Cell Cycle Disturbances and Mitotic Catastrophes in HeLa Hep2 Cells following 2.5 to 10 Gy of Ionizing Radiation

David Eriksson,1 Per-Olov Löfroth,2 Lennart Johansson,2 Katrine Ählström Riklund,3 and Torgny Stigbrand1

Abstract

Purpose: Experimental radioimmunotherapy delivering absorbed doses of 2.5 to 10 Gy has been shown to cause growth retardation of tumors. The purpose of this study was to elucidate the sequential molecular and cellular events occurring in HeLa Hep2 cells exposed to such doses.

Methods: Dose-response curves, activation of cell cycle checkpoints, and mitotic behavior were investigated in HeLa Hep2 cells following 2.5- to 10-Gy irradiation by carrying out 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays, Western blots, fluorescence-activated cell sorting analysis, and immunofluorescence stainings. Terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling staining was used to detect apoptosis.

Results: A G2-M arrest was shown by fluorescence-activated cell sorting analysis. p53 and p21 were found to be up-regulated but were not immediately related to the arrest. The G2-M arrest was transient and the cells reentered the cell cycle still containing unrepaired cellular damage. This premature entry caused an increase of anaphase bridges, lagging chromosomal material, and multipolar mitotic spindles as visualized by propidium iodide staining and immunofluorescence staining with α-tubulin and γ-tubulin antibodies. Furthermore, a dose-dependent significant increase in centromere numbers from 12.6 ± 6.6% to 67 ± 5.3% was identified as well as a dose-dependent increase of polyplody cells from 2.8 ± 1.3% to 17.6 ± 2.1% with the highest absorbed dose of 10 Gy. These disturbances caused the cells to progress into mitotic catastrophe and a fraction of these dying cells showed apoptotic features as displayed by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining 5 to 7 days after irradiation.

Conclusion: An absorbed dose of 2.5 to 10 Gy was shown to force HeLa Hep2 cells into mitotic catastrophe and delayed apoptosis. These might be important cell death mechanisms involved in tumor growth retardation following radioimmunotherapy of solid tumors.

In every cell, accurate cell division is important to maintain genomic stability and prevent mutations from being propagated. This requires correct replication of DNA and an even distribution of chromosomes to the two daughter cells. Several types of DNA lesions are induced by ionizing radiation, including changes in the bases of the nucleic acids, single-strand breaks, double-strand breaks, and abnormal cross-links in DNA or between DNA and cellular proteins (1). If not repaired, such lesions might be lethal for the cell or may impair the integrity of genomic DNA. Cells respond to these types of DNA damages by activating a network of signaling pathways, which may arrest the damaged cell at specific checkpoints. Such arrests prevent the damaged DNA to be replicated and transmitted to the next generation, either by reparation or by induction of cell death (2–5).

Radiation is known to exert significant effects on the progression of the cell cycle (6) and may activate the G1-S DNA damage checkpoint, in which the DNA damage is repaired before replication (7), and/or the G2-M DNA damage checkpoint, in which the repair of DNA occurs before initiation of mitosis (7). One of the key molecules involved in this radiation-induced cellular machinery is p53. As a tumor suppressor gene, p53 is the most important determinant of the genotoxic response and known to activate p21, a cyclin-dependent kinase inhibitor that is required for the arrest of cells in G1. p53 also affects the duration of arrest in G2 because it may prevent the activation of the cdc2-cyclin B1 complex needed for entry into mitosis, either directly or indirectly via its transcriptional targets (8). Mutation in p53 or down-regulation of its functional capacity is a common event in human carcinogenesis (9–11) and, therefore, tumors regularly display an impaired activation of the cell cycle checkpoints after irradiation (5).

During mitosis, centrosomes, the major microtubule-organizing centers, exert an important function by formation of the spindle poles. These organelles are crucial for the number of spindle poles formed during mitosis (12). The duplication of centrosomes is strictly regulated and tightly correlated to the DNA content of the cell to maintain accurate chromosome segregation to the daughter cells. Formation of numerous centrosomes may result in multipolar spindles, which cause abnormal chromosome segregation, and may
generate cells with multiple micronuclei or binucleated giant cells (13), which is defined as a mitotic catastrophe.

To achieve efficient therapy of solid tumors, it is important to understand the way tumor cells respond to different doses of radiation at the molecular and cellular levels and also the time dependency of these mechanisms. In this study, we examined the induced sequential events in HeLa Hep2 cells exposed to absorbed dose of 2.5 to 10 Gy, with special emphasis on cell cycle arrest, mitotic disturbances, and, finally, cell death.

Materials and Methods

Cell lines

HeLa Hep2 cells (ATCC no. CCL-23), effectively expressing typical HeLa cell markers, were kept in culture at 37°C in a humidified atmosphere of 5% CO2. Cells were grown in DMEM (VWR) supplemented with 1% (v/v) penicillin, streptomycin (VWR), 1% (v/v) l-glutamine (VWR), and 5% (v/v) FCS (VWR).

Irradiation

Cells were exposed to absorbed doses of 2.5 to 10 Gy using a Cobalt-60 treatment unit (Alycon II). The tumor cells, maintained in tissue culture flasks, were positioned on a 15-cm-thick solid block of a water equivalent material. The field size was constant during all the treatments. A thin plastic plate was put on top of the bottles to ensure full radiation dose to the cells. The dose rate was in the middle of the treatment period 0.45 Gy/min. Cells were then cultured for defined time intervals and analyzed by Western blotting, flow cytometry, and/or fluorescence microscopy with the intention to visualize the different cellular events occurring following irradiation.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

To generate dose-response curves for HeLa Hep2 cells following irradiation, cells were grown in 96-well plates and exposed to different doses of irradiation (as above). To check the viability following different doses of irradiation, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche Diagnostics GmbH) was done according to the manufacturer’s recommendations 2, 4, 5, 6, and 7.5 days after treatment. Briefly, 10 μL of MTT was added to each well and the plate incubated for 4 h at 37°C in a humidified atmosphere of 5% CO2. Subsequently, 100 μL of the solubilization solution were added to each well and the plate left overnight at 37°C in a humidified atmosphere of 5% CO2. The spectrophotometrical absorbance of the samples was checked in a microplate reader (570 nm).

Fluorescence flow cytometry

Fluorescence flow cytometry was used to analyze alterations in cell cycle profiles after irradiation at regular intervals for up to 1 week.

Propidium iodide.

Propidium iodide was used as a marker for DNA content. Freshly trypsinized cells were incubated for up to 30 min with 20 mmol/L Tris solution (pH 7.6), containing propidium iodide (as described above). Subsequently, 100 μL of the solubilization solution were added to each well and the plate left overnight at 37°C in a humidified atmosphere of 5% CO2. The spectrophotometrical absorbance of the samples was checked in a microplate reader (570 nm).

Western blotting

Cell extracts were prepared using an SDS lysis buffer (100 mmol/L Tris (pH 6.8), 2% SDS) containing protease inhibitors (Complete, Roche Diagnostics). Protein concentration was determined with a bichiniconinic acid protein assay kit (Pierce). Samples (50 μg) were separated on SDS-polyacrylamide gel, electrotransfered onto a polyvinylidene difluoride membrane (Millipore), and incubated with primary antibodies to p53 (DakoCytomation, clone D0-7) and p21 (Santa Cruz Biotechnology). Secondary horseradish peroxidase–conjugated goat anti-rabbit (Pierce) or horseradish peroxidase–conjugated goat anti-mouse (Pierce) polyclonal antibodies were used for detection of bound primary antibody and bands were visualized by enhanced chemiluminescence (Pierce).

Fluorescence microscopy

Propidium iodide staining. Propidium iodide staining was used to examine nuclear abnormalities occurring following irradiation. Freshly trypsinized cells were cytospun onto SuperFrost Plus microscope slides (Menzel GmbH & Co KG) fixed at -20°C in 95% methanol and 5% acetic acid for up to 30 min and then incubated with the propidium iodide solution (20 mmol/L Tris solution (pH 7.6), containing propidium iodide (50 μg/mL), NP40 (0.1%), and RNase (20 μg/mL)) for 15 min. Fluorescence staining was then examined by confocal laser scanning microscopy using a Leica SP2 confocal microscope equipped with an argon and HeNe laser.

α-Tubulin. α-Tubulin was visualized to identify the mitotic spindles. Freshly trypsinized cells (0.5 × 10^6) were resuspended in PEM buffer (80 mmol/L PIPES, 1 mmol/L EGTA, 4 mmol/L MgCl2, 6H2O, 0.2% saponin, pH 7.0) for 4 min at 37°C and then fixed by adding an equal volume of 4% paraformaldehyde in PEM buffer for 15 min at 37°C. The cells were then incubated for 10 min at 37°C in a blocking buffer (PBS, 10% FCS, 0.05% saponin). Subsequently, the cells were resuspended with an Alexa Fluor 488–conjugated α-tubulin antibody (kindly provided by P. Holmfeldt and M. Gullberg, Department of Molecular Biology, Umeå University, Umeå, Sweden). The cells were finally counterstained with propidium iodide solution (as above). Fluorescence staining was then examined under a Leica SP2 confocal laser-scanning microscope equipped with an argon and HeNe laser.

γ-Tubulin. γ-Tubulin was visualized for detection of altered amplification of centrosome numbers. Cells were fixed and blocked as described above. Subsequently, the cells were resuspended with a monoclonal antibody recognizing γ-tubulin (clone GTU-88, Sigma) diluted 1:200 in blocking buffer and incubated for 1 h at 37°C. After a washing step, the cells were incubated for 30 min at 37°C with 10 μg/mL of the secondary Alexa Fluor 488–conjugated goat anti-mouse antibody (Molecular Probes). Finally, the cells were counterstained with propidium iodide (as above). Fluorescence staining was then examined under a Leica SP2 confocal laser-scanning microscope equipped with an argon and HeNe laser.

Hypermultiplication of centrosomes was quantified by scoring mitotic cells as either having two centrosomes (normal) or more than two centrosomes (hypermultiplicated). Cells from three independent stainings were examined and a total of ~300 mitotic cells were scored for each group.

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining was done as previously described.
dUTP nick end labeling (TUNEL) was used to visualize apoptotic cell death. An In Situ Cell Death Detection Kit, TMR red (Roche Diagnostics), was used according to the instructions from the company. Cells (2 x 10^6) were transferred into a V-bottomed 96-well microplate, incubated in paraformaldehyde (4% in PBS, pH 7.4), permeabilized (0.1% Triton X-100 in 0.1% sodium citrate), and then resuspended in the TUNEL reaction mixture. The cells were finally analyzed by confocal laser scanning microscopy using a Leica SP2 confocal microscope equipped with an argon and HeNe laser.

**Statistics**

Student’s t test was used to compare control and irradiated cells and significance levels were determined. P < 0.05 (two-sided) was considered significant.

**Results**

**Dose-response curves following irradiation.** HeLa Hep2 cells were exposed to absorbed doses of 0 to 20 Gy and assayed for viability using the MTT test. Dose-response curves were obtained for 2, 4, 5, 6, and 7.5 days as shown in Fig. 1. Between 2 and 4 days after irradiation, a reduction in survival can be seen. This cell death continues to 7.5 days postirradiation. The kinetics indicates a delayed type of cell death.

**Induction of G2-M arrest.** Irradiation induces a transient G2-M arrest as seen in Fig. 2. The principle of quantifying cells in different cell cycle phases using flow cytometry is schematically presented with an unirradiated and an irradiated sample 20 h after treatment with 10 Gy (Fig. 2A). A shift from the G1-G0 phase and S phase to the G2-M phase can be seen. The shift is further documented in Fig. 2B presenting a dose-dependent decrease of cells in G1-G0 following irradiation from 57.8 ± 0.5% down to 8.3 ± 0.4% at 20 h. The relative levels of cells in the S phase follow a similar pattern, reaching low values at 20 h with subsequent restoration at 30 h (Fig. 1C). The accumulation of cells in the G2-M phase is shown in Fig. 1D with a dose-dependent significant increase from 25.9 ± 1.4% up to 77.0 ± 2.0% at 10 Gy (P < 0.0001). This arrest was transient and the fraction of cells in G2-M returned to the pretreatment level after ~30 h for cells irradiated with 2.5 and 5 Gy. When the cells were treated with 10 Gy, the increase in the G2-M population was longer-lasting compared with the cells treated with lower doses of irradiation. These results are in agreement with those obtained from the dual staining with the MPM-2 antibody and propidium iodide, showing an initial G2-M arrest after irradiation. This arrest is transient and the cells reenter the cell cycle and progress into mitosis, increasing the mitotic index (Fig. 3). The frequency of mitoses in untreated controls amounts to 2.6 ± 0.2% (Fig. 3A). Following irradiation, an initial dose-dependent significant decrease in mitotic index is seen, reaching the lowest value of 0.36 ± 0.05% at 8 h (2.5 Gy, P = 0.0041; 5 Gy, P < 0.0001; 10 Gy, P < 0.0001; Fig. 3B-D). In the time interval of 20 to 48 h, a compensatory increase in mitotic index was seen.

![Figure 1](image-url1)

**Fig. 1.** Dose-response curves for HeLa Hep2 cells exposed to 0 to 20 Gy of absorbed dose. Viability was checked using the MTT test between 2 and 7.5 d postirradiation.

![Figure 2](image-url2)

**Fig. 2.** Fluorescence flow cytometry of cells exposed to different doses of irradiation stained with propidium iodide. Percentages of cells in each phase of the cell cycle were quantified using the CellQuest software program, setting markers at appropriate fluorescence levels. The quantification of cells in different cell cycle phases using flow cytometry is schematically presented as well as representative fluorescence-activated cell sorting diagram of control cells and cells irradiated with 10 Gy 19 h after treatment (A). Initial decreases in the fraction of cells populating the G1 and S phases of the cell cycle were detected during the first 24 h (B and C). Cells in the G2-M phase of the cell cycle showed the opposite pattern with an increase at the same time point (D).

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Induction of p53. Irradiation induces an up-regulation of p53 protein in HeLa Hep2 cells, as depicted in Fig. 4A. From low levels seen in the control, the induction is observed after 1 day and persists for several days, slowly returning to control levels.

Induction of p21. Irradiation induces an up-regulation of p21 protein in HeLa Hep2 cells (Fig. 4B). Only trace expression can be visualized in the control sample. At days 2 to 4 after irradiation, significant induction of p21 is observed followed by a decline.

Induction of mitotic and nuclear aberrations. Irradiation generates mitotic and nuclear aberrations as shown in Fig. 5. Three typical patterns are observed including increase in anaphase bridges, lagging chromosomal material, and multipolar mitotic spindles (Fig. 5A). By staining with an antibody against α-tubulin, the spindles can be visualized revealing multipolar aberration patterns as exemplified by tripolar or tetrapolar divisions (Fig. 5B). In addition, an increase in the fraction of mitotic cells containing more than two centrosomes was seen in irradiated cells (Fig. 5C). As an example, γ-tubulin staining of irradiated cells with three or six centrosomes is shown. The frequency of mitotic cells with more than two centrosomes is significantly increased in a dose-dependent way from 12.6 ± 6.6% in control to 66.5 ± 5.3% in cells irradiated with 10 Gy (2.5 Gy, P = 0.012; 5 Gy, P = 0.0065; 10 Gy, P = 0.0004; Fig. 6). In addition, cells in interphase displayed an increased number of centrosomes often assembled in a group. These cells frequently contained more than one nucleus. Finally, a vast polymorphism in nuclear appearance could be detected, including micronuclei, multinucleated cells, and cells with multilobulated nuclei (Fig. 7).

Polyploid cell formation. Irradiation generates an increase in the relative amounts of polyploid cells (Fig. 8). Approximately 2 days after radiation, an increased fraction of the cells displayed >4N DNA content (polyploid cells), which could be detected by fluorescence flow cytometry on propidium iodide–stained cells. This increase was most pronounced in cells treated with the highest doses of irradiation (5 and 10 Gy) and was significant in these groups 4 days after irradiation (2.5 Gy, P = NS; 5 Gy, P = 0.0157; 10 Gy, P < 0.0001). After an initial increase up to day 3 for the lower doses and day 4 for the highest dose, the frequency of polyploid cells leveled off.

Cellular death after irradiation. From day 3, an increased fraction of cells dying by mitotic catastrophe was observed when irradiated cells were stained with propidium iodide and an α-tubulin antibody (Fig. 9A). A subgroup of these cells also stained positively for TUNEL, implying a delayed type of apoptosis involved in radiation-induced death in HeLa Hep2 cells (Fig. 9B). The TUNEL technique detects fragmented DNA, which occurs close to the final step in the apoptotic process.

Discussion

The aim of this study was to analyze cell cycle alterations and induced lethal events occurring in HeLa Hep2 cells exposed to 2.5 to 10 Gy of ionizing radiation. These doses, compared with
60 to 100 Gy given at treatment of clinical cancer, cause significant growth retardation of tumors in vivo at experimental conditions (14–17). Our results now clearly show that a number of consecutive responses with putative lethal consequences can be observed in HeLa Hep2 cells following such irradiation. Preliminary investigations of other cell lines (Jurkat, Molt-4, and HT29) indicate that these phenomena are also observed with other epithelial and lymphoid cell lines.

The p53 status in HeLa Hep2 cells is wild-type (18, 19) and the results obtained in this study indicate an increase in the expression of p53 protein as well as a correlated subsequent increase in p21. However, no detectable activation of the G1-S DNA damage checkpoint after irradiation was observed. Instead, a significant fraction of HeLa Hep2 cells responded to ionizing radiation with a transient G2-M arrest during the first 24 h. A sustained G2 arrest after DNA damage, required for correct DNA damage reparation following radiation, has been shown to require both p53 and p21. Cells either p53-deficient or p21-deficient may initiate a G2 arrest but may also rapidly escape from this arrest (20). HeLa Hep2 cells are known to be infected with human papillomavirus, which expresses E7, a protein derived from a viral oncogene and may directly interact with both p21 and the retinoblastoma protein, thereby inactivating their functions (20). This may be an explanation for the failure of HeLa Hep2 cells to induce a G1-S arrest and also for the transient G2-M arrest observed following irradiation.

Hyperamplification of centrosomes has earlier been detected after exposure to radiation during a prolonged G2 phase either dependent (21) or independent (22, 23) of a failure in cytokinesis, and may be a critical event contributing to the radiation-induced cell death. In this study, immunofluorescence stainings for centrosomes were associated with the occurrence of multiple centrosomes predominantly equal to the number of spindle poles. The frequency of mitotic cells with
more than two centrosomes was 12.6 ± 6.6% in nonirradiated cells, and the number of cells with a hyperamplified number of centrosomes increased dramatically to 66.5 ± 5.3% after irradiation in cells treated with the highest dose of radiation (10 Gy). It has previously been shown that expression of the human papillomavirus E6 and E7 proteins in epithelial cells may trigger abnormal spindle pole formation by an abundant number of centrosomes in parallel with the occurrence of anaphase bridges (24–26). This centrosome amplification has been shown to appear due to failure in cytokinesis, leading to formation of cells with a 2-fold amount of both chromosomes and centrosomes (26), explaining the relatively high numbers of control cells with abundant numbers of centrosomes observed.

Following the transient arrest now observed, irradiated HeLa Hep2 cells adapted to the G2-M DNA damage checkpoint and typically reentered the cell cycle, still harboring unrepaired cellular damages. This obviously has profound effects on the behavior of the cell and induces several mitotic disturbances including anaphase bridging, lagging chromosomal material, and multipolar mitoses.

Anaphase bridges may be generated when broken chromosomes, either induced by irradiation or triggered by abnormal shortening of the telomeres, fuse (21, 26). These anaphase bridges usually are resolved by new chromosomal breakages (27). The mitotic disturbances yielded a number of nuclear abnormalities. Irradiated cells often contained one or several micronuclei formed by nuclear membrane formation around lagging chromosomes or chromosomal material. Furthermore, an enhancement of the fraction of cells with several nuclei as well as abnormally shaped multilobulated nuclei could be identified in irradiated cells. This pronounced polymorphism in nuclear appearance has earlier been observed in experimental tumors following radioimmunotherapy (28). The fraction of cells containing >4N DNA, classified as polyploid cells, indicates an amplification of this subpopulation of cells, especially in the treatment group receiving the highest dose of radiation. It has earlier been shown that mitotic DNA damage might induce a cytokinesis failure, which will lead to binucleation. In cells containing a functional G1 checkpoint, a G1 arrest will be induced in this tetraploid cell population.

On the contrary, HeLa Hep2 cells with an impaired G1-S checkpoint will start a new DNA amplification cycle, which finally yields polyploid cells. The formation of multinuclear giant cells or cells with several micronuclei is often referred to as a “mitotic catastrophe.” These cells are destined to die and the dose-response curves indicate that the induction of cellular death starts between 2 and 4 days postirradiation and increases up to 1 week postirradiation, indicating a delayed type of cell death. In tumor cells of lymphoid origin, apoptosis occurring rapidly

Fig. 7. Nuclear abnormalities in irradiated cells. Control cells normally contained a single round nucleus. Irradiated cells displayed increased frequencies of multiple nuclei (arrowheads), micronuclei (arrow), and multilobulated nuclei (asterisk).

Fig. 8. Fluorescence flow cytometry on cells exposed to different doses of irradiation (2.5-10 Gy) and stained with propidium iodide. Top, typical fluorescence-activated cell sorting diagrams are presented showing control and treated cells 4 d after irradiation (10 Gy). Bottom, fractions of polyploid cells in control and irradiated cells (2.5-10 Gy). M1, fraction of polyploid cells.
before cell division (within 24 h) has been shown to be the major cell death mechanism following irradiation (29, 30). In cells of epithelial origin, the cell death process is initiated during or after mitosis, which is in agreement with our results.

At least a fraction of the dying cells in this study displayed a delayed form of apoptosis. This delayed form of apoptosis has earlier been observed by our group both in vitro (31, 32) and in vivo (28) following low-dose, low-dose-rate radiation. Delayed apoptosis might be a result of centrosome hyper-amplification observed in this study resulting in subsequent nuclear damage and activation of the apoptotic cascade.

In conclusion, this study shows that mitotic catastrophe and delayed apoptosis following centrosome hyperamplification might be important cell death mechanisms involved in tumor growth retardation following radioimmunotherapy of solid tumors of epithelial origin.

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Fig. 9. Staining of a control cell and an irradiated HeLa Hep2 cell dying by mitotic catastrophe with an Alexa Fluor 488–conjugated α-tubulin antibody (green), with cells counterstained with propidium iodide (red; A). TUNEL staining of a control cell and an irradiated cell dying by mitotic catastrophe via a delayed form of apoptosis (B).

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