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The Response of a Human Tumor Cell Line to Low Radiation Doses: Evidence of Enhanced Sensitivity

B. G. Wouters and L. D. Skarsgard

Department of Medical Biophysics British Columbia Cancer Research Centre, 601 West 10th Avenue, Vancouver, British Columbia V5Z 1L3, Canada

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The survival of asynchronous, exponentially growing DU-145 human tumor cells was measured after single doses of X rays in the dose range of 0.05–4 Gy using the cell sorting assay. When the response was modeled with the linear-quadratic (LQ) equation, a good fit to the data was observed for dose levels above 1 Gy; however, a region of enhanced sensitivity was observed at doses less than this. One possible explanation of this low-dose substructure is that a small, sensitive subpopulation of cells is selectively killed at low doses. Modeling of the radiation response with a two-population LQ model suggests that for these data this explanation is unlikely. Another possibility is that the whole cell population is initially hypersensitive, becoming radioresistant as damage is sustained by the cell. Conceivably this radioprotective mechanism could act in one of two ways. The cell could move from a radiation-sensitive to a radiation-resistant state by a continuous function of dose, or alternatively, only after a sufficient accumulation of damage, i.e. a "triggering dose." Both of these possibilities have been explored in the results of fitting two "induced resistance" models.

INTRODUCTION

Measurement of cell survival at low doses of radiation is complicated by the presence of random errors in counting, plating and dilution, associated with standard clonogenic assays (*1*). Two methods have been used to improve upon this resolution: the microscopic assay, which recognizes and counts cells individually (*2–4*), and the cell sorting assay, optimized in this laboratory (*5–10*). The cell sorting assay allows us to count and plate an accurately known number of cells, thereby changing the survival distribution from Poisson to binomial, and removing much of the random error associated with dilution and plating. Secondly, the assay retains the ability to make multiple measurements of survival involving a large number of cells. With this ability, data averaging can be used to effectively remove much of the other random error associated with survival measurement. Data averaging is the only way to reduce the error associated with the random distribution of the radiation event itself, and thus becomes particularly important at low doses where the initial damage level is small, and hence the variance comparatively large.

Previously with this assay (*6–8*; Skarsgard *et al.*, manuscript in preparation), a large amount of data with greater accuracy was obtained, allowing a rigorous fitting procedure. Fits to the data with the linear-quadratic (LQ) equation revealed substructure in the radiation survival response, with separate fits to the low-dose (0–4 Gy) and high-dose (4–14 Gy) regions giving significantly different values for the parameters in the LQ model. Using cell cycle synchronization procedures, we were able to show that this substructure was most likely due to subpopulations of sensitive G_1 and G_2-phase and resistant S-phase cells (*7–9*). The data also showed a very good fit to a two-population LQ model (*10*).

In the present study we have adapted the cell sorting assay to measure survival at very low doses. Evidence of an enhanced radiation sensitivity at low dose (less than 1 Gy) has been reported both for fractionated doses *in vivo* (*11*), and for single doses *in vitro* (*2, 3*) from measurements made using the Dynamic Microscope Image Processing Scanner (DMIPS) microscopic assay. The objective of the current work was to determine the effects of very low doses of radiation on DU-145 human tumor cells as measured with the cell sorting assay.

MATERIALS AND METHODS

Cell Culturing Techniques

The human prostate carcinoma cell line DU-145 used in these studies was obtained from the American Type Culture Collection (ATCC). It is a radioresistant cell line, with a surviving fraction at 2 Gy of approximately 0.70. The cells were maintained in monolayer culture *in vitro*, in McCoy's 5A medium supplemented with fetal bovine serum (10% by volume), penicillin (80 units/ml), streptomycin (80 μg/ml) and sodium bicarbonate (2.2 g/l). Cultures were grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The monolayers used for asynchronous experiments were observed to cover 50 to 70% of the growth surface of the flasks, ensuring an exponentially growing population. Cells were harvested by trypsinization, centrifuged at 90*g* for 6 min and resuspended in pH-balanced McCoy's 5A medium (lacking sodium bicarbonate) and loaded into a water-jacketed (37°C) spinner flask for a total volume of 45–55 ml at a concentration of 5 to 6 × 10^5 cells/ml. Irradiation of the cell population was then performed. Trypsinization of monolayers consisted of discarding the overlying medium, washing the flask (175 cm^2, Falcon) twice with 10 ml of 0.25% trypsin solution (Difco), incubating with the trypsin for 8 min, followed by neutralizing the trypsin with 8 ml of McCoy's medium.

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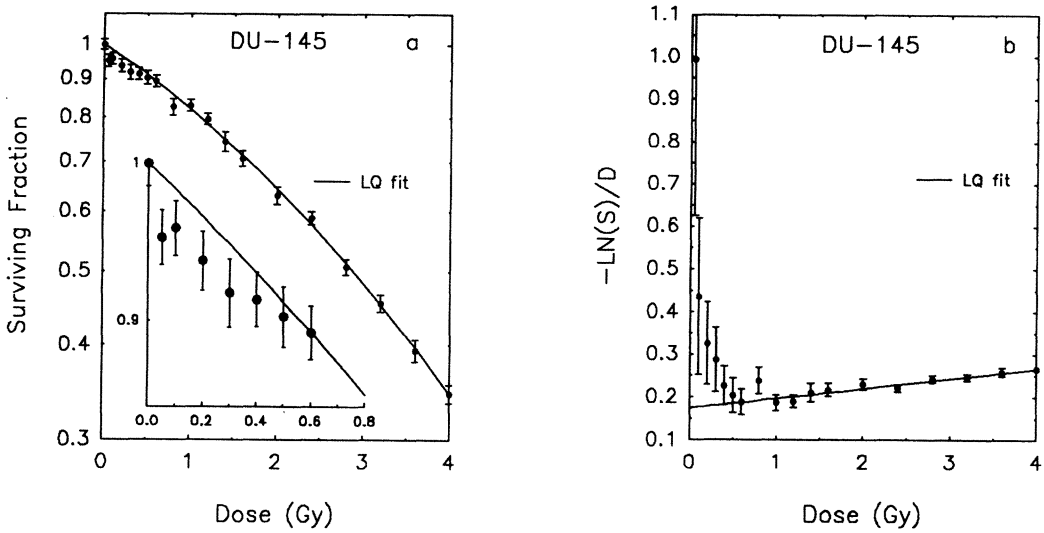

FIG. 1. Survival data for DU-145 cells irradiated at 37°C in suspension under aerobic conditions. Data are the average of eight experiments. Error bars are the standard error in the mean. Data are plotted in the form a) surviving fraction vs dose, and b) $-(\ln S)/D$ vs D. The fitted lines represent the best fit of the data to the LQ equation, $S = \exp(-\alpha D - \beta D^2)$, over the dose range (1 to 4 Gy).

Irradiation and Plating

Cell populations were irradiated in water-jacketed (37°C) spinner flasks with 250 KVp X rays (Philips RT-250,HVL 1.5 mm Cu) at a dose rate of either 0.51 Gy/min (for doses up to 1.6 Gy) or 2.21 Gy/min (for doses from 2 to 4 Gy) as determined by Fricke and ionization dosimetry. The cells were gassed for 15 min prior to and during irradiation with humidified air at 37°C. Two samples were removed after each dose increment with between 37 and 43 samples being accumulated in under 20 min, over the dose range 0 to 4 Gy. Samples were placed in a rotary shaker bath at 37°C prior to FACS counting. A cell sorter (Becton Dickinson 440) was used to deliver a known number of cells from each sample by identifying the cells on the basis of light scattering without the use of a cell stain (*5*). From each sample, the FACS machine sorted cells into three aliquots of 37°C McCoy's medium, which were subsequently plated into 100 mm petri dishes, along with 14 ml of McCoy's medium and 7×10^4 heavily irradiated (70 Gy) feeder cells. Enough cells were plated (600–1600) to produce 400–500 colonies/petri dish after 10 days of incubation. The petri dishes were then stained with malachite green and colonies containing greater than 50 cells were counted.

Data Analysis

The mean survival and the standard error in the mean were computed for each individual dose point. Parameters in different survival models were calculated by fitting this data set to the model of interest with a nonlinear curve-fitting program. This program uses the Levenberg-Marquardt algorithm to minimize the χ^2 value efficiently,

$$\chi^2 = \sum_{i=1}^{m} \frac{\left(\bar{S}_i - S_{fit}\right)^2}{\sigma_i^2}, \qquad (1)$$

for all m data points in the survival curve. $\bar{S}_i$ is the mean survival at each dose point, and

$$\sigma_i = \sqrt{\frac{\sum_{j=1}^{n}\left(\bar{S}_i - S_{ij}\right)^2}{n(n-1)}} \qquad (2)$$

is the standard error in the mean, calculated for all n measurements at each dose point. Minimization of χ^2 provides effective weighting of the data according to the scatter in the survival measurement.

RESULTS

Figure 1a shows the radiation survival response of DU-145 cells to radiation doses in the range 0 to 4 Gy. The survival points are averages of eight independent survival curves obtained on 2 separate days. Each data point represents the average survival calculated from scoring approximately 24,000 colonies from 48 petri dishes, except for zero-dose controls which are the average of about 36,000 colonies from 72 petri dishes. The entire response encompasses survival measurements calculated from scoring over 400,000 colonies. The error bars plotted are the standard error in the mean.

The solid line in Fig. 1a represents fitting the data above 1 Gy to the LQ model:

$$S_{fit} = e^{-\alpha D - \beta D^2} \qquad (3)$$

Above about 0.6 Gy there is a good fit to the data, but at doses less than this there is a clear deviation from this line, with survival levels below that predicted by the LQ fit. To illustrate this effect better, the data are plotted in the form of effect per unit dose $[-\ln(S)/D]$ versus dose. If the data follow the LQ model, then the model should yield a linear response, with a y-intercept of α and slope β:

$$\frac{-\ln(S)}{D} = \alpha + \beta D. \qquad (4)$$

TABLE I
Best-Fit Values for the Parameters in Each of Four Different Models

Model	Parameter						
	α_s (Gy^{-1})	α_r (Gy^{-1})	β (Gy^{-1})	Sensitive (%)	D_c (Gy)	c	χ^2 (minimum)
Linear-quadratic		0.174 (0.157–0.192)	0.023 (0.021–0.025)				18.5
Two-population linear-quadratic	[a] (7.08–?)	0.141 (0.127–0.156)	0.030 (0.027–0.033)	3.14 (2.1–5.0)			11.0[b]
Variable-β	0.930 (0.489–1.38)	0.173 (0.156–0.191)	0.023 (0.021–0.026)		0.137 (0.076–0.187)		6.68
Accumulated damage induced radioresistance	0.418 (0.262–0.579)	0.176 (0.158–0.193)	0.023 (0.020–0.025)			11	8.82

Notes. Parameters in the linear-quadratic equation were obtained from fitting the data above 1 Gy. Values given in parentheses below the best-fit value are the lower and upper 95% confidence limits in the fitted parameters.

[a] In the two-population linear-quadratic model the best-fit value increased without bound as the fit converged. The quoted value in parentheses is the lower 95% confidence limit.

[b] Calculated using the lower 95% confidence limit in α_s.

Figure 1b shows the data plotted in this form. The effect per unit dose expresses the effectiveness of the radiation on survival at each particular dose level. Here it is evident that this value reaches a maximum as the dose approaches zero, corresponding to the initial steep slope in the survival response of Fig. 1a. With increasing dose, the effectiveness decreases rapidly to a minimum at about 0.6 Gy, corresponding to the flat portion of the survival curve where the previous increment in dose has had a very small effect on survival. Above this dose, the data join the LQ fit to the data.

To investigate the possibility that this low dose hypersensitivity was due to a small subpopulation of cells, the data were fitted to a two-population LQ model:

$$S_{\text{fit}} = fe^{-\alpha_s D} + (1-f)e^{-\alpha_r D-\beta D^2}. \qquad (5)$$

The best fit of the data to this equation is plotted as a dashed line in Figs. 2a and 2b. In this model, the population is hypothesized to be made up of a sensitive fraction, f, which is killed exponentially with dose (characterized by α_s), and the remaining fraction, $(1-f)$, which follows a linear-quadratic response (characterized by α_r and β). Also plotted (solid curve) is the fit calculated with the lower 95% confidence limit of α_s. The fitted parameters are listed in Table I.

The data were also fitted to two "induced resistance" models. The first model is one suggested by Joiner and Johns (*11*), which is a modification of the

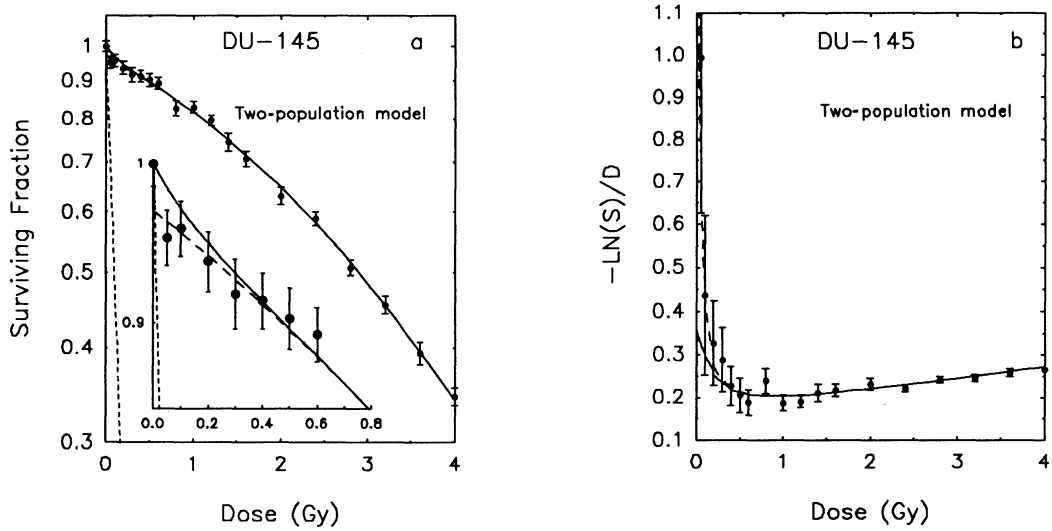

FIG. 2. The fitted lines represent the best fit of the data to the two-population LQ model over the entire dose range (0 to 4 Gy). The solid line represents the fit to the data using the lower 95% confidence limit in the fitted parameter, α_s of the sensitive population (see Discussion). Also plotted is the response calculated using the best fit value of α_s (long dashed line) and the response of the sensitive population alone (short dashed line).

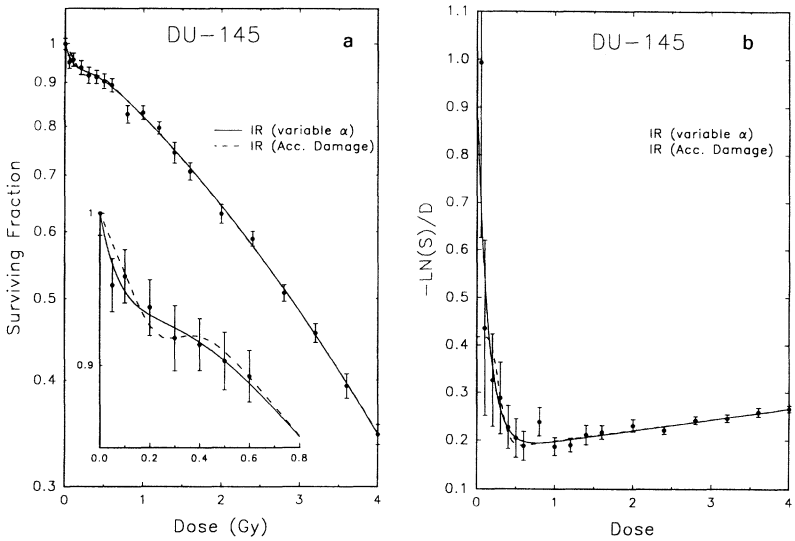

FIG. 3. The fitted lines represent the best fit of the data to the two "induced radioresistance models" over the entire dose range (0 to 4 Gy). Shown are fits to the variable-α model (solid line) and the accumulated damage induced radioresistance model (dashed line).

linear-quadratic model in which α becomes dependent on dose:

$$S_{\text{fit}} = e^{-\alpha_r[1+(\alpha_s/\alpha_r-1)e^{-D/D_c}]D-\beta D^2}. \qquad (6)$$

This fit to the data is shown as the solid line plotted in Figs. 3a and 3b. In this model the sensitivity of the cell changes from the most sensitive state to the most resistant state as a continuous function of dose. The best-fit values for these parameters are shown in Table I.

The second induced resistance model is one which assumes that at any dose level there exist two populations of cells, those with and without a radioprotective mechanism "turned on." Furthermore, it is assumed that these populations are defined by the level of damage which has accumulated within the cell. Cells which have received more than some critical level of damage reside in a protective state and follow an LQ response (characterized by α_r and β), while those cells receiving less than this level reside in the sensitive state and are inactivated exponentially (characterized by α_s). The fraction of sensitive cells is therefore dose dependent and can be calculated assuming a Poisson distribution in the level of damage. This accumulated damage induced radioresistance model can be described by the equation

$$S_{\text{fit}} = f_1(D)e^{-\alpha_s D} + f_2(D)e^{-\alpha_r D-\beta D^2}, \qquad (7)$$

where

$$f_1(D) = \sum_{n=0}^{c} \frac{a^n e^{-a}}{n!} \text{ and } f_2(D) = 1 - f_1(D).$$

a = average damage level; c = critical damage level. For example, if one considers double-strand breaks (DSBs) as the important lesion, then a would represent the average number of DSBs for a particular dose, and c would represent the number of DSBs after which the radioprotective mechanism is induced. The best fit to the data is plotted as the dashed line in Figs. 3a and 3b, and the best-fit values for the parameters are shown in Table I.

DISCUSSION

Using the FACS assay we have measured the response of DU-145 cells to low doses of radiation resulting in survival levels in the first decade of cell killing. At dose levels beyond roughly 0.6 Gy the data are consistent with an LQ response, but at doses smaller than this the survival is below that predicted by the model. We have tested two possible explanations for this observation, as evaluated by fitting the data to different models.

We have explored the possibility that the increased low dose sensitivity is due to a subpopulation of cells, perhaps in some particular phase of the cell cycle. The survival data were fitted with the two-population LQ model (Eq. 5), and the results, shown in Table I, suggest that these sensitive cells would make up about 3% of the population and be extremely sensitive. It was found that the value for α_s increased without bound, limited only by the tolerated convergence level in the χ^2 value. For this reason we quote only the lower 95% confidence limit of this parameter. Even this value, 7.08 Gy^{-1}, which when used gives a poor fit to the data, (χ^2 =11.0, solid line of Figs. 2a and 2b) would indicate a subpopulation roughly 35 times more sensitive than the remaining population (calculated at an isosurvival level of 0.67). This seems unlikely, given that it has been reported that the radiosensitivity is believed to vary by less than a factor of 10 throughout the cell cycle (*12*). Further evidence that this hypothesis is unlikely comes from fitting the higher dose data to the three-parameter equation,

$$fe^{-\alpha D-\beta D^2}. \qquad (8)$$

When the data from 0.6, 0.8 or 1 Gy onward were fitted, the best estimate for f was between 0.985 and 1.015, suggesting the survival response above 0.6 Gy was not due to only a subpopulation of cells. Here the maximum dose was limited to 4 Gy to avoid the substructure associated with mixed populations (G_1, G_2 and S-phase cells) as described elsewhere (*6–10*; Skarsgard *et al.*, manuscript in preparation).

The apparent hypersensitivity at low dose could also be a reflection of an induced radioprotective mechanism, with two methods of "induction" investigated here. The first is the variable α model suggested by Joiner (Eq. 6) in which the cell's intrinsic sensitivity is a function of dose. The second method is represented by the accumulated damage induced radioresistance model (Eq. 7), in which all cells reside in either a sensitive or a resistant state subject to their level of damage. The accumulated damage induced radioresistance model was fitted to the data assuming that DSBs were the lesion of interest, and used an estimated average of 40 DSBs/cell/Gy (*13*). The best fit occurred with the critical damage level equal to 11, meaning that any cell with 11 or fewer DSBs was sensitive to the radiation, and followed an exponential survival with slope α_s = 0.418 Gy^{-1}. This value represents a starting population which is somewhat less sensitive than that determined using the variable α model (α_s = 0.930 Gy^{-1}). As can be seen in Figs. 3a and 3b, both of these models give good fits to the data within the error associated with measurement, although the variable α model has a somewhat smaller χ^2 value (6.68 vs 8.82). The accumulated damage induced radioresistance model predicts a flatter response (dashed line) in the 0.2 to 0.5-Gy range than does the variable α model (solid line), and although there are not enough data to determine the actual trend, the point at 0.3 Gy suggests a flat region. The accumulated damage induced radioresistance model also predicts a slight rise in survival at 0.4 Gy, and although not seen in these data, Lambin has reported such a response in another human tumor line (*3*). The variable α model predicts a more gradual change in sensitivity and better predicts the survival at the lowest dose.

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