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Cytogenetic characterization of low-dose hyper-radiosensitivity in Cobalt-60 irradiated human lymphoblastoid cells

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ABSTRACT

The dose-effect relationships of cells exposed to ionizing radiation are frequently described by linear quadratic (LQ) models over an extended dose range. However, many mammalian cell lines, when acutely irradiated in G2 at doses ≤0.3 Gy, show hyper-radiosensitivity (HRS) as measured by reduced clonogenic cell survival, thereby indicating greater cell lethality than is predicted by extrapolation from high-dose responses. We therefore hypothesized that the cytogenetic response in G2 cells to low doses would also be steeper than predicted by LQ extrapolation from high doses. We tested our hypothesis by exposing four normal human lymphoblastoid cell lines to 0–400 cGy of Cobalt-60 gamma radiation. The cytokinesis block micronucleus assay was used to determine the frequencies of micronuclei and nucleoplasmic bridges. To characterize the dependence of the cytogenetic damage on dose, univariate and multivariate regression analyses were used to compare the responses in the low- (HRS) and high-dose response regions. Our data indicate that the slope of the response for all four cell lines at ≤20 cGy during G2 is greater than predicted by an LQ extrapolation from the high-dose responses for both micronuclei and bridges. These results suggest that the biological consequences of low-dose exposures could be underestimated and may not provide accurate risk assessments following such exposures.

1. Introduction

Radiation-induced damage and the associated cancer risks are well known for high doses, however substantial uncertainty still exists about the health risks associated with low-dose exposures [1–4]. Human exposure to radiation has increased considerably over the past few decades due to more frequent use of CT imaging and radiological procedures. Currently the estimates of the biological damage at low-dose exposures are based on linear extrapolation of the available high dose (>100 cGy) data. However at low-dose exposures (≤100 cGy), radiation responses have been observed to deviate from linearity [2,5].

Clonogenic cell-survival assays involving mammalian cells exposed to X-rays have identified hyper-radiation sensitivity (HRS) effects, where increased cell death is observed at low doses (≤50 cGy) accompanied by induced radiation resistance (IRR) at slightly higher doses (≥60 cGy) [6–10]. HRS has been investigated for increased tumor cell killing as a way to treat cancer and might also protect normal tissues against carcinogenesis following low-dose radiation exposures [11]. The potential clinical significance of HRS is not well understood [10], and the mechanisms underlying the HRS/IRR effect are unclear. However, evidence suggests that faithful DNA repair and effective cell cycle regulation may be diminished at very low doses [12,13]. Radiation-induced DNA damage triggers activation of checkpoints in the G1/S, S, and G2 phases of the cell cycle which allow time for repair of damaged DNA before cell cycle progression recommences [14]. The HRS/IRR effect is predominantly observed in G2 [7,15,16], suggesting that the rapidly-occurring G2 cell cycle checkpoint regulates the mechanisms of the HRS/IRR effect. During G2, radiation-induced DNA damage is verified by two distinct cell cycle checkpoints, one which arrests cells that have incurred damage in G1 or S [17] and the other (called the rapid G2/M check point) is triggered when cells in G2 are exposed to radiation [18]. The rapid G2/M checkpoint depends on the ataxia telangiectasia mutated (ATM) protein and is known to activate at ≥40 cGy [18,19]. Thus, cells exposed to doses below 40 cGy in G2 may fail to recognize the damage and enter mitosis before adequate DNA repair occurs, thereby exhibiting an HRS effect [9,15,20,21].

Here we investigated cells exposed to Cobalt-60 gamma rays in G2 by comparing the slopes of the dose-responses for micronuclei

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and nucleoplasmic bridges in the low-dose region (<50 cGy) to responses at higher doses (>60 cGy). The data for four independent normal human lymphoblastoid cell lines indicate that cells irradiated in G2 with doses ≤20 cGy show increased cytogenetic damage per unit dose compared to doses of 60 cGy and higher, and thus exhibit an HRS effect. We also evaluated the shape of the dose-responses of the G2 irradiated cells and compared them to the responses of the G1 irradiated cells for all four cell lines. The data indicate that at low doses, the responses of the G2 irradiated cells deviate from linearity, thus suggesting that back-extrapolation of the high dose responses may lead to underestimates of the actual damage. These findings suggest that the increased cell killing in the HRS region of G2 irradiated cells seen by others reflects the cytogenetic damage observed here, emphasizing the importance for better understanding of the HRS effect and the influence it can have on radiation risk assessments following low-dose exposures.

2. Materials and methods

2.1. Cell lines and cell culture

Four normal human lymphoblastoid cell lines GM15036, GM15510, GM15268, and GM15526 obtained from the Coriell Cell Repository were used in this study. The cells were cultured based on a standard protocol provided by Coriell. Briefly, the cells were grown in T-25 flasks (ISC BioExpress, Kaysville, UT) containing 10 ml of RPMI1640 medium (GIBCO, Grand Island, NY and Hyclone, Logan, UT) supplemented with 15% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA), 2 mM L-glutamine (GIBCO, Grand Island, NY), penicillin–streptomycin (100 units/ml penicillin G Sodium, 100 μg/ml streptomycin sulfate in 0.85% saline) (GIBCO, Grand Island, NY) and fungizone (amphotericin B, 2.5 μg/ml, 0.2 μm filtered) (Hyclone, Logan, UT) with loosened caps. Cell cultures were maintained at 37 °C and 5% CO_2 in a fully humidified incubator, and passaged every 3–4 days when their density reached 10^6 cells/ml.

2.2. Irradiation

Twenty-four hours after seeding the flasks, the cells were acutely exposed to Cobalt-60 γ-radiation at a dose rate of approximately 28 cGy/min at the Gershenson Oncology Center, Wayne State University, Detroit, MI. Each cell line was individually exposed to 0, 5, 10, 20, 40, 60, 80, 100, 200, 300, or 400 cGy. Each experiment was independently replicated once for each cell line.

2.3. Cell cycle kinetics and harvest

To measure cell cycle kinetics, flow cytometric analyses were performed on GM15036 and GM15510 cells by the Flow Cytometry Core facility, Wayne State University. The length of the G2 phase in GM15510 and GM15036 cells was determined to be approximately 1.5 and 2 h, respectively, and the cell cycle durations were approximately 20 and 22 h for GM15510 cells and GM15036 cells, respectively. The harvest times were based on these results to ensure that most binucleated cells scored were in G2 to observe a hyper-radiation sensitivity/induced radiation resistance (HRS/IRR) effect [22] or in G1 for the comparison experiments.

The growth rates of GM15268 and GM15526 cells were determined by a cell viability assay, using 0.4% Trypan blue (in phosphate buffered saline; Fisher Scientific, Pittsburgh, PA). Briefly, cells were seeded at an initial concentration of 3×10^5 cells/ml and cultured as described above. An aliquot of cells was taken every 24 h and the number of live cells per ml was determined using a standard hemocytometer (Fisher Scientific, Pittsburgh, PA). The growth kinetics of both the GM15268 and GM15526 cells were comparable to those of GM15510 and GM15036, thus the harvest times were established as 2 and 22 h after radiation to probe the G2 and G1 phases, respectively.

2.4. Cytokinesis-blocked micronucleus (CBMN) assay

Cytogenetic damage was measured using the CBMN assay. Immediately following irradiation, 6 μg/ml of Cytochalasin B (final concentration; Sigma Aldrich, St. Louis, MO) was added to the flasks, which were immediately returned to the incubator. At harvest, the cells were resuspended and spun onto ethanol-cleaned microscope slides using a cytocentrifuge (Statspin, Westwood, MA) at 1300 rpm for 4 min. The slides were air dried, fixed in 100% methanol (Fisher Scientific, Pittsburgh, PA) for 15 min, stained with 10% Giemsa (Sigma Aldrich, St. Louis, MO) dissolved in water for 15 min, then briefly rinsed in water and air-dried.

2.5. Data collection

Slide readers were extensively trained prior to data collection. An equal number of readers scored equivalent numbers of cells for each treatment condition for all four cell lines, both replicates and both harvest times (G1 and G2). To ensure that adequate numbers of binucleated cells were available after irradiation, the nuclear division index (NDI) was calculated as described [23]. For each treatment condition, 200 cells were counted to determine the frequency of cells with 1, 2, 3 or 4 nuclei using the formula

$$\mathrm{NDI} = \frac{M_1 + 2M_2 + 3M_3 + 4M_4}{N}$$

where M_1 to M_4 represent the number of cells with 1 to 4 nuclei and N is the total number of cells scored.

2.6. Cell scoring criteria

The slides were evaluated for micronuclei, nucleoplasmic bridges and buds simultaneously, based on established criteria [24,25] using Nikon Eclipse E200 light microscopes at 1000× magnification. Briefly, all cells scored were binucleated where the two main nuclei were separate from each other and the cell membranes were intact. The micronuclei were required to be one-third the size of the main nuclei or smaller, smooth edged, round or oval, stained similar to the main nuclei, and had to be located within the cytoplasm. The nucleoplasmic bridges were required to be colored similar to the nuclei and to be connected to both nuclei. Although buds were scored, they were relatively infrequent and did not yield meaningful results, so these data are not reported here. All slides were coded prior to scoring to avoid observer bias. At least 1000 binucleated cells were scored for each treatment condition.

2.7. Statistical analyses

The dependence of cytogenetic damage on dose was evaluated to characterize the HRS/IRR effect by comparing the responses in G1 and G2, and to evaluate the shape of the responses of G2-irradiated cells with those of the G1-irradiated cells. The slopes of the responses were evaluated by performing univariate linear regression analyses independently on each cell line and replicate, and by multivariate linear regression analyses on the combined data for both replicates and all four cell lines using JMP software, version 6.0, SAS Institute Inc. For univariate analyses the frequencies of MN and bridges per 1000 binucleated cells were independently regressed against dose. For multivariate analyses the frequencies of MN and bridges per 1000 binucleated cells were evaluated in linear regression models using dose, cell line, cell cycle phase, experimental replicate, and an interaction term for cell line