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THE EFFECT OF FRACTIONATED IRRADIATION ON CELL KINETICS

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Abstract

The effects of single and split-dose irradiation were compared by in vitro experiments on HeLa cells. Changes in rate of cell proliferation were detected by flow cytometry, simultaneously determining the DNA content and the bromodeoxyuridine incorporation of individual cells. Cell cultures were irradiated with either a single dose of 1–6 Gy or with a corresponding dose divided into multiple fractions given at 1–6-h intervals. A dose-dependent accumulation of cells in G2/M phase was observed. The method was sensitive enough for the detection of G2/M block even after 1 Gy. The block disappeared completely within a 24-h follow-up time at dose levels up to 3 Gy. Interestingly, no differences in cell kinetics were observed between the single and split-dose regiments. This approach proves to be valuable in evaluating novel fractionation models and the effects of radiation on the cell kinetics of human tumor cells.

Key words: Irradiation, x-rays, fractionation, bromodeoxyuridine-labelling, flow cytometry, cell kinetics, in vitro.

The effects of ionizing irradiation on cell proliferation are expressed in all cell cycle phases, but they are most prominent in S and G2 phases (1–4). The major manifestation of radiation-induced cell cycle redistribution in mammalian cells is G2 block (5, 6). Cells are susceptible to x-ray-induced G2 delay through most of the cell cycle (1). The target for the G2 block is located in or near the nucleus (7) or possibly in a structure associated with DNA (8). The delay in progression of cells in the cycle is not necessarily caused by the DNA damage per se (9). The increase in nuclear protein content after irradiation has been suggested to be part of the mechanisms of G2 arrest (10). It is evident that the same target, inducing the delay, is present throughout the cell cycle (11).

It is known that irradiation induces redistribution of the cell cycle and partial synchronization of previously asynchronous cells affecting and changing the sensitivity of cells to subsequent radiation doses (4, 12). The change in sensitivity is dependent on the intervals between the fractions. The interest in accelerated fractionation to increase the therapeutic gain (to minimize repopulation during conventional fractionation; 13, 14) has emphasized the need to measure cell kinetic parameters, such as potential doubling time in human tumours (15). However, until recently there has been no practical method of estimating the cell doubling time and cell kinetics in vivo. The development of a monoclonal antibody against DNA-incorporated bromodeoxyuridine and of flow cytometric methods for quantifying BrdUrd and DNA contents simultaneously (16, 17) have enabled cell kinetic analysis in vitro as well as in vivo (18–25). Since the BrdUrd technique is relatively non-toxic (as a single dose), quantitative, rapid and sensitive, it has proved to be suitable for measuring kinetic parameters of human tumours in vivo (18, 23–26). To gain information on tumours irradiated with multiple fractions per day, as in vitro model system was used for studying the effects of different irradiation doses with variable time schedules on HeLa cells.

Material and Methods

Cell culture. Asynchronously grown HeLa cells were cultured as monolayers in Dulbecco's modified Eagle's medium (Gibco, Paisley Scotland, UK) supplemented with 10% fetal bovine serum and antibiotics (Flow laboratories, UK). Cells were kept in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. In the experiment the cells were at an exponential growth phase after subculture in completely fresh medium.

Pulse labelling with bromodeoxyuridine (BrdUrd). For the determination of the passage of HeLa cells through the

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cell cycle, they were labelled with 10 μmol/l BrdUrd (Sigma Chemicals Co., St. Louis, MO) immediately before the irradiation. Cells were washed twice with PBS without Ca^{++} and Mg^{++} after a 1-h incubation period and then recultured in the fresh medium. For staining and analysis, cells were removed from flasks using trypsin-EDTA (Gibco). Samples were collected every 2 hours up to 24 h or in multiple fractions after the beginning of G2/M block.

Irradiation. Irradiation was carried out at room temperature using 6 MV photons from a 6 MeV linear accelerator, at a dose rate of 2 Gy/min. Doses of 1–6 Gy were given as a single dose or as a corresponding dose divided into multiple fractions at 1–6 h intervals. In the meanwhile the control cells were kept at room temperature.

Cytochemistry. Cells were prepared for analysis of DNA and BrdUrd contents according to Dolbeare et al. (17), with slight modifications. Briefly, the cells were fixed at 70% ethanol cooled to −20°C. DNA was denaturated with 2N HCL and neutralized with 0.05 mol/l $Na_2B_4O_7$. Thereafter the cells were incubated in Tween-BSA (0.05% Tween-20, 0.5% bovine serum albumin in PBS) supplemented with 1.0% rabbit serum for 15 min followed by incubation with mouse monoclonal antibodies to BrdUrd (Becton-Dickinson, Mountain View, Ca) diluted 1:20 in Tween-BSA. The cells were washed with Tween-BSA and incubated with FITC-labelled $F(ab)_2$ fragments of rabbit anti-mouse immunoglobulins (Dakopatts, Glostrup, Denmark) for 1 h. The level of non-specific binding of mouse IgG was tested by incubating cells with normal mouse serum followed by FITC-labelled $F(ab)_2$ fragments of rabbit anti-mouse immunoglobulins. Finally, the cells were incubated with propidium iodide (PI; 5 μg/ml in PBS; Sigma) and kept in ice for 1 h before the analysis.

Flow cytometry. Two-color fluorescence was measured using an EPICS'C flow cytometer (Coulter Electronics LTD, Hialeah, FLA) equipped with a 2 W-argon ion laser. The excitation wavelength was 488 nm at a light output of 200 mW. The green signal from FITC-conjugated antibody (measured on logarithmic scale) was collected between 515–530 nm and the red signal from PI (measured on linear scale), over 610 nm. The slight cross-over fluorescence, caused by PI and recorded by the green detector, was corrected by fluorescence subtraction. At least 2×10^4 cells were analyzed from each sample. The G1 peak was centered at channel number 50. The FCM data were visualized in two-parameter histograms, using a Coulter computer and software. The changes in cell kinetics were studied in two ways. First, the duration of the G2/M phase and the irradiation-induced G2/M block was monitored by the entry of cells in the G1 phase as a function of time. A window with a width of three channels was centered on the G1 peak and the number of green fluorescent (BrdUrd-labelled) cells in the window was compared with the total cell number (27). The proportion of BrdUrd-labelled cells in the G1 phase was expressed as percentages of the total population and plotted against the time after irradiation. Second, the movement of cells through the cell cycle was followed by calculating the mean BrdUrd content in the mid S-phase as a function of time. A region of 4 channels was set midway between the G1 and G2 peaks. The proportion of labelled cells in the mid S phase window was expressed as a percentage of the total number of the S-phase cells in the window and plotted against the time after irradiation. The duration of the S phase was estimated by measuring the midpoint between the maximum and minimum values for the green fluorescence (Ts/2). The movement of the cells through the cell cycle (Tc) was followed by calculating the mean BrdUrd content in the mid S phase as a function of time (28, 29).

Results

The cell cycle of unirradiated and irradiated HeLa cells. The effect of irradiation on the cell cycle of HeLa cells is demonstrated in Fig. 1. In Fig. 1a the radiation induced effects (at 2 Gy and 6 Gy) are presented as two parameter histograms, with unirradiated cells as controls. A clear separation of BrdUrd-labelled (S phase) and unlabelled (BrdUrd-negative; G1 and G2/M phases) populations was observed both in unirradiated and irradiated cells 1 h after the BrdUrd pulse. As early as 6 h after the pulse the rate of progression of the irradiated cells in the cell cycle was slower than that of the unirradiated cells. At that time point in unirradiated samples, a fraction of labelled late S phase cells had gone through mitosis and were entering the G1 phase, whereas the irradiated cells were still accumulating in the late S and G2/M phases. Ten hours after the BrdUrd pulse, a dose-dependent delay was observed as increasing accumulation of labelled cells into the G2/M phase at 6 Gy, whereas cells irradiated with 2 Gy had already started to divide. DNA histograms showed a clear dose-dependent accumulation of irradiated cells in the late S and G2/M-phases (Fig. 1b).

Cell cycle parameters of unirradiated and irradiated HeLa cells. For unirradiated cells the cell cycle (Tc) was 22 h and the labelling index 33%. Ts was about 10 h and the duration of the G2/M about 5 h (Figs 2 and 3). The movement of irradiated HeLa cells through the mid S phase was equal or slightly delayed compared with the unirradiated cells. After the division there was a dose-dependent decrease in the rate of proliferation of irradiated cells in the cell cycle (Fig. 2). The irradiation-induced G2/M delay in labelled cells was demonstrated by the entry of the cells into the G1 phase and calculated from the midpoint of the minimum and maximum values of green fluorescence in the G1 phase (Fig. 3). The irradiation-induced G2/M delays were 2.2 h, 3.5 h, 5.5 h, and

7.5 h for doses of 1 Gy, 2 Gy, 3 Gy, and 6 Gy respectively. The formation of the G2/M block began immediately after irradiation with doses of 1–3 Gy and 1.5 h later with 6 Gy (data not shown). The maximum accumulation of labelled cells in the G2/M phase was achieved at about 6 h, 8 h, 10 h, and 14 h after 1 Gy, 2 Gy, 3 Gy and 6 Gy irradiation

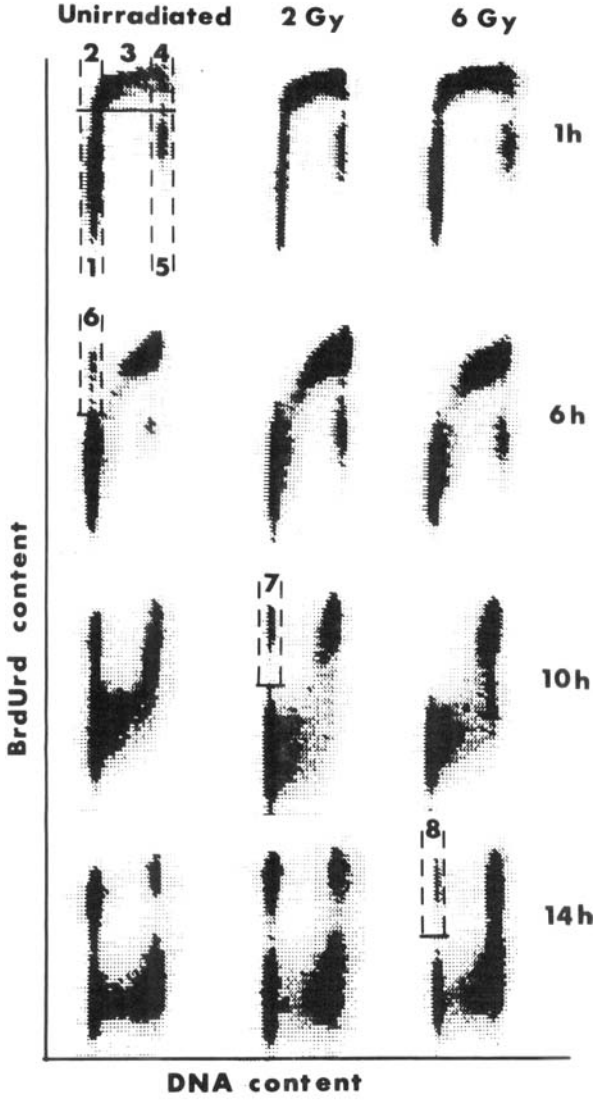

Fig. 1a. The progression of HeLa cells in the cell cycle before (column unirradiated) and after irradiation (columns 2 Gy and 6 Gy) as seen in two parameter histograms [x-axis, DNA content in a linear scale; y-axis, bromodeoxyuridine (BrdUrd) content in a logarithmic scale]. The analysis was performed 1 h, 6 h, 10 h, and 14 h after a 1-h BrdUrd pulse. BrdUrd-labelled cells are seen above the solid line (column unirradiated at 1 h). Dotted lines separate the G1, S, and G2/M phases on the basis of their DNA content. The following categories of cells are seen: Box 1 = unlabelled (BrdUrd-negative) G1 phase cells; Box 2 = labelled early S phase cells, Box 3 = labelled mid-S phase cells; Box 4 = labelled late S phase cells; Box 5 = unlabelled G2/M phase cells. Note that unirradiated cells that were initially labelled in S phase have progressed into G1 phase within 6 h (Box 6), whereas irradiated cells arrive in G1 within 10 h (2 Gy, Box 7) and 14 h (6 Gy, Box 8).

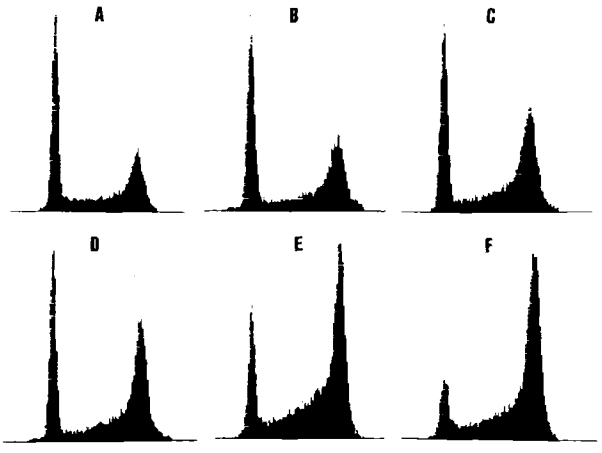

Fig. 1b. DNA histograms from HeLa cells 14 h after split-dose irradiation. Y-axis = relative cell number; X-axis = DNA amount (red fluorescence from PI). A = Unirradiated; B = 1 Gy; C = 2 Gy; D = 3 Gy; E = 4 Gy; F = 6 Gy.

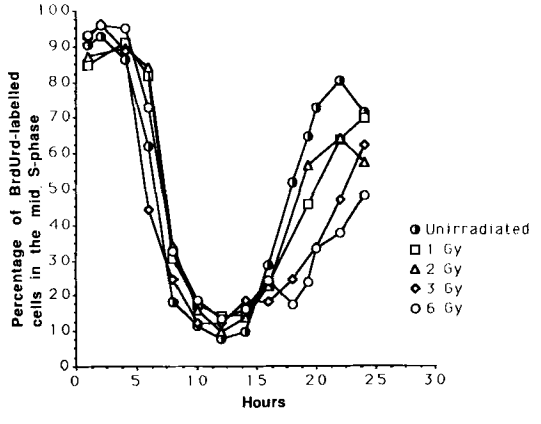

Fig. 2. The cell cycle of unirradiated and irradiated (1–3 Gy and 6 Gy) HeLa cells. (Values are means from two independent experiments).

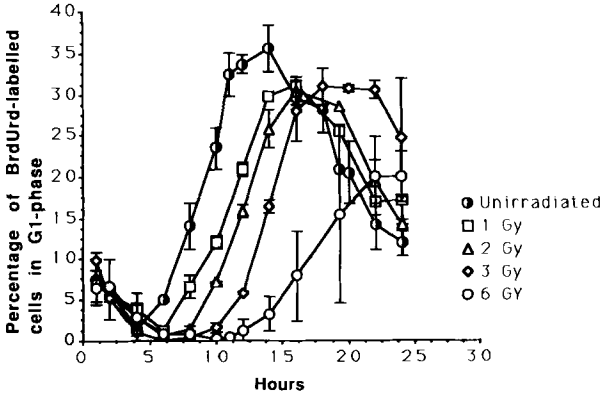

Fig. 3. Irradiation-induced G2/M block monitored by the entry of labelled S phase cells into the G1-phase. The measurement points represent X ± SD from two experiments.

respectively. The delay (h)/Gy was about 1.7 with smaller doses (1–3 Gy) and about 0.8 with the higher dose (6 Gy). In addition, after the division of irradiated cells, a dose-dependent delay in the progression of labelled G1 phase cells into the S phase was observed.

Fractionation. No major difference was found in cell kinetics between the single doses (1–6 Gy) and the corresponding doses divided into two equal parts given at a 4-h interval, measured 14 h and 18 h after irradiation (Fig. 4a). The arrival of cells in the G1 phase was in linear correlation to the radiation dose. It was interesting that no major differences in cell kinetics was found whether the 6 Gy dose was divided into 3 (3 × 2 Gy) or 4 (4 × 1.5 Gy) fractions given at either 2- or 4-h intervals (Fig. 4b). The effect of the time interval between the fractions was more thoroughly investigated with total doses of 2 and 3 Gy divided into two equal fractions given at 1 h, 2 h, 4 h and 6 h. During short-term study (24-h follow-up), no clear difference was observed in cell kinetics (Fig. 5).

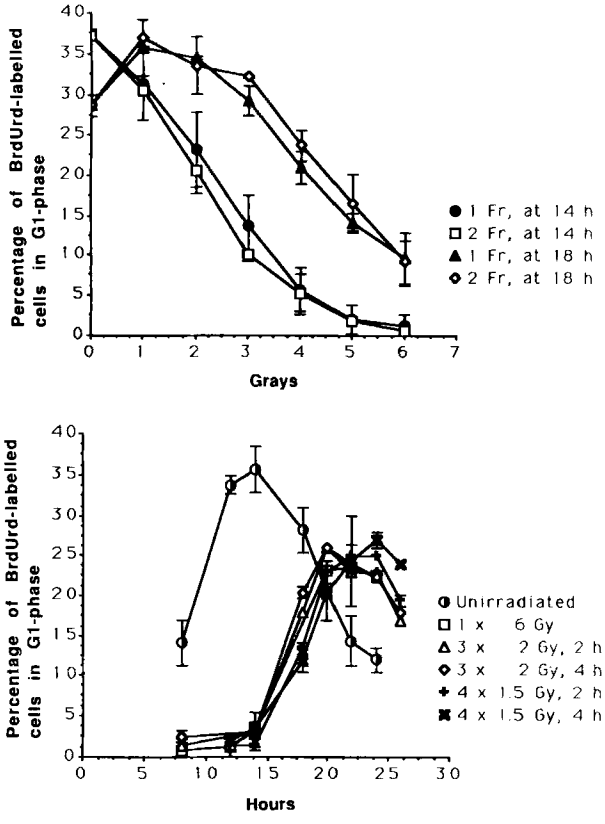

Fig. 4. The effect of split-dose irradiation on HeLa cell kinetics analyzed on the basis of the entry of labelled cells into G1 phase.

a) A single dose (1–6 Gy) and a split-dose divided into two equal fractions given at a 4-h interval were compared. The measurements were carried out at 14 h and 18 h after irradiation. The measurement points represent X ± SD from two experiments.

b) Split-dose regimens (3 × 2 Gy and 4 × 1.5 Gy) and a corresponding single dose. In each case doses were separated either by 2 h or 4 h. The measurement points represent X ± SD.

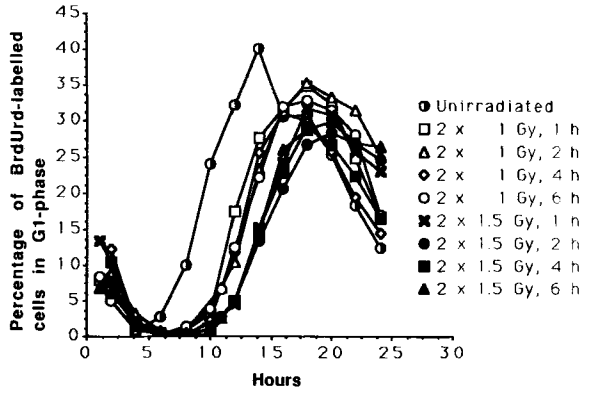

Fig. 5. The effect of time interval (1–6 h) between two equal fractions analyzed on the basis of the entry of labelled cells into G1 phase. The doses were 2 × 1 Gy and 2 × 1.5 Gy.

Discussion

Split-dose irradiation has been extensively used for examining the repair of sublethal injury in vitro and in vivo (1, 5, 30–32). The proportion of surviving cells is greater after split-dose irradiation than after single-dose irradiation, as the cells are able to repair the damage to some extent within a few hours. Prolongation of the interval up to 6 h between the two equal 3.5 Gy fractions increases the proportion of surviving HeLa cells (12), due to sublethal damage repair and to the fact that a proportion of the cells have progressed into a less sensitive phase of cell cycle (5, 12). As cells continued cycling they passed into a more sensitive phase which was observed as decreased survival at 12 h after the first dose. In the present cell kinetic study, in contrast to cell survival studies, no differences in the effect were observed between single and split-dose irradiation given at various time intervals up to 6 h during the short 24-h follow-up time. We cannot, however, exclude the possibility that changes in the kinetics of asynchronously cultured HeLa cells are manifested very slowly during the following cell cycles.

Ionizing radiation causes dose-dependent delay in the cell cycle (5). In mammalian cells this is mostly due to a prolongation of the S and G2 phases (G2/M blockage; 1, 5, 6, 10, 27, 33, 34) or, in density-inhibited and slowly proliferating cultures, to a prolongation of the time during which the cells stay at the border of the G1/S phase (3, 4 35–37). We observed an irradiation-induced, linear, dose-dependent increase in delay of the late S and G2/M phases and the concomitant depletion of cells in the G1 phase. By the sensitive flow cytometric BrdUrd method we were able to demonstrate the G2/M delay even after 1 Gy. The sensitivity and usefulness of the BrdUrd method in radiobiological studies has earlier been shown in animal tumor model (27).

Human tumors have relatively long cell cycle times (38), and the mitotic delay (39), which is approximately 1/10 of the cell cycle time increases with the length of the cell cycle. This may explain why the irradiation-induced G1 or G2 delay in human tumors is much longer than in animal tumors (40, 41). In the present study with the near tetraploid HeLa cells, the duration of the delay was unlinearly dose-dependent. With lower doses (1–3 Gy) it was about 1.7 h/Gy, which is near that reported by Denekamp (39) and with higher dose, about 0.8 h/Gy.

Irradiation of exponentially growing asynchronous cells results in partial dose-dependent synchronization of surviving cells due to redistribution and G2 block (42–44). As different doses synchronize the cell cycle into different time scales and since the maximum radiosensitivity of most mammalian cells is in the G2/M phase, the optimal time for the second dose would be during the period when there is a maximal number of cells in the G2/M phase. With the HeLa cells the optimal time after 1 Gy, 2 Gy and 3 Gy would be about 6 h, 8 h, and 10 h, respectively, and after 6 Gy, about 14 h. This may indicate that in multiple dose fractionation more attention should be focused on optimizing the intervals between the fractions at different dose levels. This may be an important aspect in research on optimal fractionation of radiotherapy. This should be further examined in model systems as well as in clinical situations.

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