^[3]	4.76 ± 0.44	4.8 ± 0.2	n.s.
PFGE ^[2]	1.6 ± 0.2	1.6 ± 0.2	n.s.
PFGE (high salt) ^[3]	0.24 ± 0.03	0.08 ± 0.01	<i>p</i> < 0.01
Halo ^[3]	143.2 ± 15.2	85.9 ± 7.9	<i>p</i> < 0.0001

[1] McMillan *et al.* (1990); [2] Woudstra *et al.* (1996); [3] this study.

reveal differences in initial DNA damage (Whitaker *et al.* 1995), while we did not (Woudstra *et al.* 1996, this study).

Our results should not be confused with those data showing that chromatin proteins protect against the induction of lesions, as, for example, found by step-wise removal of these proteins before irradiation of these remaining structures (Ljungman 1991, Warters and Lyons 1992, Oleinick *et al.* 1994). In our experiments cells were irradiated *in situ* and the amount of lesions induced in the DNA (with most [all] proteins removed: PFGE, AU, comet) was found to be the same.

4.2. Possible causes for the fragile chromatin structure of HX142

The halo assay revealed a greater inhibition of DNA loop rewinding in the radiosensitive cell line after the cells were treated with ionizing radiation. This loss of DNA supercoiling ability could be due to differences in the anchorage of the DNA loops to the nuclear matrix. In case of unstable DNA-matrix protein interactions, loss of superhelical density may not be confined to the radiation damaged loop only, but may affect neighbouring loops as well. So, the *same* amount of DNA damage may lead to a *different* 'presentation' of the damage, which is measurable only in some assays (NDE, halo) which are sensitive to DNA structure. Similar hypothetical models have been proposed before (Olive 1992, Schwartz and Vaughan 1993, Malyapa *et al.* 1994, 1996).

The proteins involved in DNA-matrix interactions and responsible for confinement of supercoiling within one loop domain are yet unidentified (Baskin 1995). Only a few proteins like topoisomerase II (Adachi *et al.* 1989), attachment region binding protein (ARBP; von Kries *et al.* 1991), lamin B1 (Ludérus *et al.* 1992), and the

120-kD protein SP120 (Tsutsui *et al.* 1993) have been proposed but not proven to play a role in loop-matrix interaction. Moreover, it is as yet unclear whether, upon DNA strand breakage, additional proteins are recruited for possible stabilization of the damaged loop. It is hard to determine, therefore, what may have caused the instability of such interactions after DNA strand breakage in HX142 cells. Various proteins that remained in the triton X-100/high salt isolated nucleoids from RT112 cells were absent in those isolated from HX142 cells. It is tempting to speculate that (one of) these proteins are the cause for differences in stability of the nuclear architecture of HX142.

In preliminary experiments, we focused on the ~38 kD protein that was absent in HX142 nucleoids. Recently a ~37 kD protein was identified as a nuclear matrix protein involved in binding DNA via Cu^{++} (Oleinick, personal communication). A likely candidate for this ~38 kD protein could have been the ~37 kD hRad51, the human homologue of *E. coli* RecA and yeast Rad51, involved in homologous recombination (Benson *et al.* 1994). However, hRad51 was not found in nucleoids isolated from either HX142 or RT112 cells (data not shown). Cellular extracts of both cell lines did contain the protein and it seems to be functioning normally in both our cell lines as its amount was found to increase with time after irradiation and as it appeared in distinct foci in nuclei of both irradiated HX142 and RT112 cells (data not shown). So, unfortunately the identity of the proteins lacking in nucleoids from HX142 cells and possible causes for the more fragile chromatin structure of HX142 cells as yet remain obscure.

4.3. Damage presentation and radiosensitivity

As with all assays used so far no differences in

repair rates nor in the amounts of residual DNA strand breaks could be detected (Woudstra *et al.* 1996, this study). Preliminary data further revealed that hyperthermia can cause substantial radiosensitization in HX142 cells (Woudstra *et al.* unpublished data). Also, repair fidelity as revealed by the plasmid reconstitution assay was not significantly different for HX142 and RT112 cells (Powell and McMillan 1994). At the cellular level, previous data already showed proficient split-dose recovery in HX142, also suggesting no major repair defect (Peacock *et al.* 1989). Finally, the pattern of chromosomal aberrations seen in HX142 is distinct from that seen in a clear double-strand break repair deficient cell like *xrs-5* (Jones *et al.* 1994) indicating that the radiosensitivity of HX142 is not related to double-strand break rejoining. The question remains, therefore, how putative differences in chromatin structure and in 'presentation' of the damage can lead to differences in cellular radiosensitivity.

Here, we like to hypothesize that the ratio between lethal lesions (irreparable) and potentially lethal lesions (repairable) is elevated in HX142 cells. When comparing the number of initial DNA double-strand breaks/Gy/cell with the number of initial breaks found with the PCC technique, a mean ratio of far above 1 has been observed (Bedford 1991 for a review). This indicates that, without (biochemical) DNA repair, only few double-strand breaks give rise to a (lethal) chromosome break. Apparently, the chromatin structure around the majority of the double-strand breaks is such that they do not appear as a break in the PCC technique. The latter double-strand breaks might be those that are potentially repairable.

Support for such a hypothesis may come from data obtained with the radiosensitive ataxia-telangiectasia (A-T) cells. These cells also show no differences in the number of initial double-strand breaks (Pandita and Hittelman 1992, Hittelman and Pandita 1994, Wurm *et al.* 1994; our unpublished data). Yet, the amount of initial damage detected by the halo assay is higher in A-T fibroblasts than in normal human fibroblasts (Taylor *et al.* 1991). Intriguingly, also the number of initial PCC fragments for a given dose is higher in A-T cells than in normal fibroblasts (Hittelman and Pandita 1994) and inadequate 'stabilization' of DNA damage was proposed as a tentative cause for these findings (Thacker 1994). So far, accurate assessment of PCCs in RT112 and HX142 has been unsuccessful due to technical problems. Yet, it is speculated that the ratio of double-strand breaks to PCC fragments may be higher

in HX142 cells due to their fragile chromatin structure.

In summary, X-irradiation leads to the induction of an equal number of strand breaks per Gy per dalton DNA in the radiosensitive HX142 and radioresistant RT112 cells. HX142 cells seem to have a more fragile chromatin structure compared to RT112 cells, leading to an altered 'presentation' (and detectability) of the DNA lesions. This fragility might be due to a lack of functional nuclear matrix proteins involved in DNA attachment. The higher fragility of the nuclear structure does not affect DNA repair rates nor fidelity, but might 'convert' potentially repairable strand breaks into non-repairable (lethal) breaks, leading to the higher cellular radiosensitivity of HX142 cells.

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References

- ADACHI, Y., KÁS, E. and LAEMMLI, U. K., 1989, Preferential, cooperative binding of DNA topoisomerase II to scaffold-associated regions. *EMBO Journal*, **8**, 3997-4006.
- BASKIN, Y., 1995, Mapping the cell's nucleus. *Science*, **268**, 1564-1565.
- BEDFORD, J. S., 1991, Sublethal damage, potentially lethal damage, and chromosomal aberrations in mammalian cells exposed to ionizing radiations. *International Journal of Radiation Oncology, Biology, Physics*, **21**, 1457-1469.
- BENSON, F. E., STASIAK, A. and WEST, S. C., 1994, Purification and characterization of the human Rad51 protein, an analogue of *E. coli* RecA. *EMBO Journal*, **13**, 5764-5771.
- BEREZNEY, R., 1991, The nuclear matrix: a heuristic model for investigating genomic organization and function in the cell nucleus. *Journal of Cellular Biochemistry*, **47**, 109-123.
- BLÖCHER, D., EINSPENNER, M. and ZAJACKOWSKI, J., 1989, CHEF electrophoresis, a sensitive technique for the determination of DNA double strand breaks. *International Journal of Radiation Biology*, **56**, 437-448.
- FLENTJE, M., ASADPOUR, B., LATZ, D. and WEBER, K. J., 1993,

- Sensitivity of neutral filter elution but not PFGE can be modified by non-dsb chromatin damage. *International Journal of Radiation Biology*, **63**, 715–724.
- FRANKENBERG-SCHWAGER, M., 1989, Review of repair kinetics for DNA damage induced in eukaryotic cells in vitro by ionizing radiation. *Radiotherapy and Oncology*, **14**, 307–320.
- GORDON, D. J., MILNER, A. E., BEANY, R. P., GRDINA, D. J. and VAUGHAN, A. T. M., 1990, The increase in radioresistance of Chinese hamster cells cultured as spheroids is correlated to changes in nuclear morphology. *Radiation Research*, **121**, 175–179.
- HITTELMAN, W. N. and PANDITA, T. K., 1994, Possible role of chromatin alteration in the radiosensitivity of ataxia-telangiectasia. *International Journal of Radiation Biology*, **66**, S109–113.
- JACKSON, D. A., DOLLE, A., ROBERTSON, G. and COOK, P. R., 1992, The attachments of chromatin loops to the nucleoskeleton. *Cell Biology International Reports*, **16**, 687–696.
- JONES, L. A., CLEGG, S., BUSH, C., MCMILLAN, T. J. and PEACOCK, J. H., 1994, Relationship between chromosome aberrations, micronuclei and cell kill in two human tumour cell lines of widely differing radiosensitivity. *International Journal of Radiation Biology*, **66**, 639–642.
- JORGENSEN, T. J., PRASAD, S. C., BRENNAN, T. P. and DRITSCHILO, A., 1990, Constraints to DNA unwinding near radiation-induced strand breaks in Ewing's sarcoma cells. *Radiation Research*, **123**, 320–324.
- KAMPINGA, H. H., WRIGHT, W. D., KONINGS, A. W. T. and ROTI ROTI, J. L., 1989, Changes in the structure of nucleoids isolated from heat-shocked HeLa cells. *International Journal of Radiation Biology*, **56**, 369–382.
- KENT, C. R. H., EADY, J. J., ROSS, G. M. and STEEL, G. G., 1995, The comet moment as a measure of DNA damage in the comet assay. *International Journal of Radiation Biology*, **67**, 655–660.
- LJUNGMAN, M., 1991, The influence of chromatin structure on the frequency of radiation-induced DNA strand breaks: a study using nuclear and nucleoid monolayers. *Radiation Research*, **126**, 58–64.
- LUDÉRUS, M. A. E., DE GRAAF, A., MATTIA, E., DEN BLAAUWEN, J. L., GRANDE, M. A., DE JONG, L. and VAN DRIEL, R., 1992, Binding of matrix attachment regions to lamin B1. *Cell*, **70**, 949–959.
- LYNCH, T. H., ANDERSON, P., WALLACE, D. M. A., KONDRATOWICZ, G. M., BEANEY, R. P. and VAUGHAN, A. T. M., 1991, A correlation between nuclear supercoiling and the response of patients with bladder cancer to radiotherapy. *British Journal of Cancer*, **64**, 867–871.
- MALYAPA, R. S., WRIGHT, W. D. and ROTI ROTI, J. L., 1994, Radiation sensitivity correlates with changes in DNA supercoiling and nucleoid protein content in cells of three Chinese hamster cell lines. *Radiation Research*, **140**, 312–320. Erratum in *Radiation Research*, 1995, **141**, 233.
- MALYAPA, R. S., WRIGHT, W. D. and ROTI ROTI, J. L., 1996, DNA supercoiling changes and nucleoid protein composition in a group of L5178Y cells of varying radiosensitivity. *Radiation Research*, **145**, 239–242.
- MCMILLAN, T. J., CASSONI, A. M., EDWARDS, S., HOLMES, A. and PEACOCK, J. H., 1990, The relationship of DNA double-strand break induction to radiosensitivity in human tumour cell lines. *International Journal of Radiation Biology*, **58**, 427–438.
- OLEINICK, N. L., BALASUBRAMANIAM, U., XUE, L. and CHIU, S., 1994, Nuclear structure and the microdistribution of radiation damage in DNA. *International Journal of Radiation Biology*, **66**, 523–529.
- OLIVE, P. L., 1992, DNA organization affects cellular radiosensitivity and detection of initial DNA strand breaks. *International Journal of Radiation Biology*, **62**, 389–396.
- OLIVE, P. L., BAN...TH, J. P. and DURAND, R. E., 1990, Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the 'Comet' assay. *Radiation Research*, **122**, 86–94.
- OLIVE, P. L., BAN...TH, J. P. and MACPHAIL, H. S., 1994, Lack of a correlation between radiosensitivity and DNA double-strand break induction or rejoining in six human tumor cell lines. *Cancer Research*, **54**, 3939–3946.
- OLIVE, P. L., HILTON, J. and DURAND, R. E., 1986, DNA conformation of Chinese hamster V79 cells and sensitivity to ionizing radiation. *Radiation Research*, **107**, 115–124.
- PANDITA, T. K. and HITTELMAN, W. N., 1992, Initial chromosome damage but not DNA damage is greater in ataxia telangiectasia cells. *Radiation Research*, **130**, 94–103.
- PEACOCK, J. H., EADY, J. J., EDWARDS, S., HOLMES, A., MCMILLAN, T. J. and STEEL, G. G., 1989, Initial DNA damage or repair as the major determinant of cellular radiosensitivity? *International Journal of Radiation Biology*, **56**, 543–547.
- POWELL, S. N. and MCMILLAN, T. J., 1994, The repair fidelity of restriction enzyme-induced double strand breaks in plasmid DNA correlates with radioresistance in human tumor cell lines. *International Journal of Radiation Oncology, Biology, Physics*, **29**, 1035–1040.
- RADFORD, I. R., 1986, Evidence for a general relationship between the induced level of DNA double-strand breakage and cell-killing after X-irradiation of mammalian cells. *International Journal of Radiation Biology*, **49**, 611–620.
- ROSEMANN, M., KANON, B., KONINGS, A. W. T. and KAMPINGA, H. H., 1993, An image analysis technique for the detection of radiation-induced DNA fragmentation after CHEF electrophoresis. *International Journal of Radiation Biology*, **64**, 245–249.
- ROTI ROTI, J. L. and WRIGHT, W. D., 1987, Visualization of DNA loops in nucleoids from HeLa cells: assays for DNA damage and repair. *Cytometry*, **8**, 461–467.
- SCHWARTZ, J. L., COWEN, J. M., MOAN, E., SEDITA, B. A., STEPHENS, J. and VAUGHAN, A. T. M., 1993, Metaphase chromosome and nucleoid differences between CHO-K1 and its radiosensitive xrs-5. *Mutagenesis*, **8**, 105–108.
- SCHWARTZ, J. L. and VAUGHAN, A. T. M., 1993, DNA–nuclear matrix interactions and ionizing radiation sensitivity. *Environmental and Molecular Mutagenesis*, **22**, 231–233.
- SMEETS, M. F. M., MOOREN, E. H. M. and BEGG, A. C., 1993, Radiation-induced DNA damage and repair in radio-sensitive and radioresistant human tumour cells measured by field inversion gel electrophoresis. *International Journal of Radiation Biology*, **63**, 703–713.
- TAYLOR, Y. C., DUNCAN, P. G., ZHANG, X. and WRIGHT, W. D., 1991, Differences in the DNA supercoiling response of irradiated cell lines from ataxia-telangiectasia versus unaffected individuals. *International Journal of Radiation Biology*, **59**, 359–371.
- THACKER, J., 1994, Cellular radiosensitivity in ataxia-telangiectasia. *International Journal of Radiation Biology*, **66**, S87–96.

- TSUTSUI, K., TSUTSUI, K., OKADA, S., WATARAI, S., SEKI, S., YASUDA, T. and SHOHMORI, T., 1993, Identification and characterization of a nuclear scaffold protein that binds the matrix attachment region DNA. *Journal of Biological Chemistry*, **268**, 12886–12894.
- VOGELSTEIN, B., PARDOLL, D. M. and COFFEY, D. S., 1980, Supercoiled loops and eucaryotic DNA replication. *Cell*, **22**, 79–85.
- VON KRIES, J. P., BUHRMESTER, H. and STRÄTLING, W. H., 1991, A matrix/scaffold attachment region binding protein: identification, purification, and mode of binding. *Cell*, **64**, 123–135.
- WARD, J. F., 1990, The yield of DNA double-strand breaks produced intracellularly by ionizing radiation: a review. *International Journal of Radiation Biology*, **57**, 1141–1150.
- WARTERS, R. L. and LYONS, B. W., 1992, Variation in radiation-induced formation of DNA double-strand breaks as a function of chromatin structure. *Radiation Research*, **130**, 309–318.
- WHITAKER, S. J., UNG, Y. C. and MCMILLAN, T. J., 1995, DNA double-strand break induction and rejoining as determinants of human tumour cell radiosensitivity. A pulsed-field gel electrophoresis study. *International Journal of Radiation Biology*, **67**, 7–18.
- WOUDSTRA, E. C., BRUNSTING, J. F., ROESINK, J. M., KONINGS, A. W. T. and KAMPINGA, H. H., 1996, Radiation induced DNA damage and damage repair in three human tumour cell lines. *Mutation Research*, **362**, 51–59.
- WURM, R., BURNET, N. G., DUGGAL, N., YARNOLD, J. R. and PEACOCK, J. H., 1994, Cellular radiosensitivity and DNA damage in primary human fibroblasts. *International Journal of Radiation Oncology, Biology, Physics*, **30**, 625–633.
- ZDZIENICKA, M. Z., 1995, Mammalian mutants defective in the response to ionizing radiation-induced DNA damage. *Mutation Research*, **336**, 203–213.