Low-Dose Radiation Hypersensitivity in Human Tumor Cell Lines: Effects of Cell–Cell Contact and Nutritional Deprivation

Sudhir Chandna,[a,1] B. S. Dwarakanath,[a] Divya Khaitan,[a] T. Lazar Mathew[a] and Viney Jain[b,c]

[a] *Institute of Nuclear Medicine & Allied Sciences, Brig. S. K. Mazumdar Road, Delhi, 110054, India;* [b] *Dr. B. R. Ambedkar Centre for Biomedical Research, University of Delhi, Delhi, 110007, India; and* [c] *Wallace-Kettering Neuroscience Institute, Kettering Medical Center and Department of Emergency Medicine, Wright State University, 3525 Southern Boulevard, Kettering, Ohio 45429*

Chandna, S., Dwarakanath, B. S., Khaitan, D., Mathew, T. L. and Jain, V. Low-Dose Radiation Hypersensitivity in Human Tumor Cell Lines: Effects of Cell–Cell Contact and Nutritional Deprivation. *Radiat. Res.* 157, 516–525 (2002).

The hyper-radiosensitivity at low doses recently observed *in vitro* in a number of cell lines is thought to have important implications for improving tumor radiotherapy. However, cell–cell contact and the cellular environment influence cellular radiosensitivity at higher doses, and they may alter hyper-radiosensitivity *in vivo*. To confirm this supposition, we investigated the effects of cell density, multiplicity and nutritional deprivation on low-dose hypersensitivity *in vitro*. Cell survival in the low-dose range (3 cGy to 2 Gy) was studied in cells of two human glioma (BMG-1 and U-87) and two human oral squamous carcinoma (PECA-4451 and PECA-4197) lines using a conventional macrocolony assay. The effects of cell density, multiplicity and nutritional deprivation on hyper-radiosensitivity/induced radioresistance were studied in cells of the BMG-1 cell line, which showed prominent hypersensitivity and induced radioresistance. The induction of growth inhibition, cell cycle delay, micronuclei and apoptosis was also studied at the hyper-radiosensitivity-inducing low doses. Hyper-radiosensitivity/induced radioresistance was evident in the cells of all four cell lines to varying extents, with maximum sensitivity at 10–30 cGy, followed by an increase in survival up to 50 cGy–1 Gy. Both the glioma cell lines had more prominent hyper-radiosensitivity than the two squamous carcinoma cell lines. Low doses inducing maximum hyper-radiosensitivity did not cause significant growth inhibition, micronucleation or apoptosis in BMG-1 cells, but a transient G_1/S-phase block was evident. Irradiating and incubating BMG-1 cells at high density for 0 or 4 h before plating, as well as irradiating cells as microcolonies, reduced hyper-radiosensitivity significantly, indicating the role of cell–cell contact-mediated processes. Liquid holding of BMG-1 cells in HBSS + 1% serum during and after irradiation for 4 h significantly reduced hyper-radiosensitivity, suggesting that hyper-radiosensitivity may be due partly to active damage fixation processes at low doses. Therefore, our findings suggest that the damage-induced signaling mechanisms influenced by (or mediated through) cell–cell contact or the cellular environment, as well as the lesion fixation processes, play an important role in hyper-radiosensitivity. Further studies are required to determine the exact nature of the damage that triggers these responses as well as for evaluating the potential of low-dose therapy. © 2002 by Radiation Research Society

[1] Author to whom correspondence should be addressed at Department of Biocybernetics, Institute of Nuclear Medicine & Allied Sciences, Brig. S. K. Mazumdar Road, Delhi, 110054, India; e-mail: sudhirchandna@yahoo.com.

INTRODUCTION

The classical clonogenic cell survival dose response in irradiated mammalian cells is generally characterized by a variable shoulder region extending up to approximately 1 Gy, followed by an exponentially decreasing surviving fraction at higher doses (*1*). The response at doses below 50 cGy is generally extrapolated from the data obtained at higher doses ($\geq$1 Gy) by employing the linear-quadratic (LQ) equation. Experiments examining low-dose effects in animal models, however, showed radiosensitivities in certain tissues that were higher than expected, indicating deviations from the LQ model (*2–4*). In recent years, attempts have been made to obtain reliable cell survival data at low doses by employing special techniques and instruments to improve precision, for example, by using the Dynamic Microscope Image Processing System [DMIPS; ref. (*5*)] or by using a flow sorter for accurate cell seeding (*6*). Interestingly, several such studies have confirmed that in normal and transformed cells (derived from various animal and human tissues), cell survival at absorbed radiation doses below 50 cGy is significantly lower than that predicted by the LQ model (*7–17*). This low-dose hyper-radiosensitivity is followed by an increase in survival at slightly higher doses, resulting in a multiphasic dose–response survival curve.

Two major hypotheses have been put forward to explain this intriguing low-dose response. The widely accepted ''induced repair'' model postulates the induction of cellular repair processes only above a critical threshold dose of radiation. Very low radiation doses (below the critical threshold) are not sufficient to stimulate cellular repair processes, resulting in high cell lethality (*4*), whereas doses above this threshold trigger repair processes, leading to an increase in cell survival (often referred to as induced radioresistance). The second hypothesis proposes that hyper-radiosensitivity

is due to the presence of sensitive subpopulations of cells (e.g. in specific cell cycle phases) that might be contributing to the enhanced sensitivity in the low-dose region. Models based on this assumption suggested an increase in the sensitivity of these putative subpopulations to an extent that is quite unlikely based on the maximum variation in sensitivity actually observed among cells from different phases of the cell cycle (*10, 14*).

The molecular mechanisms underlying hyper-radiosensitivity/induced radioresistance remain to be elucidated. DNA damage and repair have been implicated as playing a role (*18–21*), although direct evidence showing that DNA damage is the primary determinant of hyper-radiosensitivity at these low doses has not been obtained. In addition, the roles of various experimental conditions as well as environmental and genetic factors that may influence hyper-radiosensitivity and induced radioresistance need to be investigated.

Extrapolating to the situation *in vivo*, the implications of hyper-radiosensitivity/induced radioresistance for risk estimates and radiotherapy have often been discussed in the literature, and it has been suggested that megafractionation or superfractionation with small fractions (<50 cGy) may be better than the conventional fractionation regimen using 1.5–2 Gy/fraction (*22*). However, quantitative comparisons between relatively simple *in vitro* experiments and the more complex situation *in vivo* have revealed some discrepancies at higher doses; e.g., animal tissues and human tumor xenografts, when irradiated *in vivo*, have been reported to be more radioresistant than expected on the basis of *in vitro* data (*23–27*). This may be due to critical differences in the microenvironment and intercellular contact and communication. Similar results reported in studies with spheroids, which represent a better model for the response of solid tumors than do monolayer cell cultures, also revealed the importance of cell–cell contact and nutritional heterogeneity in modulating radiosensitivity (*28, 29*). Recent work using sparse and dense monolayer cultures indicates that cell communication can significantly influence the cellular radiation responses at doses of 1 Gy and above (*30*). It is likely that these variations in cell density and the microenvironment may also influence hyper-radiosensitivity/induced radioresistance.

In the present study, low-dose survival responses in cells of four human tumor cell lines (two malignant gliomas and two oral squamous carcinomas) differing in their origins, TP53 (previously known as p53, and also called the guardian of the genome) status, and radiosensitivities have been compared. The effects of cell density, multiplicity (to modulate cell–cell contact and communication), and nutrient deprivation (to retard cell progression) were investigated in one of the glioma cell lines (BMG-1) showing prominent hyper-radiosensitivity/induced radioresistance. Induction of growth inhibition, cell cycle delay, micronuclei and apo-

ptosis at low doses was also studied in these cells. Preliminary results from this study have been presented.[2,3]

MATERIALS AND METHODS

Cell Lines

The BMG-1 glioma cell line (DNA index = 0.96; wild-type TP53) was established from a human glioma[4] (*31*). The U-87 malignant glioma cells (DNA index = 1.0; wild-type TP53 but deficient in TP53 induction after irradiation) were obtained from the National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore. Cells of the PECA-4451 (DNA index = 1.50; mutated TP53) and PECA-4197 (DNA index = 1.00; wild-type TP53) oral squamous carcinoma cell lines were obtained from the Institut für Medizinische Strahlenbiologie, Universitätsklinikum Essen, Germany. All the cells were maintained as monolayers at 37°C in 25-cm² tissue culture flasks (Tarsons, India) in Dulbecco's modified Eagle's medium (DMEM; D5523, Sigma) supplemented with fetal calf serum (Biologicals, Israel; 5% for BMG-1, 10% for U87 and PECA-4451, and 20% for PECA-4197), 10 mM Hepes buffer (Sigma) and antibiotics (Sigma). Cells were passaged routinely in exponential growth phase twice a week using 0.05% trypsin-EDTA solution in phosphate-buffered saline (PBS).

Irradiation

Cells were irradiated at room temperature using a cobalt-60 teletherapy unit (Theratron-780 C, Canada) at a dose rate of 1.9–2.1Gy min^{-1}.

Low-Dose Survival Response of BMG-1 Cells under Various Conditions

Experiments were conducted using four different experimental designs to study the influence of cell density, multiplicity and nutritional conditions on cell survival.

Cells irradiated at low cell density. Cells were trypsinized from the exponentially growing stock cultures, counted with a hemocytometer, and diluted to a density of approximately 1–2 × 10⁵ cells/ml in fresh DMEM. An average of two to three independent counts of this dilution were taken, and known numbers of cells were added to fresh growth medium to obtain the required plating density (150 cells/4 ml per Petri dish). Cells from this suspension were then dispensed into the Petri dishes to achieve uniform seeding for colony formation. Cultures were maintained overnight (18–20 h) at 37°C in a humidified 96% air/4% CO₂ atmosphere prior to irradiation. The mean multiplicity at this time was close to one. After irradiation, the cells were returned to the same atmosphere and incubated for 5–7 days before the colonies were fixed and stained with 1% crystal violet. Colonies containing more than 50 cells were scored as survivors.

Cells irradiated at high cell density. Cells were grown in tissue culture flasks for 48–72 h and were irradiated at a cell density of 35–40,000 cells/cm². After irradiation, cells were trypsinized and plated at 5–6 cells/cm² at appropriate times (0 h and 4 h) and were further incubated at 37°C for 5–7 days before the colonies were fixed and stained.

Cells irradiated as microcolonies. Effect of cell multiplicity on the low-dose response was studied by plating cells at a low density (5–6 cells/cm²) and allowing them to multiply for 24 h and 48 h before irradiation. Cell multiplicity was measured at each time by counting cell numbers per colony in 100 colonies for each group. The observed plating efficiencies of microcolonies were corrected according to the equation of Elkind and Whitmore (*32*):

$$\text{Corrected PE} = 1 - n(1 - \text{PE}_m),$$

where n is the average number of cells in microcolony, PE_m is the observed plating efficiency of the microcolony, and PE is the predicted plating efficiency of single cells based on the assumption that intercellular interaction does not have any effect on the survival of cells within a microcolony (*30, 33*).

Irradiation under nutrient-deficient conditions. The growth medium of preplated cells at low density (5–6 cells/cm²) was replaced with Hanks' balanced salt solution (HBSS; Himedia, India) containing 1% serum 1 h prior to irradiation. After irradiation, cells were incubated under the same conditions for 4 h, and HBSS was then replaced with fresh growth medium. In the control group where HBSS was not used, the growth medium was replaced with fresh growth medium 4 h postirradiation. The pH of the medium was unchanged during these treatments due to the presence of Hepes buffer as well as due to the very small number of cells seeded.

Proliferation Kinetics and Cell Cycle Distribution Studies

BMG-1 cells were irradiated 18–20 h after seeding at 7000–8000 cells/cm², and their proliferation kinetics was measured at 24-h intervals by trypsinizing and counting total cells per flask using a hemocytometer. For studying cell cycle distribution, cells were washed in PBS and fixed in 70% ethanol at 4°C at least overnight. Fixed cells were again washed with PBS, treated with 200 µg/ml RNase A for 30 min at 37°C, and stained with 25 µg/ml propidium iodide (Sigma) in PBS. The distribution of DNA contents was measured using a FACSCalibur flow cytometer (Becton Dickinson) with CellQuest software (version 3.0.1; Becton Dickinson) for data acquisition and ModFit LT software (version 2.0; Verity Software House Inc.) for cell cycle analysis.

Micronucleus Test

The micronucleus test was carried out with BMG-1 cells at low (150 cells/cm²) and high cell densities (3700 cells/cm²) using the cytochalasin B-induced cytokinesis block method (*34*). After irradiation at room temperature, cells were incubated with 2 µg/ml of cytochalasin B (Sigma) for 24 h before fixation. In the case of high-density cultures, the cells were washed with PBS *in situ*, trypsinized, fixed in Carnoy's fixative for 2–4 h, and spread on clean microscope slides. The slides were air-dried overnight before staining with Hoechst 33258 as described previously (*35*). Cells plated at low density were resuspended in PBS after trypsinization and transferred onto slides using cytospin2 (Shandon, UK). The slides were air-dried overnight, fixed in Carnoy's fixative, and stained with Hoechst 33258. Cytochalasin B treatment in unirradiated cells as well as irradiated cells under these conditions yielded 80–90% binucleated cells. Only micronuclei from binucleated cells were scored.

Apoptosis

Morphological analysis using fluorescence microscopy of samples prepared for the micronucleus test and the Annexin-V binding assay with flow cytometry (*36*) were used to detect apoptosis in BMG-1 cells. Standard criteria were used for identifying apoptotic cells under the microscope. For flow cytometry, cells were irradiated as high-density exponential cultures and gently scraped from the surface 24 h later. After two washes in PBS, the cells were incubated with a kit containing Annexin-V-FITC and propidium iodide in binding buffer (BD Pharmingen, catalog No. 556547) for 20–25 min at room temperature before analysis by flow cytometry.

Statistical Analysis

Each experiment was repeated three times with triplicate samples. Mean values and standard deviations were calculated. Student's *t* test was used to examine the statistical significance of differences between groups.

RESULTS

Cell Survival Curves at Low Cell Density

The cell survival curves of cells of all four cell lines showed a distinct pattern of hyper-radiosensitivity/induced radioresistance, resulting in a multiphasic survival curve, primarily at doses below 1 Gy. The survival curve for BMG-1 cells, for example, deviated from the expected response with a shoulder at an absorbed radiation dose as low as 3 cGy, with the maximum sensitivity [surviving fraction (SF) = 0.56; SD = 0.06] observed at 10 cGy (Fig. 1a). This dose at which maximum sensitivity was observed will hereafter be referred to as D_{hyp}, and the corresponding surviving fraction will be called SF_{hyp}. As the dose was increased further, the SF increased to a value close to 1.0 around doses of 30–50 cGy. This maximum SF (possibly as a result of induced radioresistance) will be referred to as SF_{irr}, and the corresponding dose will be called D_{irr}. U87 glioma cells were observed to be most sensitive at low doses among all the four cell lines, with a surviving fraction (SF_{hyp}) as low as 0.33 (SD = 0.06) at the D_{hyp} of 20 cGy (Table 1). The SF increased in these cells beyond 20 cGy to a maximum of 0.70 (SD = 0.04) at a D_{irr} of 1.0 Gy (Fig. 1b).

The two squamous cell carcinoma cell lines, PECA-4451 and PECA-4197, had SF_{hyp} values of 0.56 (SD = 0.05) and 0.48 (SD = 0.04), respectively, at the common D_{hyp} of 30 cGy (Fig. 1c, d). The induced radioresistance in both PECA-4451 and PECA-4197 cells was less pronounced than that observed in glioma cells, with the SF_{irr} in these cell lines reaching values of 0.81 (SD = 0.06) and 0.85 (SD = 0.03) at a D_{irr} of 0.5 Gy and 1 Gy, respectively.

The SF per gray calculated at 2 Gy as well as at the respective D_{hyp} values showing minimum survival for each of the four cell lines is shown in Table 1. In BMG1 and U87 glioma cells, the SF per gray at D_{hyp} was 0.003 and 0.004, whereas at 2 Gy it was 0.90 and 0.75, respectively. Therefore, the cell killing per unit dose is about two orders of magnitude higher at D_{hyp} than at 2 Gy in the two glioma cell lines. On the other hand, the SF per gray at D_{hyp} was about six to nine times higher in PECA-4451 and PECA-4197 cells. The ratio of [–log (e) SF/Gy] values for respective D_{hyp}'s and 2 Gy is also shown for all four cell lines in Table 1. In the glioma cell lines, this value is about 20–50 times higher at D_{hyp} than at 2 Gy, whereas this difference is about 10 times smaller in the case of squamous carcinoma cell lines. The above results confirm that there is considerable heterogeneity in the extent of hyper-radiosensitivity among these human tumor cell lines, with glioma cell lines showing greater hyper-radiosensitivity. Previous
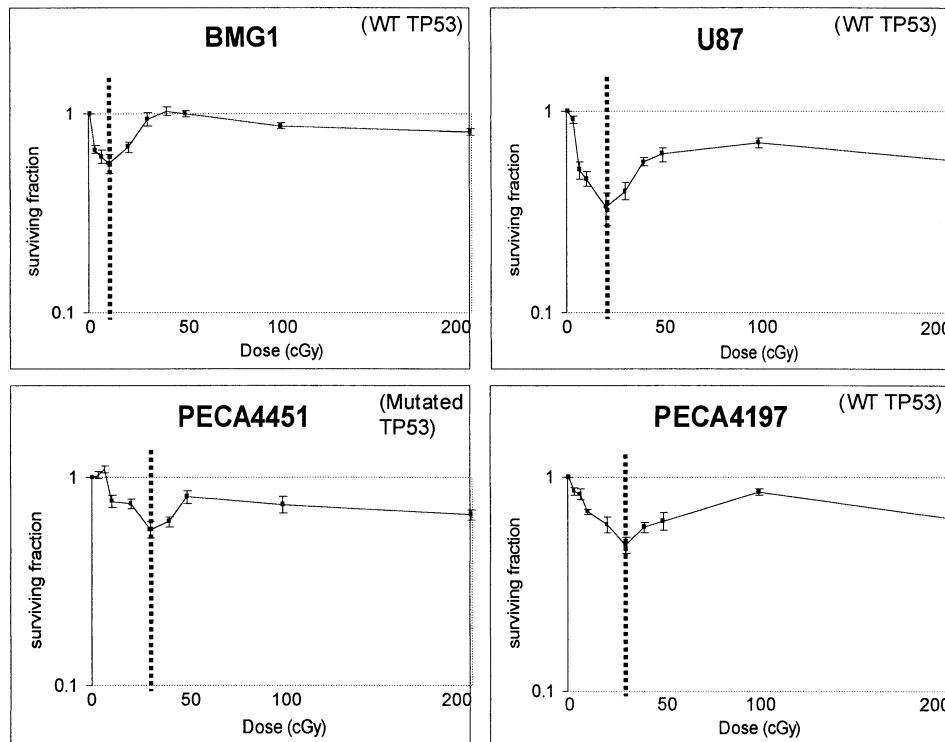
FIG. 1. Multiphasic dose–response curves for the four cell lines investigated, showing hypersensitivity and induced radioresistance. Cells plated at low density 18–20 h prior were irradiated in growth medium. The hypersensitive dose region varies among these cell lines (left of the dotted vertical line). Each data point is mean of nine observations from three independent experiments, and error bars represent standard deviations.

studies have also shown that hyper-radiosensitivity is common, although not universal, among glioma cell lines (*17*).

Induced radioresistance is usually estimated by comparing the slope obtained in the low-dose region (α_s) with the slope obtained from linear-quadratic fit of the high-dose data (α_r). In the present study, the α_s/α_r values for both the glioma cell lines were higher than those for the two squamous carcinoma cell lines, but they had no significant correlation with SF2 ($r = 0.17$; Table 1), which is in agreement with the recently published analysis of Joiner *et al.* (*37*).

Effect of High Cell Density on Hyper-radiosensitivity

Since cell–cell contact is known to influence cellular radiation responses, we studied hyper-radiosensitivity in BMG-1 cells irradiated at high density ($>35,000$ cells/cm²) and plated at low density (5–6 cells/cm²) for colony formation immediately after irradiation or 4 h later (to allow damage processing under conditions of high cell density). Compared to cells irradiated at low density, the hypersensitivity at 10 cGy was reduced significantly in cells plated at 0 h ($P < 0.01$) or 4 h after irradiation ($P < 0.0001$) (Fig. 2). The radiation-induced cell death, however, increased significantly at 50 cGy and 2 Gy in cells that were plated after 4 h of incubation at high cell density. These results indicate that high cell density differentially protects against low-dose hyper-radiosensitivity.

Effect of Cell Multiplicity on Hyper-radiosensitivity

The influence of cell multiplicity on hyper-radiosensitivity/induced radioresistance was investigated by seeding BMG-1 cells at low density (5–6 cells/cm²) and allowing them to multiply into microcolonies for 24 h and 48 h before irradiation. After cell plating, the average cell multiplicity in these microcolonies was 1.62 ± 0.02 at 24 h and 6.89 ± 0.05 at 48 h. Colonies were harvested 5–7 days postirradiation in both groups. The survival data were corrected for cell multiplicity based on the assumption that the survival of a single cell within a microcolony is independent of the survival of all other cells within that microcolony (*30, 33*). Both the uncorrected and corrected sets of data shown in Fig. 3 indicate that hyper-radiosensitivity was significantly reduced at doses below 40 cGy when cells were irradiated 48 h after plating compared to cells irradiated 24 h after plating. However, no significant difference in the sensitivity of the two groups was apparent at 40 cGy, and interestingly, above 40 cGy the relative sensitivity was significantly higher ($0.0001 < P < 0.01$) in cells irradiated as 48-h microcolonies.

Effect of Liquid Holding on Hyper-radiosensitivity

In these experiments, preplated BMG-1 cells (at 5–6 cells/cm²) were incubated in HBSS containing 1% serum during and after irradiation. The HBSS was replaced with

TABLE 1
Summary of Various Cell Survival and Derived Parameters in the Four Cell Lines
Investigated

Cell line	SF2[a]	SF$_{hyp}$[a]	α_s	α_r	α_s/α_r	SF/Gy[b] at D$_{hyp}$	SF/Gy[b] at 2 Gy	$[-\ln S/D$ at D$_{hyp}]/[-\ln S/D$ at 2 Gy]
BMG-1	0.81	0.56	5.36	0.07	73.4	0.003	0.90	53
U-87	0.57	0.33	8.68	0.11	78.9	0.004	0.75	20
PECA-4451	0.66	0.56	1.77	0.14	12.6	0.14	0.81	9
PECA-4197	0.64	0.48	2.30	0.18	12.8	0.09	0.80	11

[a] Standard deviations (mentioned in the text) were less than 10% in most cases.

[b] SF per gray was measured by extrapolating the cumulative effect of fractions of the respective doses, while assuming an absence of any induced resistance.

fresh growth medium 4 h after irradiation. In the control group of cells, HBSS was not used, but the growth medium was replaced with fresh growth medium 4 h postirradiation. Replacing the growth medium with fresh growth medium a few hours after irradiation had no effect on hyper-radiosensitivity/induced radioresistance. The survival curve of cells incubated under liquid holding conditions shows a significant reduction in hyper-radiosensitivity below 20 cGy, while there was no significant effect on cell survival at the higher doses (Fig. 4). This reduction of low-dose hypersensitivity under low-serum conditions in HBSS indicates a role of proliferation-linked cellular processes in the expression of hyper-radiosensitivity.

Growth Inhibition and Cell Cycle Delay

The data on postirradiation changes in the proliferation of BMG-1 cells irradiated at high cell density are summarized in Table 2. The proliferation kinetics of BMG-1 cells did not indicate any significant growth inhibition at 10 cGy, a dose that caused maximum hypersensitivity in cell survival studies. However, studies of cell cycle distributions using flow cytometry revealed a transient G_1/S-phase block

at 8–12 h after irradiation with 10 cGy that was released by 16 h (Fig. 5).

Micronuclei and Apoptosis

The frequency of BMG-1 cells with micronuclei after the first postirradiation mitosis, analyzed using the cytochalasin B method, was not significantly different in cells irradiated with 10 cGy and in the unirradiated cells at high or low cell densities. Data on induced micronuclei and apoptosis at high cell density are presented in Table 2. The low doses did not induce apoptosis in these cells at high and low cell
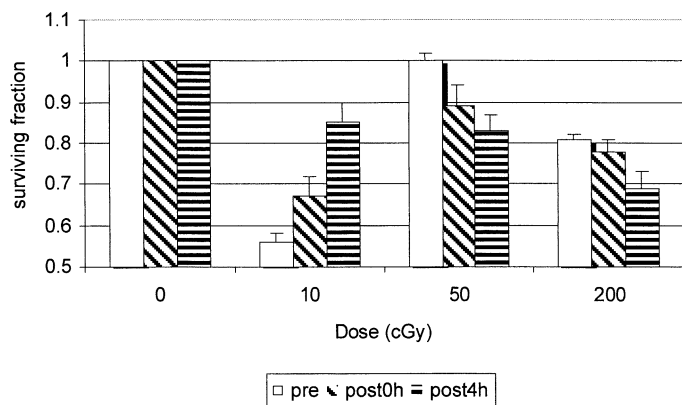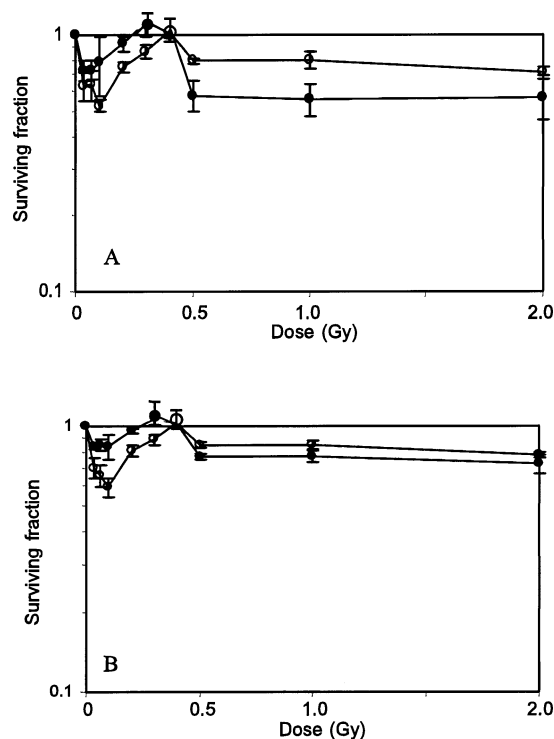

FIG. 2. Effect of irradiation of high-density cultures and delayed plating on hyper-radiosensitivity/induced radioresistance in BMG-1 cells. Cells were either irradiated as high-density exponential cultures and plated 0 h (post0h) or 4 h (post4h) later, or irradiated after 18–20 h incubation after low-density plating (pre). Data are the means of nine observations from three independent experiments, and error bars represent standard deviations.

FIG. 3. Effect of cell multiplicity on hyper-radiosensitivity/induced radioresistance in BMG1 cells. The mean multiplicity number was 1.62 ± 0.02 at 24 h (○) and 6.89 ± 0.05 at 48 h (●) after plating. Panel A: Data corrected for cell multiplicity according to Elkind and Whitmore (*32*). Panel B: Uncorrected data. Each data point is the mean of nine observations from three independent experiments, and error bars represent standard deviations.
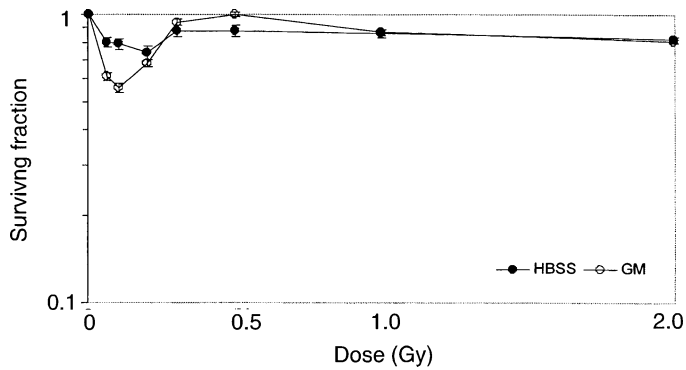
FIG. 4. Effect of liquid holding on hyper-radiosensitivity/induced radioresistance in BMG-1 cells. Cells plated at low density 18–20 h prior to irradiation were either irradiated in growth medium (○) or incubated in HBSS + 1% serum during and for 4 h after irradiation before being transferred to growth medium (●). Each data point is the mean of nine observations from three independent experiments, and error bars represent standard deviations.

TABLE 2
Relative Growth, Induced Micronucleus Fraction (Δ MF) and Apoptosis in High-Density BMG-1 Cells Observed at D_{hyp} (10 cGy) and 2 Gy

Dose	Relative growth at 72 h postirradiation ($PR_\gamma/PR_{control}$[a])	Induced micronucleus fraction (%) (Δ MF[b])	Percentage apoptotic cells[c]
0	1.0	0	0.07 ± 0.02
10 cGy	0.94 ± 0.08	0.7 ± 0.4	0.05 ± 0.03
200 cGy	0.90 ± 0.02	13.8 ± 2.7	0.04 ± 0.03

[a] PR (proliferation rate) = N_{Time}/N_{0h}.
[b] Δ MF = %MF_γ − %$MF_{control}$.
[c] Data from morphological analysis at 24 h postirradiation.

densities, as observed by morphological analysis. Using the flow cytometric Annexin-V binding assay at high cell density, no significant increase in the number of Annexin-V positive cells was observed at the hyper-radiosensitivity-inducing dose of 10 cGy, although an increase was observed at 2 Gy.

DISCUSSION

The present study demonstrates that low-dose hyper-radiosensitivity and induced radioresistance can be observed in mammalian cells using the conventional colony-forming assay when the cells are irradiated at low densities similar to those usually used in studies using DMIPS. Therefore, application of sophisticated and expensive techniques such as DMIPS or flow sorting may not always be necessary for studying hyper-radiosensitivity/induced radioresistance. The conventional assay may be particularly useful for studying low-dose responses in cell lines that form irregular colonies, since DMIPS is not suitable in such cases (*16*). The cell sorter-based plating method has its own disadvantages, because the exposure of cells to a laser pulse is likely to induce certain stress response signals (*22*).

All the cell lines investigated in this study demonstrated a multiphasic radiation survival curve at doses below 1 Gy, corroborating the earlier observations that hyper-radiosensitivity/induced radioresistance is quite common in human tumor cell lines, although some exceptions have been reported (*17, 37*). The extent and pattern of hyper-radiosensitivity and induced radioresistance varied considerably among the four cell lines investigated, although the maximum hypersensitivity was observed in the dose range of 10–30 cGy, in agreement with reports published previously (*15*). The occurrence of these effects within the same dose range indicates the possible existence of a highly conserved mechanism.

A positive, although weak, correlation was reported previously between the extent of induced radioresistance (ex-
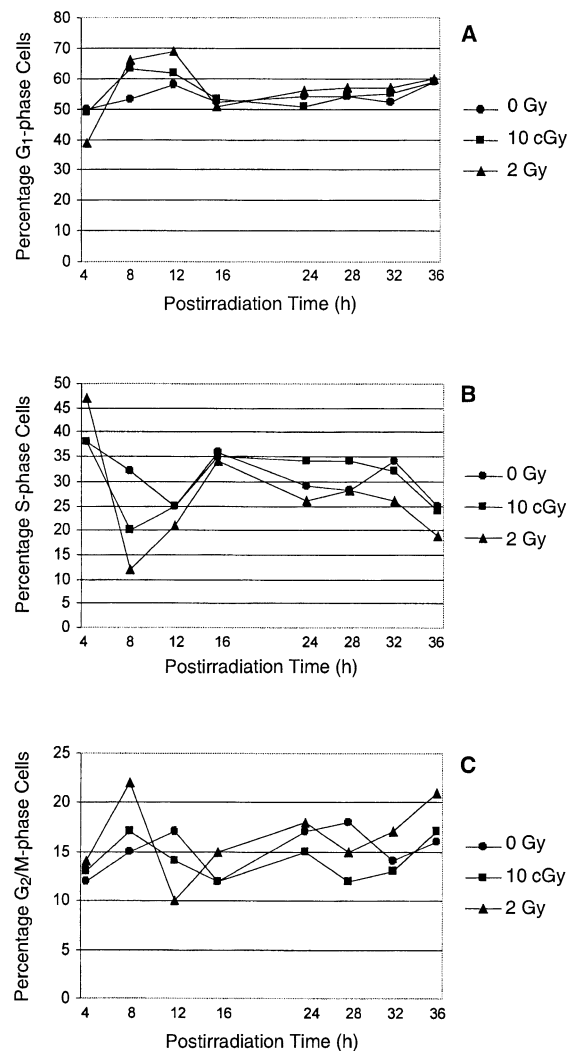

FIG. 5. Effect of D_{hyp} (10 cGy) and 2-Gy doses on cell cycle progression in BMG-1 cells irradiated at high cell density. Data on the cell cycle distribution as a function of postirradiation time are from a representative experiment for control cells (●) and cells irradiated with 10 cGy (■) or 2 Gy (▲).

pressed as the α_s/α_r ratio) and the SF2 values in a number of cell lines, suggesting that this response may be more pronounced in cell lines that have a relatively higher radioresistance at 2 Gy (*17*). However, the present observations with the four cell lines investigated here failed to support this concept (Table 1). This finding is in line with a recent review of published data for 28 cell lines (*37*). Based on these previous observations relating induced radioresistance to the SF2, it has also been speculated that hyper-radiosensitivity/induced radioresistance may be more prominent in cell lines originating from certain radioresistant tumors such as gliomas. In this study, the α_s values were distinctly higher in both glioma cell lines than in the two squamous carcinoma cell lines, although we did not find any relationship between induced radioresistance and radioresistance at 2 Gy (Table 1). Similarly, the increase in [–log (e) SF/Gy] values at the respective D_{hyp}'s compared to 2 Gy was also distinctly higher for the glioma cell lines than for the squamous carcinoma cell lines (Table 1). The relative efficacy of cell killing at doses below 50 cGy compared to that in the therapeutically relevant dose of 2 Gy is higher in glioma cells. Possibly, therefore, the gliomas might be better treated with radiation therapy given in low doses per fraction.

There has been considerable concern regarding the influence of intercellular interaction on the response of cells to radiation, since cell density varies considerably in tumors *in vivo*. Our results show that environmental factors such as the presence of other cells influence the expression of low-dose hypersensitivity. For example, when the cells were allowed to multiply for 48 h and were irradiated as small microcolonies, hyper-radiosensitivity was significantly reduced, and an enhanced induced radioresistance was evident at doses below 40 cGy. Irradiation and incubation at higher cell densities followed by trypsinization and plating at low cell densities for colony formation also reduced hyper-radiosensitivity, suggesting that cell–cell contact and intercellular communication protect cells against hyper-radiosensitivity. The fate of cells after irradiation (survival, death, mutation and transformation) is determined by competition between pathways involved in the induction of DNA and non-DNA lesions, error-free repair, misrepair and fixation of the induced lesions (*38*). These pathways are intimately connected with cell proliferation kinetics and damage-sensing and signal transduction processes. The molecular mechanisms underlying cell–cell contact-induced modulation of hyper-radiosensitivity or the contact effect remain to be explored. Alterations in the induction and repair processes by intercellular communication through gap junctions, cytoskeleton-/cell shape-induced changes, and alterations in chromatin organization have been discussed in connection with radioresistance observed at higher doses in human tumor xenografts and spheroids (*28, 29, 39*). The cell surface receptors and signaling cascades triggered by reactive oxygen species at the membrane can influence the regulatory networks controlling cell proliferation and/or DNA repair processes, and they therefore may be involved

in modulating cell contact-induced radioresistance. It is interesting to note in this context that tyrosine phosphorylation and dephosphorylation (through specific kinases and phosphatases) of certain signaling molecules have been observed to be influenced by cell density (*40, 41*). Such changes in the phosphorylation status of proteins can lead to changes in the activities of enzymes and transcription factors, leading to alterations in the expression of selected genes. Alterations in the expression of certain genes (such as *CDKN1A, GADD45, FOS, XIP8, DIR1*) have been observed after exposure to low radiation doses (*20, 42–46*). Our results suggest that studies to identify molecular signals triggered in response to low doses specifically under conditions of low cell density may provide important clues to the mechanisms underlying hyper-radiosensitivity/induced radioresistance.

It remains to be determined whether the radiation-induced signals associated with hyper-radiosensitivity are triggered primarily by DNA damage or by non-DNA damage. Recent observations on genomic instability and bystander effects have raised serious concerns about whether DNA damage is the major determinant of radiosensitivity, especially at doses below 0.5 Gy (*47, 48*). In the present study, a significant increase in the formation of micronuclei was not observed at the hyper-radiosensitivity-inducing low doses (Table 2), although the absence of cytogenetic damage does not necessarily rule out the involvement of subtle DNA damage in the induction of hyper-radiosensitivity. Vaganay-Juery *et al.* (*21*) reported that cell lines showing prominent hyper-radiosensitivity have reduced DNA-PK activity, indicating a possible direct involvement of DNA damage-sensing mechanisms. Vral *et al.* (*49*) observed enhanced micronucleation at doses below 50 cGy in B lymphocytes. This effect at low doses was not linked with the expression and activity of XRCC5 (also known as Ku86) protein.

In view of the importance of induction and repair of DNA double-strand breaks in radiation-induced cell lethality, it has been hypothesized that the non-homologous end joining (NHEJ) repair pathway plays an important role in induced radioresistance and that its absence or reduced functioning may be responsible for hyper-radiosensitivity (*21, 37*). The NHEJ pathway for DSB repair involves a crucial role of DNA-PK comprising the DNA-binding heterodimeric protein Ku (consisting of 70- and 86-kDa subunits) and the catalytic unit PRKDC (also known as DNA-PKcs). A significant proportion of the 70-kDa subunit has been shown to be localized at the cell surface with a membrane-spanning element, which could be part of a signal transduction system (*50, 51*). In sparsely growing cell cultures, the immunoreactive p70 antigen was observed to be predominantly cytoplasmic. Interestingly, however, enhancement of cell–cell contact was able to induce a redistribution of Ku antigen from the cytoplasm to the nucleus in primate cell cultures (*51*). Such a redistribution could explain the modulation of radiosensitivity as a function of cell multiplicity and density in cell cultures observed in the

present study, since binding of Ku proteins with the DNA damage sites is necessary for the functioning of the repair enzymes. Experiments to verify this possibility further are therefore warranted.

Differences observed in hyper-radiosensitivity at high and low cell densities could also be due to variations in the cell cycle distribution at the time of irradiation. However, in all the three conditions (cells plated at low density over-night before irradiation, high-density cultures plated after irradiation, and preplated cells grown into microcolonies before irradiation), cells were essentially in the exponential phase of growth at the time of irradiation. Further, similar frequencies of binucleated cells were observed in the cy-tochalasin B-treated BMG-1 cells seeded at low and high densities, implying that the cells in both cases have similar proliferation kinetics immediately after irradiation. There-fore, alterations of the low-dose sensitivity of cells ob-served under these three conditions in this study do not appear to arise from variations in the cell cycle distribution, and could be largely due to cell–cell contact.

The hyper-radiosensitivity-inducing low doses did not cause any significant cell growth inhibition, micronucleus for-mation, or apoptosis in BMG-1 cells (Table 2). The cell cycle distribution showed a transient G_1/S-phase block between 8 h and 16 h after exposure to 10 cGy. This block was less pro-nounced, however, than at 2 Gy (Fig. 5). Earlier studies have also reported G_1/S-phase transition delay in TP53 wild-type cells (52) coupled with up-regulation of the *CDKN1A* (*CIP1*/*WAF1*) gene (46), which in turn is known to be dependent on TP53. It is pertinent to note that BMG-1 cells showing a similar response also carry the wild-type *TP53* gene (31). However, hyper-radiosensitivity may not be linked to TP53 status, since two of the cell lines in the present study, U-87 and PECA4451, have nonfunctional and mutated TP53, re-spectively, and still show hyper-radiosensitivity/induced radio-resistance. The transient G_1/S-phase transition block observed at 10 cGy implies the stimulation of cell cycle regulatory processes in response to the damage induced at hypersensitive doses, although this cell cycle block is not unique to doses inducing hyper-radiosensitivity (and therefore may not repre-sent hyper-radiosensitivity).

Conditions that delay cell cycle progression after radia-tion exposure have been reported to enhance cell survival by promoting repair of potentially lethal damage (PLD) (53, 54). Such conditions may be provided to the cells through conditioned medium or isotonic balanced salt solutions that reduce cell metabolism and cell cycle progression (54–57). Decreased metabolic status of cells is thought to enhance cell survival by reducing damage fixation processes gen-erally associated with cell proliferation (38, 58). Reduction of hyper-radiosensitivity under nutrient deficient (liquid-holding) conditions in the present study suggests the in-volvement of cell progression-linked damage fixation pro-cesses in this phenomenon. These results suggest that while the nature and/or extent of damage at low doses causing hyper-radiosensitivity (below 0.5 Gy) may not be able to

stimulate error-free repair (as suggested by the "IR" mod-el), the lesion fixation processes are nevertheless activated, thereby causing increased cell lethality. Therefore, we pro-pose that hyper-radiosensitivity could arise partly because of the active fixation of radiation-induced lesions in the absence of a competitive repair process at low doses.

In summary, our findings suggest that hyper-radiosensi-tivity/induced radioresistance is common among tumor cell lines, as observed previously in other laboratories, and that glioma cells may have distinctly higher sensitivity at low doses. The damage-induced signal transduction processes influenced by (or mediated through) cell–cell contact and the cellular environment may play an important role in hy-per-radiosensitivity, and the exact nature of the damage pri-marily responsible for these responses needs to be investi-gated. Further studies on cell systems and experimental tu-mors are necessary for evaluating the effects of low doses *in vivo* as well as for understanding the exact mechanisms underlying hyper-radiosensitivity/induced radioresistance. The present study was carried out at a dose rate that com-pares well with the higher dose rates used earlier for low-dose studies (10) and that is also close to the dose rates used for radiotherapy. Studies are currently in progress in our laboratory on the effects of metabolic modifiers on hy-per-radiosensitivity/induced radioresistance, and prelimi-nary results indicate that induced radioresistance may have a strong dependence on the cellular energy status.

Irrespective of the precise molecular mechanisms in-volved, present results on the cell density-dependent alter-ations in hyper-radiosensitivity imply that the latter may be considerably reduced under *in* vivo conditions due to high cell density and intercellular contacts in the tissues. How-ever, hyper-radiosensitivity has been reported in normal tis-sues where a good cell–cell contact exists, although this response is less pronounced than that in tumor cell lines *in vitro* (17). Some reports have shown that the experimen-tally observed hyper-radiosensitivity/induced radioresis-tance *in vitro* and *in vivo* translates to the clinical situation for normal human skin (59, 60), although cell repopulation obscured the expression of hyper-radiosensitivity/induced radioresistance (37). However, *in vivo* effects still remain to be completely established (61). Normal and malignant tissues are likely to differ in this respect since the cell sur-face characteristics are altered in the transformed cells, and cell–cell contact as well as its influence on cell prolifera-tion, signal transduction, gene expression and cellular repair processes may vary. Systematic studies on animal tumor models are therefore essential for evaluating the effects of low-dose radiation.

REFERENCES

1. T. T. Puck and P. I. Marcus, Action of X-rays on mammalian cells. *J. Exp. Med.* **103**, 653–666 (1956).

2. C. S. Parkins and J. F. Fowler, The linear quadratic fit for lung function after irradiation with X-rays at smaller doses per fraction than 2 Gy. *Br. J. Cancer* **7** (Suppl.), 320–323 (1986).

3. M. C. Joiner, J. Denekamp and R. L. Maughan, The use of 'top-up' experiments to investigate the effect of very small doses per fraction in mouse skin. *Int. J. Radiat. Biol.* **49**, 565–580 (1986).

4. M. C. Joiner and H. Johns, Renal damage in the mouse: The response to very small doses per fraction. *Radiat. Res.* **114**, 385–398 (1988).

5. B. Palcic and B. Jaggi, The use of solid state sensor technology to detect and characterize live mammalian cells growing in tissue culture. *Int. J. Radiat. Biol.* **50**, 345–352 (1986).

6. L. D. Skarsgard, D. J. Wilson and R. E. Durand, Survival at low dose in asynchronous and partially synchronized Chinese hamster V79–171 cells. *Radiat. Res.* **133**, 102–107 (1993).

7. P. Lambin, B. Marples, B. Fertil, E. P. Malaise and M. C. Joiner, Hypersensitivity of a human tumor cell line to very low radiation doses. *Int. J. Radiat. Biol.* **63**, 639–650 (1993).

8. P. Lambin, B. Fertil, E. P. Malaise and M. C. Joiner, Multiphasic survival curves for cells of human tumor cell lines: Induced repair or hypersensitive subpopulation? *Radiat. Res.* **138** (Suppl.), S32–S36 (1994).

9. P. Lambin, B. Marples and M. C. Joiner, The effect of very low radiation doses on the human bladder carcinoma cell line RT112. *Radiother. Oncol.* **32**, 63–72 (1994).

10. B. Marples and M. C. Joiner, The response of Chinese hamster V79 cells to low radiation doses: Evidence of enhanced sensitivity of the whole cell population. *Radiat. Res.* **133**, 41–51 (1993).

11. M. C. Joiner, Induced radioresistance: An overview and historical perspective. *Int. J. Radiat. Biol.* **65**, 79–84 (1994).

12. B. Singh, J. E. Arrand and M. C. Joiner, Hypersensitive response of normal human lung epithelial cells at low radiation doses. *Int. J. Radiat. Biol.* **65**, 457–464 (1994).

13. B. G. Wouters and L. D. Skarsgard, The response of a human tumor cell line to low radiation doses: Evidence of enhanced sensitivity. *Radiat. Res.* **138** (Suppl.), S76–S80 (1994).

14. B. G. Wouters, A. M. Sy and L. D. Skarsgard, Low-dose hypersensitivity and increased radioresistance in a panel of human tumor cell lines with different radiosensitivity. *Radiat. Res.* **146**, 399–413 (1996).

15. B. Marples, P. Lambin, K. A. Skov and M. C. Joiner, Low dose hyper-radiosensitivity and increased radioresistance in mammalian cells. *Int. J. Radiat. Biol.* **71**, 721–735 (1997).

16. S. C. Short, C. Mayes, M. Woodcock, H. Johns and M. C. Joiner, Low dose hypersensitivity in the T98G human glioblastoma cell line. *Int. J. Radiat. Biol.* **75**, 847–855 (1999).

17. S. C. Short, S. A. Mitchell, P. Boulton, M. Woodcock and M. C. Joiner, The response of human glioma cell lines to low dose radiation exposure. *Int. J. Radiat. Biol.* **75**, 1341–1348 (1999).

18. B. Marples and M. C. Joiner, The elimination of low dose hypersensitivity in Chinese hamster V79–39A cells by pretreatment with X rays or hydrogen peroxide. *Radiat. Res.* **141**, 160–169 (1995).

19. B. Marples and M. C. Joiner, Modification of survival by DNA repair modifiers: A probable explanation for the phenomenon of increased radioresistance. *Int. J. Radiat. Biol.* **76**, 305–312 (2000).

20. T. Robson, M. E. Price, M. L. Moore, M. C. Joiner, V. J. McKelvey-Martin, S. R. McKeown and D. G. Hirst, Increased repair and cell survival in cells treated with DIR1 antisense oligonucleotides: implications for induced radioresistance. *Int. J. Radiat. Biol.* **76**, 617–623 (2000).

21. S. Vaganay-Juery, C. Muller, E. Marangoni, B. Abdulkarim, E. Deutsch, P. Lambin, P. Calsou, F. Eschwege, B. Salles and J. Bourhis, Decreased DNA-PK activity in human cancer cells exhibiting hypersensitivity to low-dose irradiation. *Br. J. Cancer* **83**, 514–518 (2000).

22. A. Dasu and J. Denekamp, Inducible repair and intrinsic radiosensitivity: A complex but predictable relationship? *Radiat. Res.* **153**, 279–288 (2000).

23. P. J. Deschavanne, M. Guichard and E. P. Malaise, Radiosensitivity of mouse kidney cells determined with an *in vitro* colony method. *Int. J. Radiat. Oncol. Biol. Phys.* **6**, 1551–1557 (1980).

24. R. T. Mulcahy, M. N. Gould and K. H. Clifton, The survival of thyroid cells: *In vivo* irradiation and *in situ* repair. *Radiat. Res.* **84**, 523–528 (1980).

25. C. A Wallen, S. M. Michaelson and K. T. Wheeler, Evidence for an unconventional radiosensitivity of rat 9L subcutaneous tumors. *Radiat. Res.* **84**, 529–541 (1980).

26. M. Guichard, H. Dertinger and E. P. Malaise, Radiosensitivity of four human tumor xenografts: Influence of hypoxia and cell–cell contact. *Radiat. Res.* **95**, 602–609 (1983).

27. Y-M. Jen, C. M. L. West and J. H. Hendry, The lower radiosensitivity of mouse kidney cells irradiated *in vivo* than *in vitro*: A cell contact effect phenomenon. *Int. J. Radiat. Oncol. Biol. Phys.* **20**, 1243–1248 (1991).

28. R. E. Durand and R. M. Sutherland, Effects of intercellular contact on repair of radiation damage. *Exp. Cell Res.* **71**, 75–80 (1972).

29. P. L. Olive and R. E. Durand, Drug and radiation resistance in spheroids: Cell contact and kinetics. *Cancer Metast. Rev.* **13**, 121–138 (1994).

30. H. Moussa, S. A. Mitchell, R. Grenman and M. C. Joiner, Cell–cell contact increases radioresistance in head and neck carcinoma cell lines. *Int. J. Radiat. Biol.* **76**, 1245–1253 (2000).

31. B. S. Dwarakanath, F. Zolzer, S. Chandna, T. Bauch, J. S. Adhikari, W-U. Muller, C. Streffer and V. Jain, Heterogeneity in 2-deoxy-D-glucose induced modifications in energetics and radiation responses of human tumor cell lines. *Int. J. Radiat. Oncol. Biol. Phys.* **50**, 1051–1061 (2001).

32. M. M. Elkind and G. F. Whitmore, *The Radiobiology of Cultured Mammalian Cells.* Gordon & Breach, New York, 1967.

33. R. J. Cummins, C. Mothersill, C. B. Seymour, H. Johns and M. C. Joiner, The effect of microcolony size, at time of irradiation, on colony forming ability. *Int. J. Radiat. Biol.* **75**, 225–232 (1999).

34. M. Fenech and A. Morley, Solutions to the kinetic problem in the micronucleus assay. *Cytobios* **43**, 233–246 (1985).

35. B. S. Dwarakanath and V. K. Jain, Enhancement of the radiation damage by 2-deoxy-D-glucose in organ cultures of brain tumors. *Indian J. Med. Res.* **82**, 266–268 (1985).

36. G. Koopman, C. P. M. Reutelingsperger, C. A. M. Kuijten, R. M. J. Keehnen, S. T. Pals and M. H. J. van Oers, Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* **84**, 1415–1420 (1994).

37. M. C. Joiner, B. Marples, P. Lambin, S. C. Short and I. Turesson, Low-dose hypersensitivity: Current status and possible mechanisms. *Int. J. Radiat. Oncol. Biol. Phys.* **49**, 379–389 (2001).

38. V. K. Jain, V. K. Kalia, R. Sharma, V. Maharajan and M. Menon, Effects of 2-deoxy-D-glucose on glycolysis, proliferation kinetics and radiation response of human cancer cells. *Int. J. Radiat. Oncol. Biol. Phys.* **11**, 943–950 (1985).

39. H. Dertinger, M. Guichard and E. P. Malaise, Relationship between intercellular communication and radiosensitivity of human tumor xenografts. *Eur. J. Cancer Clin. Oncol.* **20**, 561–566 (1984).

40. D. B. Batt and T. M. Roberts, Cell density modulates protein-tyrosine phosphorylation. *J. Biol. Chem.* **273**, 3408–3414 (1998).

41. G. Pani, R. Colavitti, B. Bedogni, R. Anzevino, S. Borrello and T. Galeotti, A redox signaling mechanism for density-dependent inhibition of cell growth. *J. Biol. Chem.* **275**, 38891–38899 (2000).

42. A.V. Prasad, N. Mohan, B. Chandrasekar and M. L. Meltz, Induction of transcription of immediate early genes by low-dose ionizing radiation. *Radiat. Res.* **143**, 263–272 (1995).

43. T. Robson, H. Lohrer, D. G. Hirst, M. C. Joiner and J. Arrand, Modulation of gene expression by low-dose ionizing radiation. *Radiat. Res.* **141**, 112–113 (1995).

44. S. Sadekova, S. Lehnert and T. Y. Chow, Induction of PBP74/mor-

talin/Grp75, a member of the hsp70 family, by low doses of ionizing radiation: Possible role in induced radioresistance. *Int. J. Radiat. Biol.* **72**, 653–660 (1997).

45. D. A. Boothman, E. Odegaard, C. R. Yang, K. Hosley and M. S. Mendonca, Molecular analysis of adaptive survival responses (ASRs): Role of ASRs in radiotherapy. *Hum. Exp. Toxicol.* **17**, 448–453 (1998).

46. S. A. Amundson, K. T. Do and A. J. Fornace, Jr., Induction of stress genes by low doses of gamma rays. *Radiat. Res.* **152**, 225–231 (1999).

47. C. Mothersill and C. Seymour, Genomic instability, bystander effects and radiation risks: Implications for development of protection strategies for man and environment. *Radiat. Biol. Radioecol.* **40**, 615–620 (2000).

48. C. B. Seymour and C. Mothersill, Relative contribution of bystander and targeted cell killing to the low-dose region of the radiation dose–response curve. *Radiat. Res.* **153**, 508–511 (2000).

49. A. Vral, H. Thierens, P. Bryant and L. De. Ridder, A higher micronucleus yield in B- versus T-cell after low-dose γ-irradiation is not linked with defective Ku86 protein. *Int. J. Radiat. Biol.* **77**, 329–339 (2001).

50. B. S. Prabhakar, G. S. Allaway, J. Sriniwasappa and A. L. Notkins, Cell surface expression of the 70-kD component of Ku, a DNA-binding nuclear autoantigen. *J. Clin. Invest.* **86**, 1301–1305 (1990).

51. J. W. Fewell and E. L. Kuff, Intracellular redistribution of Ku immunoreactivity in response to cell–cell contact and growth modulating components in the medium. *J. Cell Sci.* **109**, 1937–1946 (1996).

52. A. S. Hendrikse, A. J. Hunter, M. Keraan and G. H. Blekkenhorst, Effects of low dose irradiation on TK6 and U937 cells: Induction of p53 and its role in cell-cycle delay and the adaptive response. *Int. J. Radiat. Biol.* **76**, 11–21 (2000).

53. R. A. Philips and L. J. Tolmach, Repair of potentially lethal damage in X-irradiated HeLa cells. *Radiat. Res.* **29**, 413–432 (1966).

54. G. Iliakis, Radiation-induced potentially lethal damage: DNA lesions susceptible to fixation. *Int. J. Radiat. Biol.* **53**, 541–584 (1988).

55. J. A. Belli and M. Sheldon, Potentially lethal radiation damage: Repair by mammalian cells in culture. *Science* **165**, 490–492 (1969).

56. J. B. Little, Repair of potentially lethal radiation damage in mammalian cells: Enhancement by conditioned medium from stationary cultures. *Int. J. Radiat. Biol.* **20**, 87–91 (1971).

57. T. T. Kwok and R. M. Sutherland, The influence of cell–cell contact on radiosensitivity of human squamous carcinoma cells. *Radiat. Res.* **126**, 52–57 (1991).

58. H. Utsumi and M. M. Elkind, Two forms of potentially lethal damage have similar repair kinetics in plateau- and in log-phase cells. *Int. J. Radiat. Biol.* **47**, 569–580 (1985).

59. C. S. Hamilton, J. W. Denham, M. O'Brien, P. Ostwald, T. Kron, S. Wright and W. Dörr, Underprediction of human skin erythema at low doses per fraction by the linear quadratic model. *Radiother. Oncol.* **40**, 23–30 (1996).

60. I. Turesson and M. C. Joiner, Clinical evidence of hypersensitivity to low doses in radiotherapy. *Radiother. Oncol.* **40**, 1–3 (1996).

61. H. H. Dubben, B. Roper and S. Brackrock, Is there sufficient evidence of hypersensitivity to low doses in radiotherapy? *Radiother. Oncol.* **43**, 324–325 (1997).