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Summary Recent studies have suggested a correlation between the rate and incidence of apoptosis and the radiation response of particular cell lines. However, we found that increasing the rate of induction of apoptosis in the fibroblast line 208F, by transfecting it with human c-myc, did not lead to a change in its clonogenic survival dose—response for either γ-irradiation or 121I-induced DNA damage. It was also found that expression of mutant (T24) Ha-ras in the 208F line appeared to decrease the level of apoptosis per mitosis after irradiation and inhibited the formation of nucleosomal ladders, but did not affect either the onset of the morphological features of apoptosis or the clonogenic survival dose—response of the cells to either γ-irradiation or 121I-induced DNA damage. Our findings suggest that it may be incorrect to make predictions about the radiosensitivity of cells based only on knowledge of their mode of death.

Keywords: apoptosis; fibroblasts; radiosensitivity; c-myc; ras; endonuclease

The sensitivity of both normal and transformed cell types to killing by ionising radiation can differ markedly. For example, haemopoietic cell types are often radiation sensitive, while hepatocytes are relatively radiation resistant (Hendry, 1985). Similarly, lymphomas are typically responsive to irradiation, while melanomas often respond poorly (Steel, 1989). Although such generalisations about the dependence of radiation response on cell lineage have been useful in devising tumour radiotherapy regimens, a significant confounding factor is inter-individual differences in normal tissue and tumour response (Rofstad, 1986; Burnet et al., 1992). Accordingly, there is considerable interest in understanding the mechanisms that produce such differences and thus devising assays that will more accurately predict tumour radioresponsiveness.

Recent work has focused attention on the possibility that susceptibility of normal or transformed cells to radiation-induced apoptosis may be an important indicator of radiosensitivity. Using a panel of mouse lymphoid or myeloid cell lines, all of which underwent apoptosis after irradiation, a correlation between the rapidity of induction of apoptosis and the clonogenic survival dose—response of a particular cell line was shown (Radford, 1994a). The greater sensitivity of these haemopoietic lines to radiation-induced DNA double-strand breaks as compared with fibroblasts lines such as V79, which die by necrosis, suggested that radiosensitivity may be related to the mode of death (Radford, 1991, 1994a). Similarly, in vivo studies with transplantable murine tumours showed that elevation of both spontaneous and radiation-induced apoptosis correlated positively with growth delay and negatively with TCD50 (dose to cure 50% of animals) (Mein et al., 1993). However, these studies also showed a correlation between the incidence of apoptosis and tumour type, with several adenocarcinomas found to display increased apoptosis and radiosensitivity (longer growth delay and lower TCD50) when compared with several sarcomas. Interestingly, one sarcoma was highly radiation sensitive in spite of showing no apoptosis following irradiation.

Because the cell lines used in the above studies have different origins, we were interested in investigating the relationship between mode and rapidity of cell death and radiosensitivity in cell lines with a common origin. Accordingly, we have examined the response to γ-ray and DNA-associated 121I decay-induced damage of two transfected of the rat lung fibroblast line 208F which express either human c-myc (cell line RBM7) or activated Ha-ras (cell line T1). Expression of these oncogenes has been shown to differentially alter susceptibility to both 'spontaneous' and serum withdrawal-induced apoptosis, with activated Ha-ras reducing the incidence of apoptosis over a 48 h period and c-myc increasing the incidence of apoptosis over this time (Arends et al., 1993). A similar pattern of high and low levels of apoptosis was seen in vivo using solid fibrosarcomas formed by the RBM7 and T1 cell lines (Arends et al., 1994). Increased levels of apoptosis following growth factor withdrawal have also been demonstrated in Rat-l fibroblasts expressing c-myc (Evan et al., 1992) and in murine myeloid cells constitutively expressing c-myc (Askev et al., 1991). It was therefore of interest to determine how the expression of these genes affected the mode and rapidity of cell death following irradiation, and whether cellular sensitivity to DNA damage was altered.

Materials and methods

Cell lines: growth conditions and p53 status

The derivation of the 208F, T1 and RBM7 cell lines used in this study is described elsewhere (Spandidos and Wilke, 1984; Arends et al., 1993). Cells were grown in the alpha modification of Eagle's medium (ICN Flow) supplemented with 10% fetal calf serum (Commonwealth Serum Laboratories, Melbourne, Australia). Cells were cultured at 37°C in sealed flasks that had been flushed with 5% carbon dioxide, 5% oxygen and 95% nitrogen. Cells were subcultured by treatment with pronase (Calbiochem). All experiments were performed with asynchronous cell cultures in log-phase growth. Under these conditions, the cell lines had population doubling times of 16 (208F), 16 (T1) and 17 h (RBM7).

The p53 status of each of the cell lines was examined by immunoprecipitation as described in Radford (1994a). A
positive result was found for extracts of all three cell lines using PAβ 421 antibody, which binds to both wild-type and mutant p53 protein, whereas a negative result was found with PAβ 240 antibody, which binds specifically to mutant p53 (Gannon et al., 1990).

γ-irradiation, 125I labelling and clonogenic assay

 Cultures were irradiated at room temperature in a 137Cs source at a dose rate of approximately 0.9 Gy min\(^{-1}\). Prior to irradiation, cultures were rinsed to remove any dead cells and fresh growth medium was added. Cell monolayers for clonogenic assay were treated with 0.02% pronase in phosphate-buffered saline (PBS) plus 0.2 mm EDTA for 5 min at room temperature and then dispensed by pipetting. After washing, cell suspensions were counted using a Coulter counter and appropriate cell numbers were plated to give 50–100 colonies in each of five replicate Petri dishes. Colonies of ≥ 50 cells were scored after 8 days' incubation at 37°C. Mean (± s.e.) cloning efficiencies for 208F, T1 and RRM7 were respectively 0.33 ± 0.03, 0.42 ± 0.03 and 0.40 ± 0.04.

125I labelling was performed by incubating cultures for approximately 24 h in growth medium containing around 2.5 kBq ml\(^{-1}\) [125I]iododeoxyuridine (NEN/DuPont) and 2 μM thymidine. Cells were then washed and incubated in growth medium containing 20 μM thymidine and 20 μM deoxyctydine for 3 h. After chasing, incorporated 125I was measured by pelleting cells and counting radioactivity in a Compugamma CS (LKB) gamma counter. Pellets were then resuspended in a known volume and aliquots were taken for counting the number of 125I decays cell per day. Cells were then aliquoted and frozen (at −1°C per min in a controlled-rate freezer) in growth medium plus 10% dimethylsulphoxide (DMSO). Cell aliquots were removed from liquid nitrogen storage at various time, after known numbers of 125I decays per cell had occurred, and assayed for clonogenic survival. Further details are given in Radford (1991). Freezing and thawing did not significantly affect the cloning efficiency of these cell lines as evidenced by values of 0.33 ± 0.02, 0.35 ± 0.01 and 0.40 ± 0.02 for 208F, T1 and RRM7 respectively.

A least-squares fit, using the criterion \((S_0 - S_2)^2/S_0\), where \(S_0\) is the estimated survival and \(S_2\) is the observed survival for each experimental point, was obtained for cell survival data using the KaleidaGraph (Abelbeck Software) program on a Macintosh computer. The γ-ray survival data were fitted to the equation \(S = \exp [-(\alpha D + \beta D^2)]\) where \(S\) is survival, \(D\) is dose and \(\alpha\) and \(\beta\) are constants, \(\alpha\) and \(\beta\) are dose and decay survival constants were fitted to the simple exponential \(S = A_2\exp(-D/D_0)\), where \(S\) is survival, \(D\) is the number of 125I decays and \(A_2\) and \(D_0\) are constants.

Electron microscopy and gel electrophoresis

At 24 h intervals after irradiation, non-adherent cells were collected by giving flasks several sharp taps, removing the growth medium, and then rinsing the monolayer once with PBS-EDTA. The growth medium and PBS-EDTA wash were pooled and centrifuged and the cell pellets were then fixed on ice for 30 min in growth medium (without serum) containing 0.25% glutaraldehyde and 45 min in 2.5% glutaraldehyde before post fixation with osmium tetroxide. Cells were then embedded in Spurr's resin and sectioned. Sections of control cells were obtained by fixing and embedding cells in situ on glass coverslips.

PAβ A migration samples were processed as described previously (Radford et al., 1994). Approximately 2 μg of DNA from each sample was electrophoresed on a 1.5% agarose gel using SPF-1/EcoRI DNA (Brescat, Adelaide, Australia) as size markers. Pulsed-field gel electrophoresis (PFGE) was carried out on a CHEF apparatus with a hexagonal array of electrodes (Chu et al., 1986) for 24 h at 150 V with a pulse time of 80 s. Plugs were prepared by mixing 1 × 10⁶ (unless otherwise stated) non-adherent or adherent cells (removed from flasks by pronase treatment as described above) in growth medium with an equal volume of 1% low gelling temperature agarose (SeaPlaque, FMC) in balanced salt solution (BSS). Plugs were then placed in NDS (10 mm Tris, 0.5 mm EDTA, 1% lauroylsarcosine, pH 9.5) plus 1 mg ml\(^{-1}\) proteinase K for 60 min on ice followed by overnight incubation at 37°C before electrophoresis. Yeast chromosomes from Saccharomyces cerevisiae strain YP148 (Pyle et al., 1988) and λ DNA (BRL) were used as markers. Following electrophoresis, gels were stained with ethidium bromide.

Measurement of apoptosis, population expansion and mitotic fraction

Non-adherent cells were collected over 24 h intervals as described above. They were then pelleted and counted using a haemocytometer. The level of apoptosis was determined by mixing equal volumes of non-adherent cell suspension and growth medium containing 10 μg ml\(^{-1}\) ethidium bromide and 3 μg ml\(^{-1}\) acridine orange and then scoring approximately 500 cells for apoptotic nuclear morphology by fluorescence microscopy. The number of adherent cells present was determined by treating the monolayer with pronase and Coulter counting the suspension. The fraction of non-adherent cells which was apoptotic was multiplied by the total number of non-adherent cells, divided by the total number of cells in both adherent and non-adherent fractions, and then multiplied by 100 to give the percentage apoptosis.

After irradiation, all flasks were rinsed and had fresh growth medium added at 24 h intervals. The non-adherent cells collected thus represented cells released from the monolayer over a 24 h period. A combination of 24 h medium changes and seeding flasks at appropriate cell numbers ensured that cell death was attributable to the effects of irradiation and not to medium depletion. Population expansion was defined as the number of cells in the monolayer at a given time divided by the number at the time of irradiation.

The level of post-irradiation mitotic activity was determined by incubation of cultures with the mitotic spindle poison nocodazole at 0.1 μg ml\(^{-1}\) for 3 h and then scoring the fraction of metaphase-arrested cells. Further details are given in Radford and Murphy (1994).

Results

Clonogenic survival dose–response is not markedly changed by over-expression of c-myc or T24-ras

Clonogenic survival curves were obtained for the parent and transfected cell lines exposed to either γ-irradiation or DNA-associated 125I decays. The γ-ray survival curves suggest that the overexpression of c-myc or an activating mutation of Ha-ras in these cells does not markedly affect their radiosensitvity (Figure 1). The similarity in the response of these cell lines is particularly evident at high doses. At low doses, the Ha-ras transfected cells may show increased resistance leading to a slightly larger shoulder region as compared with the parent line. An activated ras-induced change in the shoulder region of the survival curve of a transfected line has been reported by others (Hermens and Bentvelzen, 1992).

In order to quantify more readily the level of DNA damage required for cell killing, the sensitivity of the three cell lines to DNA-associated 125I decays was measured. Radiative decay of 125I atoms incorporated into cellular DNA produces high linear energy transfer (LET)-type DNA damage (Charlton, 1986) and results in approximately one DNA double-strand break per decay event (Kirsch and Sauri, 1975). The 125I data shown in Figure 2 suggest that there is no significant difference in the number of DNA double-strand breaks required to produce a lethal event in each of the cell lines. D\(_0\) values of 50 ± 2.5, 53 ± 1.4 and 56 ± 2.5 125I decays were obtained for 208F, T1 and RRM7 respectively. These D\(_0\) values suggest that around 50–56 125I
Clonogenic survival of 208F (---), T1 (---□---) and RBM7 (---○--) fibroblasts as a function of γ-radiation dose. Data were fitted to a quadratic function of dose as described in Materials and methods. The calculated values of α(Gy⁻¹) and β(Gy⁻²) were: 208F, 0.40 ± 0.05 and 0.012 ± 0.004; T1, 0.27 ± 0.03 and 0.019 ± 0.003; RBM7, 0.38 ± 0.003 and 0.009 ± 0.004. Each data set was obtained from at least four independent experiments.

Decays per cell

Clonogenic survival

Decays per cell

Figure 1. Clonogenic survival curves of 208F (---), T1 (---□---) and RBM7 (---○--) fibroblast cells that had accumulated DNA-associated ¹²⁵I decays during liquid nitrogen storage. The data were fitted to a simple exponential function of accumulated decays and D₀ values of 50 ± 3, 53 ± 1 and 56 ± 3 ¹²⁵I decays were obtained for 208F, T1 and RBM7 respectively. Each data set was obtained from at least three independent experiments.

Figure 3. Electron micrographs of control (unirradiated) and γ-irradiated (12 Gy plus incubation at 37°C for 24 h) cells. (a) 208F control, (b) 208F irradiated, (c) T1 control, (d) T1 irradiated, (e) RBM7 control and (f) RBM7 irradiated. Scale bars represent 5 μm.
decay-induced DNA double-strand breaks are required to produce a lethal event in each of these cell lines.

All cell lines used show apoptotic death following irradiation

The morphology associated with cell death in each of the three cell lines, following exposure to a γ-ray dose (12 Gy) that would reduce clonogenic survival to 0.5% of the control value, was determined. Both light microscopy of stained sections and fluorescence microscopy (after staining with ethidium bromide and acridine orange) suggested that dying cells showed cytoplasmic shrinkage and condensation and margination of nuclear chromatin (data not shown). These features are diagnostic for apoptosis (Arends and Wyllie, 1991). The conclusion that radiation induces apoptosis in each of the three cell lines was confirmed by electron microscopy (Figure 3). These electron micrographs showed characteristic features of apoptosis such as chromatin condensation and margination to the nuclear periphery (Figure 3f), convolutions of the nuclear membrane (Figure 3b) and eventual cellular break-up into apoptotic bodies (Figure 3d). Cells collected over 0–24 or 48–72 h after irradiation were also examined by electron microscopy for features characteristic of necrotic cell death, such as cellular or organelle swelling. Over these time intervals, no evidence indicative of necrotic cell death was found in any of the three cell lines.

The mechanism of cell death after irradiation was further examined by analysis of the pattern of DNA degradation occurring in dying cells. One of the features often associated with apoptosis is the digestion of nuclear DNA into fragments that are multiples of the 180 bp nucleosome unit (Wyllie, 1980; Arends et al., 1990). When electrophoretically separated on an agarose gel, these DNA fragments produce a characteristic ‘ladder’ pattern. At various time points after irradiation, DNA was extracted from control (non-irradiated) monolayers and separately from the adherent and non-adherent cell fractions of irradiated cultures. The parent line 208F and the c-myc-transfected line RBM7 both showed clear DNA ladders characteristic of apoptosis in the non-adherent cell fraction 24 h after irradiation (Figure 4a). However, the Ha-ras-transfected line T1 showed no ladder after 24 h (Figure 4a). DNA extracted and analysed from non-adherent T1 cells at 36 or 48 h after irradiation still did not show laddering of the DNA (Figure 4b). DNA was also extracted from all three cell lines at time points prior to 24 h post irradiation, but ladders were not detected (data not shown). DNA degradation in T1 cells was then examined further using PFGE of cells incubated for 24 or 48 h at 37°C after 12 Gy of γ-irradiation. In each case, the DNA from 1 × 10⁶ cells was examined, except for the 24 h non-adherent fraction where only 0.5 × 10⁶ cells were available. The adherent fraction of the irradiated cell cultures, and to a lesser extent the control sample, showed a broad range of DNA sizes, indicating random degradation. DNA from the non-adherent fraction of irradiated T1 cultures incubated for 48 h, showed more marked degradation and the possible appearance of a 100 kbp intermediate that is subsequently degraded further (Figure 4c).

Elevated expression of c-myc increases the incidence of apoptosis following γ-irradiation

Previous work using these cell lines had shown a difference in the incidence of apoptosis at 48 h after serum withdrawal (Arends et al., 1993). It was therefore of interest to examine the relative time of onset of apoptosis for asynchronous cultures of each of the cell lines following a 0.5% clonogenic survival γ-ray dose. At 24 h periods following irradiation, non-adherent cells were collected, counted, and the percentage of cells undergoing apoptotic death was scored by fluorescence microscopy. Apoptotic cells were not found in adherent cell populations and non-adherent, non-apoptotic cells were found to account for less than 3.5% of non-apoptotic cells. The results of this experiment showed that the c-myc-transfected cell line RBM7 had a markedly greater incidence

Figure 4 Ethidium bromide-stained agarose gel separations, using conventional (a and b) or pulsed-field gel electrophoresis (c) of DNA extracted from the control (C), irradiated adherent cells (Ad) and irradiated non-adherent cells (N). Cells were incubated at 37°C after γ-irradiation (12 Gy) for the indicated time periods.
of radiation-induced apoptosis, over the time interval examined, than the other lines (Figure 5a). However, the expression of activated Ha-ras (T1) did not alter the frequency of apoptosis compared with the parent line (208F) up to 96 h post irradiation (Figure 5a). Adherent cells were also counted and RBM7 was found to maintain a similar number of cells on the monolayer as the parent line in spite of its higher rate of apoptosis. Cell numbers of T1, however, continued to increase up to 72 h post irradiation (Figure 5b).

In order to determine the level and time of resumption of mitotic activity, replicate cultures of the three cell lines were incubated with the mitotic spindle poison nocodazole for successive 3 h intervals following 12 Gy of γ-irradiation (Figure 5c). Following an initial marked decrease of mitotic activity, a wave of mitosis was noted, which suggested a partial cell cycle-synchronising effect of irradiation on each of the lines. Cultures of both the myc- and ras-transfected lines (RBM7 and T1) resumed cycling more rapidly and showed a higher fraction of cells entering mitosis than the parental 208F line. In toto, the data in Figure 5 suggest that, relative to the parental cell line, the level of apoptosis per mitosis is similar in the myc-transfected line but might be decreased in the ras-transfected line. Assuming that radiation-induced apoptosis occurs after mitosis in these cell lines, the increased incidence of apoptosis in the myc-transfected line would then be a consequence of its higher (relative to the parental line) level of post-irradiation mitotic activity. Time-lapse cinemicroscopy studies will be required in order to confirm these conclusions.

Discussion

The 208F rat fibroblast line and its myc- and ras-transfected derivatives RBM7 and T1 all undergo radiation-induced apoptosis as evidenced by morphology. This response is broadly in keeping with previous observations of spontaneous cell death of these cell lines in culture by apoptosis, albeit at high and low levels respectively for RBM7 and T1 (Arends et al., 1993) and also the pattern of death observed in solid tumours: RBM7 fibrosarcomas showed a high level of apoptosis and very little necrosis, but T1 demonstrated widespread necrosis with low levels of apoptosis (Arends et al., 1994). This occurrence of radiation-induced apoptosis in all three fibroblast lines examined in this study differs from some reports in which normal and transformed fibroblasts were found to undergo necrosis after irradiation (e.g. Afanas'ev et al., 1986; Radford, 1991). However, Tomei et al. (1988) using C3H-10T1/2 mouse fibroblasts and Lowe et al. (1988) using adenovirus E1A gene-expressing mouse embryonic fibroblasts both reported induction of apoptosis after irradiation.

In an effort to explain differences in response to irradiation between cell types, many groups have looked at the possible link between oncogene expression and radiosensitivity (reviewed in Kasid et al., 1993). The consequences of transfection of members of the ras family of oncogenes have been widely studied. However, these studies have yielded conflicting results. Expression of mutationaly activated ras or overexpression of normal ras have been reported to increase the radiosensitivity of mouse 3T3 fibroblasts and of rat rhabdomyosarcoma cells (Sklar, 1988; Samid et al., 1991; Hermens and Bentvelzen, 1992), while studies using transfected normal or immortalised human cells found that expression of activated ras does not, by itself, lead to an increase in radiosensitivity (Mendonça et al., 1991; Su and Little, 1992). We found that expression of activated Ha-ras in the T1 derivative of the 208F fibroblast line had no marked effect on its clonogenic survival dose–response for γ-ray or 125I decay-induced DNA damage. Activated ras did not appear to alter the fraction of the total cell population undergoing apoptosis up to 96 h after irradiation as compared with the parent line. However, irradiated cultures of the T1 line showed greater residual proliferation, suggesting that activated ras protein may decrease the level of apoptosis per mitosis. This effect may be related to the inhibition of serum withdrawal-induced apoptosis and to the decrease in susceptibility to apoptosis under both in vitro and in vivo growth conditions observed previously for this cell line (Arends et al., 1993, 1994).

The expression of activated Ha-ras inhibited the endonuclease activity responsible for the 180 bp nucleosomal ladder that is commonly associated with apoptosis (Arends et al., 1990). This finding is consistent with that of Arends et al.
(1993), who showed that this endonuclease(s) is downregulated by the expression of activated ras. Despite lacking detectable nucleosomal ladder-producing endonuclease activity, irradiated T1 cells showed extensive DNA degradation to relatively high molecular weight fragments and normal apoptotic morphology. Apoptosis without the appearance of nucleosomal ladders has been reported previously (Cohen et al., 1992), and it has been shown that the characteristic apoptotic nuclear morphology is associated with initial cleavage of nuclear DNA into 300 kb fragments and then into 50 kb fragments and does not require the production of 180 kb nucleosomal ladders (Brown et al., 1993; Obourn et al., 1993).

The RBM7 cell line, which overexpresses c-myc, showed an increase in the incidence of radiation-induced apoptosis, over the time period examined, with respect to the parent line. This finding is consistent with the observations of Arends et al. (1993, 1994) that the RBM7 line shows increased susceptibility to serum withdrawal-induced apoptosis and increased intrinsic apoptosis when growing as a solid fibrosarcoma or in culture, and with the hypothesis that c-myc expression induces a state in which cells are 'primed' for apoptosis (Arends and Wyllie, 1991). The increase in apoptosis may reflect a more rapid post-irradiation resumption of mitotic activity in the RBM7 cell line.

However, despite their increased rate of γ-irradiation-induced apoptotic death, the sensitivity of RBM7 cells to either γ-irradiation or DNA-associated 125I decay-induced cell killing is not significantly different from that of the parent line. This contrasts with previous data from mouse lymphoid lines, which revealed a correlation between rate of radiation-induced apoptotic death and radiosensitivity (Radford, 1994a). However, it should be noted that, even in the RBM7 line, apoptosis is induced considerably more slowly in irradiated fibroblasts than in the more radiosensitive lymphoid lines. For example, apoptotic cells were not detected in RBM7 cultures until at least 8 h after irradiation (data not shown), as compared with 1–2 h in radiosensitive lymphoid lines (Radford, 1994a). The comparatively lengthy time period available to irradiated RBM7 cells, prior to possible induction of apoptosis, may be adequate for DNA repair.

The reason for the difference in the rate of induction of apoptosis between irradiated fibroblasts and some lymphoid lines is currently unclear. Studies with mouse lymphoid lines have suggested that rapid induction of apoptosis after irradiation is dependent upon the presence of wild-type p53 protein (Radford, 1994b). Although all three fibroblast lines used in this study appear to contain non-mutant p53 protein, a definitive conclusion awaits DNA sequencing (see Materials and methods). Studies by other investigators, although not defining the mode of cell death occurring, have also generally concluded that overexpression of c-myc does not alter radioresistance (reviewed in Kasid et al., 1993).

These findings lead us to question the hypothesis that a cell's radiosensitivity can be directly related to its mode of death. Indeed, the number of 125I decay-induced DNA double-strand breaks required to produce a lethal event in the 208F line and its transfected derivatives was similar to the number required to kill the V79 fibroblast line (D0 = 61 ± 2), which undergoes necrotic cell death (Radford, 1991). This suggests that differences in radiosensitivity between cell lines may be related more to the intrinsic characteristics of the cell type of origin than to mode of death and that radiosensitivity is a phenotypic property distinct from susceptibility to apoptosis and that it may be independently genetically influenced. However, there is a need to study the relationship between radiosensitivity and mode of cell death in a wider range of cell types before such conclusions can be confirmed. It should also be noted that the significant difference in residual post-irradiation proliferation between the cell lines studied may complicate the extrapolation of our results to the in vivo situation.

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