Radiation-induced Apoptosis: Effects of Cell Age and Dose Fractionation

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Abstract

The cell cycle dependence of radiation-induced apoptosis was measured using mitotically synchronized REC:myc(ch1) and Rat1:myc_v cells. Cells in S and G2 phases were more susceptible; the apoptotic fraction was about 0.7—0.8 as compared to about 0.4 for G1 cells at a dose of 10 Gy. Two-dimensional cytofluorimetric analysis of cells, pulsed-labeled with bromodeoxyuridine and then irradiated with 10 Gy, showed both G1 and G2 blocks (6—8 h) for REC:myc(ch1) cells but only G2 block for Rat1:myc_v cells. Consistent with these results, wild-type p53 and WAF1 (or p21), known to play a role in G1 delay, was induced by radiation in REC:myc(ch1) but not in Rat1:myc_v cells. The cell cycle dependence of radiation-induced apoptosis and the absence of a G1 block for Rat1:myc_v cells led to the prediction and observation of the novel “inverse split-dose effect,” i.e., a radiation dose given in two equal halves separated by a few hours yielded a higher level of apoptosis relative to that resulting from the same total dose given all at once. This effect is due to cell cycle progression from G1 to the more sensitive S-G2 phase during the interval between the split doses. In contrast, the inverse split-dose effect for apoptosis is absent for REC:myc(ch1), due presumably to the radiation-induced G1 delay. Parallel split-dose experiments, but using clonogenic survival as end points, show recovery for REC:myc(ch1) cells but not for Rat1:myc_v cells, reflecting the influence of split-dose, radiation-induced apoptosis.

Introduction

Since the observation of Kerr and Wyllie more than 20 years ago, it is now recognized that apoptosis, or programmed cell death, is important in embryonic development, homeostatic control of normal tissues, carcinogenesis, tumor development, and cancer therapy (1, 2). This mode of cell death, characterized by distinct steps of membrane blebbing, chromatin condensation, and DNA fragmentation, can also be induced in various cell types by insults such as UV, ionizing radiation, and hyperthermia (3—5). The genetic basis and signal transduction pathway of apoptosis is now under intensive investigations (6—12). Several laboratories have studied the phenomenon of radiation-induced apoptosis and suggested its potential relevance to cancer radiotherapy (13—17). Recently, we have reported on the enhancement and attenuation of radiation-induced apoptosis in rat cells by transfected c-myc and Ha-ras oncogenes, respectively (15). In addition, we quantified the dose response at different dose rates and concluded that, in susceptible cell lines, radiation-induced apoptosis contributes significantly in the shoulder portion of the survival curve, a region of importance for both fractionated radiotherapy and low-dose-rate brachytherapy (17). In this investigation, we studied the cell age dependence of radiation-induced apoptosis and the postirradiation cell cycle progression in REC:myc(ch1) and Rat1:myc_v cells. Using mitotically synchronized cells, we observed that the S- and G2-phase populations were more sensitive, relative to the cells in G1, for both REC:myc(ch1) and Rat1:myc_v. Two-dimensional cytofluorimetry data at various times after irradiation demonstrated both G1 and G2 blocks for REC:myc(ch1) but only a G2 block for Rat1:myc_v. Immunoblot analysis showed radiation-induced increase of wild-type p53 and WAF1 (p21) in REC:myc(ch1) but not in Rat1 or Rat1:myc_v. DNA sequence data, however, indicated that wild-type p53 was present in all three cell lines. The relative sensitivity of S-phase cells and the absence of a G1 block led to the novel observation of an “inverse split-dose effect” for radiation-induced apoptosis in Rat1:myc_v cells [but not in REC:myc(ch1) cells], i.e., a split-dose delivery yielded a higher level of apoptosis (and therefore less survival) than a single dose. Consistent with these results, split-dose experiments, with clonogenic survival as end points, show recovery for REC:myc(ch1) cells but not for Rat1:myc_v cells.

Materials and Methods

Cell Culture. As described previously, cell lines Rat1:myc_v and REC:myc(ch1) were derived by transfecting Rat1 and rat embryo cells with a retroviral vector pMV7/c-myc containing the human c-myc cDNA and the neomycin phosphotransferase gene (15). The presence and expression of the transfected oncogenes were verified by Southern and Northern analysis (15). For experiments using synchronized populations, cells were harvested by mitotic shake-off, plated, and held in a 37°C incubator for specific time periods before irradiation.

Irradiation and Assays of Cell Death. A Co-60 unit was used for irradiating cells in exponential growth phase, plated in tissue culture plates approximately 24 h previously. The decreased adherence of apoptotic cells to the culture plates was relied upon to estimate the fraction of cells dying by this mode (6). At about 40 h postirradiation, an optimum time as determined previously (15), the floating cells were collected, and the attached cells were trypsinized; then both fractions were quantified with a Coulter cell counter. The fraction of apoptotic cells was expressed as the ratio of the number of the floating cells to the total cell number. To validate this assay, we separated the floating cells from the attached cells and stained them with acridine orange or performed fluorescence analysis from nuclear extracts using methods described previously (15).

For colony formation assay, irradiated cells were trypsinized, appropriately diluted, plated into Petri dishes, and incubated at 37°C for 7—10 days before fixation and staining. Colonies contained >50 cells were considered as arising from surviving cells.

Two-dimensional Cytofluorimetry Analysis. Exponentially growing cells were labeled in 10 μM BrdUrd1 for 1 h at 37°C, rinsed twice, irradiated, and then incubated at 37°C for various periods. Then the cells were fixed sequentially with 70% ethanol and 2 N hydrochloric acid, centrifuged, and resuspended in 1 ml of 0.1 mM Na2B4O7 (pH 8.5) to neutralize the acid. For immunofluorescence staining, cells were resuspended in TWEEN 20/BSA/PBS and 20 μl of anti-BrdUrd added per 106 cells and incubated for 30 min. The cells were again centrifuged and resuspended in 50 μl of 0.5% Tween 20/BSA/PBS solution with 1 mg of FITC-conjugated F(ab')2 GAM IgG and incubated at room temperature for 30 min. For DNA staining, the cells were resuspended in 1 ml of sheath fluid containing 5 μg/ml of propidium iodide. The cells were then analyzed on a FACStar system.

Induction of p53 and p21WAF1 Protein by Radiation. Protein was prepared from control cells and from irradiated cells at various times after...
irradiation. Cells were lysed in 0.4 ml buffer [70 mM Tris-HCl (pH 6.8), 10% glycerol, 3% SDS, 10% β-mercaptoethanol, and 1% bromophenol blue]. Lysates were electrophoresed in 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane. Equivalent protein loading was verified by staining of the nitrocellulose membrane with Ponceau red. The membrane was destained and blocked in 5% milk in TTBS (10 mM Tris-HCl (pH 8.0), 0.1% Tween 20, and 150 mM NaCl) for 1 h, incubated with either p53 monoclonal antibody (Oncogene Science) or p21 polyclonal antibody (Santa Cruz Biotechnology, CA) in TTBS for 3 h, and then with sheep antimouse IgG for p53 and antirabbit IgG for p21, respectively (Boehringer Mannheim). After additional incubation in ECL reagents (Amersham Life Science) the membranes were exposed to X-ray films.

Results

Fig. 1 shows Rat1:myc, cells stained with acridine orange and photographed with a fluorescent microscope: Fig. 1a, control cells; Fig. 1b, irradiated but attached cells, 48 h after a 10-Gy treatment; and Fig. 1c, irradiated and detached cells. The irradiated and detached cells (Fig. 1c) exhibited considerable chromatin condensation, which was largely absent in control (unirradiated) cells or irradiated but attached cells (Fig. 1a and b, respectively). DNA fragmentation analysis of nuclear extracts revealed the characteristic "laddering" pattern for the detached cells but not for the attached cells (Fig. 1d). Thus, the use of reduced adherence as a criteria for quantifying the apoptotic fraction was valid, as was documented previously (15).

The cell cycle distribution of REC:myc(ch1) cells at various times after the mitotic shake-off, as determined by cytofluorimetric analysis, is given in Fig. 2a. At 2 and 4 h after harvest, the cells were predominantly in G1 (>95%), as compared to a G1 fraction of about 45% in an asynchronous population (Fig. 2a first column). At 6 h, the G1 population decreased to about 55% as S-phase cells increased to 44%. Between 8 and 10 h after harvest, the number of cells in S decreased, whereas the G2 population increased slightly. The observed cell phase progression was consistent with the cell cycle of about 12 h for this cell line. Similar results on cell cycle distribution after mitotic shakeoff were obtained for Rat1:myc, cells (data not shown). The fraction of apoptotic cells for samples irradiated at various times after the synchronization procedure are shown in Fig. 2, B and C, for REC:myc(ch1) and Rat1:myc, respectively. For both cell lines, samples irradiated 2–4 h after the mitotic shake-off yielded apoptotic fractions of 40–50%, significantly smaller than that (70–80%) of samples irradiated at the 8–10 h time points. Thus, cells in G1 (corresponding to the 2–4 h time points; Fig. 2a) were more resistant to radiation-induced apoptosis relative to cells in the S and G2 phases.

Two-dimensional cytofluorimetry data at various times after BrdUrd labeling and irradiation are shown in Fig. 3a for control (first row, sham-irradiated) and irradiated Rat1:myc, cells (second row, 10 Gy). In the control sample, at the 2-h time point, much of the BrdUrd-labeled cells have moved into G2. By 4–6 h, a significant
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Fig. 2. A, cycle distribution of REC:myc(ch1) cells at various times after mitotic shake-off. B, apoptotic cells induced in synchronized REC:myc(ch1) samples by 10 Gy delivered at various times after mitotic shake-off. C, apoptotic cells induced in synchronized Rat1:myc(ch1) samples by 10 Gy delivered at various times after mitotic shake-off. C, apoptotic cells induced in synchronized Rat1:myc(ch1) samples by 10 Gy delivered at various times after mitotic shake-off. In contrast to the obvious G2 delay, a G1 block was not observed in the irradiated Rat1:myc(ch1) cells. Experiments with REC:myc(ch1) cells demonstrated a G2 block (the 2-, 4-, and 6-h time points in the third row of Fig. 3A), similar to that observed in Rat1:myc(ch1). However, a G1 block was also observed; the unlabeled G1 cells of REC:myc(ch1) remained in G1 for at least 6–8 h longer after irradiation (the 2-, 4-, 6-, and 8-h time points in the third row of Fig. 3), relative to sham-irradiated cells (data not shown). A similar G1 block was observed for REC:ras(ch1) cells (data not shown).

Fig. 3b shows immunoblot analysis of p53 and WAF1 (p21) proteins in Rat1, Rat1:myc(ch1), and REC:myc(ch1) cells. Data are shown for control samples and for irradiated samples at 0.5, 1, and 2 h after treatment with 6 Gy. The same amount of proteins was used in each lane. Radiation induced enhanced expression of p53 in REC:myc(ch1) at 1 h after irradiation, and the WAF1 level was elevated at 2 h and beyond (data for longer times not shown). In contrast, neither p53 nor WAF1 was induced in the Rat1 and Rat1:myc(ch1) samples. When the p53 gene in each of the cell lines was sequenced, identical DNA sequences were obtained, corresponding to those of the wild type (data not shown).

Given the cell cycle dependence of apoptosis and the existence of a radiation-induced G1 block for REC:myc(ch1), but not for Rat1:myc(ch1), we postulated that split-dose apoptosis experiments would lead to different results for the two cell lines. Specifically, the level of apoptosis will increase for split-dose delivery in Rat1:myc(ch1) because of progression of G1 cells to the more sensitive S-G2 phase, whereas the G1 block in REC:myc(ch1) will minimize its split-dose apoptotic response. As shown in Fig. 4A, split-dose delivery (two fractions of 3, 5, or 7 Gy, separated by 0–8 h) produced approximately the same magnitude of apoptotic cells in REC:myc(ch1). In contrast, a significantly higher percentage of apoptotic cells was observed in Rat1:myc(ch1) for split-dose delivery than a single dose of 6, 10, or 14 Gy (Fig. 4B), as was postulated.

With colony formation as the end point, the surviving fraction of REC:myc(ch1) increased almost 300% as the time period between the split-dose increased from 0 to 4 h (Fig. 4C). In contrast, there was hardly any increase for Rat1:myc(ch1). The different fractionation effect of radiation-induced apoptosis (Fig. 4, A and B) most likely contributes to the difference in the observed split-dose recovery of these two cell lines.

Discussion

This is the first report on the cell cycle dependence of radiation-induced apoptosis, and such dependence has both biological and clinical significance. In terms of basic understanding, our data on the relative resistance of G1 cells explain in part the observation, by us and others (3, 13, 15), that a certain fraction of an asynchronous cell population is refractory to radiation-induced apoptosis. Although as yet not understood, the molecular basis for this cell cycle dependence would be of considerable importance. The radiation-induced G1 checkpoint delay, thought to be a cellular defense measure against damage (5, 9, 18, 19), is unlikely to be the basis, since only REC:myc(ch1) exhibited a G1 block, and yet the same cell cycle dependence was observed for both REC:myc(ch1) and Rat1:myc(ch1). The sensitive S phase of Rat1:myc(ch1) and REC:myc(ch1) cells to
radiation-induced apoptosis may appear to be in contradiction to the classical S-phase radioresistance as assayed by clonogenic survival. However, S-phase radioresistance is not universal, and some cell lines are not differentially radioresistant in the S phase (20, 21). In addition, most of the previous experimental cell survival data was obtained with cells that lacked an apoptotic response (13) such that the cell cycle dependence of radiation-induced apoptosis was not a factor. These considerations suggest additional studies on cell age dependence of radiosensitivity using cell lines of different responsiveness to radiation-induced apoptosis. The high sensitivity of S-phase cells to radiation-induced apoptosis may have significance for radiotherapy. For tumors susceptible to this mode of cell death, the S-phase cells would be relatively sensitive to apoptosis, and this may counterbalance the classical late-S phase radioresistance in the absence of apoptosis. That ectopic c-myc expression enhances apoptosis and that many human cancers involve c-myc amplification also suggest the potential importance of radiation-induced apoptosis in cancer radiotherapy. This is particularly relevant at the doses used in fractionated radiotherapy because radiation-induced apoptosis may be significant in the shoulder region of the survival curve (16, 17). Thus, it would be important to use a variety of human tumor cell lines to investigate the cell cycle dependence of both radiation-induced apoptosis and overall clonogenic survival at clinical relevant doses so as to understand their relative importance in fractionated radiotherapy.

We have shown previously that c-myc expression enhanced, and Ha-ras expression suppressed, radiation-induced apoptosis in the oncogene-transfected cell lines (15). In this study, two-dimensional cytofluorometric results showed that the postirradiation cell kinetics were not altered in Rat1:myc5, relative to the Rat1 cells. Similar data (not presented) was obtained for ras-transfected cells. Also, primary REC and oncogene-transfected REC exhibited the same postirradiation cycle progression kinetics (results not shown). Thus, in these cell lines, the modulating effects of c-myc and Ha-ras of radiation-induced apoptosis are not related to the G1 and G2 blocks (18, 22). Relative to ras-induced radioresistance as assayed by clonogenicity, McKenna et al. (22) have suggested that increased G2 delay may be the underlying reason.

Whereas there was no difference in the postirradiation cell cycle kinetics between the parental and the myc- or ras-transfected cells, a difference existed between the cell lines of REC and Rat1 origin. Specifically, a radiation-induced G1 block was observed for REC and its derivatives but not for Rat1 or cells of Rat1 origin. This difference appeared to be due to the induction of p53 and WAF1 in the former but not in the latter cell lines. Both wild-type p53 and its dependent transcript WAF1 are known to be involved in G1 arrest (18, 23, 24). Sequencing data, however, revealed that Rat1 and Rat1:myc6 contained the same wild-type p53 gene as REC:myc(ch1). Thus, the Rat1 and its derivatives appear to be like the human Burkitt's lymphoma cell line P119, which contains endogenous wild-type p53, but do not exhibit X-ray-induced p53, WAF1 expression or G1 arrest (25). Thus, although there exists a general relationship between endogenous wild-type p53 and radiation-induced p53 elevation, WAF1 activation, and G1 arrest, there are cell-specific exceptions, as has been documented by other investigators (25, 26).

Previous studies have implicated a role of wild-type p53 in radiation-induced apoptosis of thymocytes (5, 9) and in c-myc-mediated apoptosis in mouse and rat fibroblasts (27). That endogenous wild-type p53 is present in the apoptosis-susceptible, c-myc-transfected cell lines is consistent with this notion (although our data do not provide direct support for this relationship). Assuming that wild-type p53 is involved in radiation-induced apoptosis for both of our c-myc-transfected cell lines, the absence of G1 delay for Rat1:myc5 supports the premise that apoptosis is not secondary to p53-induced growth arrest but that there is a more direct relationship between wild-type p53 activity and cell death (23, 28).

The inverse split-dose effect of radiation-induced apoptosis, which
were given, separated by time intervals of 0—8 h. C, split-dose effect in REC:myc(ch1) and Rat1:myc, with clonogenic survival as the end point for two equal doses of 5 Gy each.

Fig. 4. Effect of split-dose radiation delivery on radiation-induced apoptosis for REC:myc(ch1) (A) and Rat1:myc(ch1) (B). Two equal doses (3, 5, and 7 Gy, respectively) were given, separated by time intervals of 0—8 h. C, split-dose effect in REC:myc(ch1) and Rat1:myc, with clonogenic survival as the end point for two equal doses of 5 Gy each.

leads to increased cell inactivation by apoptosis, is diametrically opposite to the Elkind-Sutton split-dose effect attributed to the repair of sublethal damage, which increases survival (29). The inverse split-dose effect is predicated upon the absence of a G1 block (as in Rat1:myc cells), permitting the progression of the “apoptosis-resistant” G1 cells to the “apoptosis-sensitive” S-G2 phase. Thus, it is not observed in cells with a G1 block, such as the REC:myc(ch1) cells. The putative influence of this phenomenon on split-dose clonogenic survival is consistent with our results (Fig. 4C); the presence of an inverse split-dose effect of radiation-induced apoptosis corresponds to reduced split-dose recovery assayed by colony formation.

The inverse split-dose effect may be an exploitable phenomenon in clinical radiotherapy. For the large variety of tumors with mutated p53, there would not be a G1 block, and two doses given several hours apart (as in hyperfractionation) would yield enhanced cell killing due to the inverse split-dose effect of apoptosis. In contrast, normal tissue with wild-type p53 expression would be preferentially spared, due in part to the presence of the G1 block and in part to sublethal damage repair (Fig. 4C).

References


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