RADIATION – INDUCED APPOPTOSIS IN TP53 – DEFICIENT HUMAN LEUKEMIA CELL LINE HL – 60

P. Petrunov[1], M. Sceridan[2]
Military Medical Academy, Department of Radiobiology and Radiation Protection
Sofia, Bulgaria[1]
Institute of Technology – Dublin, Ireland[2]

ABSTRACT
Human promyelocytic leukemia (HL – 60) cells were irradiated with 0.5- 110 Gy gamma irradiation and studied for 48 h post irradiation to determine the mode of death. HL – 60 cells are much more sensitive to radiation-induced loss of clonogenicity (D_0 = 2.2 Gy) than to induction of apoptosis at 6 h (D_0 for nonapoptotic cells = 32.6 Gy). Maximum apoptosis occurred 48 h after irradiation with doses 3.5-10 Gy, and cells that died by necrosis were found in 8-42%.

Introduction

In 1996 Reffray and Cohen (8) compared three basic models, when the cells die by apoptosis or necrosis: 1) totally separate death modes 2) a continuum, whereby there are extremes of biochemical overlapping death pathways or 3)essentially distinct processes with only limited molecular and cell biology overlap. Experimental results support the third model. The established axiom that, even when considering same toxicant, injury amplitude (dose) is a primary determinant of whether cells die via active cells death (apoptosis) or failure of homeostasis (necrosis), remains valid.

In cancer, the increase in tumor burden can be regarded as a result of too much growth and too little death of the tumor cells. Therefore, the aim of chemotherapy and radiotherapy is to block proliferation and to enhance cell death. Tumor cells have often advantages due to genetic translocations, which lead to increased surviving. Many studies proved that DNA damaging agents (cytostatic drugs, ionizing radiation) cause genetic alterations related to protein TP53 (formerly known as p53). Increased level of TP53 leads to growth suppression and is involved in DNA repair via three main functions: 1. increase of TP53 causes cell cycle arrest of damaged cells in G1 phase 2. high amount of TP53 leads cells to apoptosis 3. TP53 is involved in reparation of DNA. Synthesis of all three functions is essential for maintenance of undamaged genetic information and, therefore TP53 is called " guardian of genome"(4). MOLT-4 cells of pre-T lymphoma line TP53 Wild/mutant are very sensitive to ionizing radiation similarly as HSB-2 cells of pre-T lymphoma line with type TP53. Apoptosis is induced in all cell cycle phases as soon as 6 hours after 5 Gy (57%) and after 24 hours 97% of cells are apoptotic. TP53 gene is oftentimes mutated or missing in cells of human tumors. These cells have some proliferative advantages to normal cells. Some authors (4, 7) have proved that ionizing radiation and chemotherapeutic drugs initialize increase of ceramide concentrations which lead to cell apoptosis. This

pathway is initiated by changes on cell membrane. Sphingomyelin (plasma membrane main lipid) hydrolysis by sphingomyelinase (specific fosfolipase C) leads to ceramide production. Ceramide then serves as second messenger leading to induction of the stress-activated protein kinases (JNK/SAPK). Herr et al (8) proved that ceramide accumulated as a response to cellular stress (chemo and radiotherapy) increases expression of CD95-L. CD95-L reacts with CD95-R on cell membrane and activates CD95 signaling pathway leading consequently to caspase splitting and apoptosis. Ceramide is called "tumor suppressor lipid". Both molecules- TP53 and ceramide – are important in the regulation of cell cycle, aging and apoptosis. T lymphoma lines CEM and JURKAT have mutated TP53 and they are much more radioresistant, apoptosis could be detected only after cell cycle arrest in G_2 phase (1,8). Acute myeloid leukemia is in 40% caused by chromosomal translocation and the TP53 gene is usually mutated or missing. HL-60 cells of human promyelocytic leukemia do not have TP53 gene, they have normal or slightly increased expression of Bcl-2 and minimumal expression of Bcl-x_l (9). They are relatively radioresistant and after doses to 10 Gy apoptosis could be observed after cell cycle arrest in G_2 phase.

In our study we compared clonogenicity loss and apoptosis in HL-60 cells induced by ionizing radiation. Mode of death of cell irradiated by dose up to 10 Gy and doses 20-100 Gy is discussed.

Materials and Methods

Cell culture and culture conditions. Human leukemia HL-60 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK) and were cultured in Iscove's Dulbeco's medium (Sigma) supplemented with 20% fetal calf serum in a humidified incubator at 37 C and controlled 5 % CO atmosphere. The cultures were divided every 3rd day by dilution to a concentration of 2×10^5 cells/ml. Cell counts were performed with a hemocytometer, cell membrane integrity was determined using the Trypan blue exclusion technique. HL-60 cells in the maximal range of 20 passages were used for this study.

Gamma irradiation. Exponentialy growing HL-60 cells were suspended at a concentration of 2×10^5 cells/ml in complete medium. 10 ml of aliquots were plated into 25 cm^2 flasks (Nunk) and irradiated at room temperature using ^{60}Co gamma-ray source with a dose rate 0.87 Gy/min or 3 Gy/min (doses 20-100 Gy). After irradiation, flasks were placed in a 37^0 C incubator for up to 72 h, and aliquots of cells were removed at various times after irradiation for analysis.

The cells were counted and cell viability was determined with Trypan blue assay.

In vitro clonogenic assay. Radiation survival curve was generated using an in vitro clonogenic assay. Untreated control (10^2 ml) and irradiated (10^2 -10^5/ml) HL-60 cells were grown in 0.9% methylcellulose in Iscove's modified Dulbecco's medium with 30% fetal calf serum. All semisolid cultures were performed in duplicates and stimulated with 10% conditioned medium from the 5637 human bladder carcinoma cell line and 4units/ml erythropoietin. Colonies (containing 40 or more cells) were counted after 14 days of incubation in 5 % CO_2 and 5 % O_2 at 37 °C and curve was generated. Two independent experiments were performed with cell irradiated by increasing dose 0.5-10 Gy.

Cell morphology. To calculate the percentage of cells showing morphology of apoptosis, cell aliquots were removed from control and irradiated cell cultures at various times of incubation, and usually 400 cells

were counted on Diff-Quik (Date Behring, Switzerland) stained cytospin preparations. Apoptosis cells were identified by the condensed and fragmented state of their nuclei and focal protrusions of the cell surface. Three independent experiments were performed.

Statistical analysis. The value represents mean ± SD (standard deviation of the mean)

Results and Discussion

The mode of cell death in HL-60 cell after irradiation is dependent on the dose. In our work we want to point to different modes of death in cells irradiated by doss 20-100 Gy and doses up to 10 Gy. Cell morphology was assessed 6 hours after irradiation with 20-100 Gy (**Fig.1**).

Within 6 h after irradiation with 20-100Gy (Fig1) most of cells had apoptotic morphology. Flow cytometric analysis (from our unpublished results) of cells containing sub-diploid amount of DNA also conformed that most of the cells irradiated with these high doses are apoptopic. Six hours after irradiation with increasing doses of radiation (10, 20, and 50 Gy) 15, 30 and 49% of cells were apoptotic. Surprisingly, living, (nonapoptotic) cells were in G_1/G_0 phase of the cell cycle. **Fig. 2** and **Fig. 3** shows the effect of radiation with 0.5-10 Gy on the proliferative rate

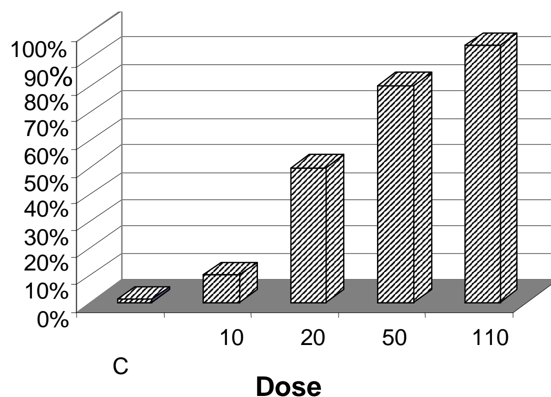

Fig. 1. Irradiation induced apoptosis in HL-60 cells 6 h after increasing doses of ^{60}Co gamma rays. Apoptosis was determined by Diff-Quick stained cytospin preparation.

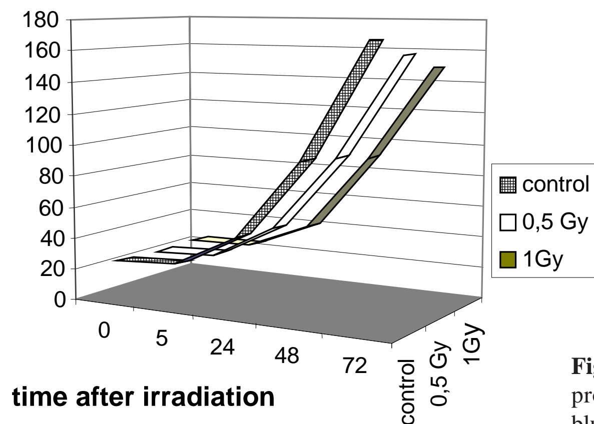

Fig. 2. Kinetics of irradiation effect on the proliferative rate of the viable (trypan blue-negative) HL-60 cells.

of the HL-60 cell line. Irradiation with 0.5 and 1 Gy had a small effect on the proliferation of HL-60 cells. Irradiation with 2 Gy induced a slight decrease of proliferation. 3.5-10 Gy irradiation of the rate of HL-60 growth. After 72 hours all cells irradiated with 10 Gy were dead.

Cell viability (trypan blue) and apoptosis level (Diff-Quik stained cytospin preparations) were assessed 48 hours after irradiation after irradiation with 3.5-10 Gy. During this time interval after 3.5-10 Gy maximum apoptosis was observed, yet cell death by necrosis was also apparent (**Fig. 4**).

In another experimental setup the clonogenic survival of HL-60 cells after irradiation was investigated.The cells have D_0 =2.2 Gy. The dose response curves show that HL-60 cells are 15-fold more sensitive radiation-induced loss of clonogenicity than to induction of apoptosis at 6 h measured by cell morphology on cytospin preparations. 48 h after irradiation with dose up to 10 Gy cells die from both apoptosis and necrosis.

The mode of death in HL-60 cells as well as

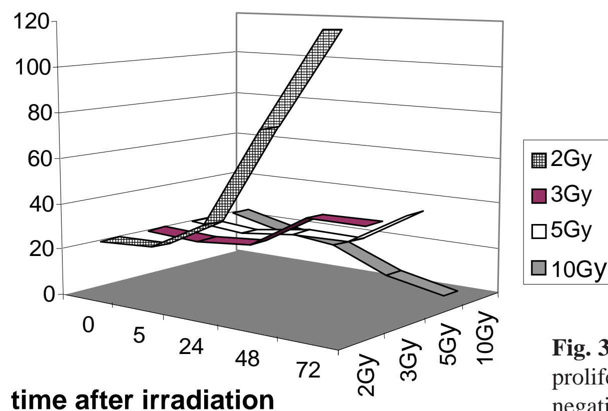

Fig. 3. Kinetics of irradiation effect on the proliferative rate of the viable (trypan blue-negative) HL-60 cells.

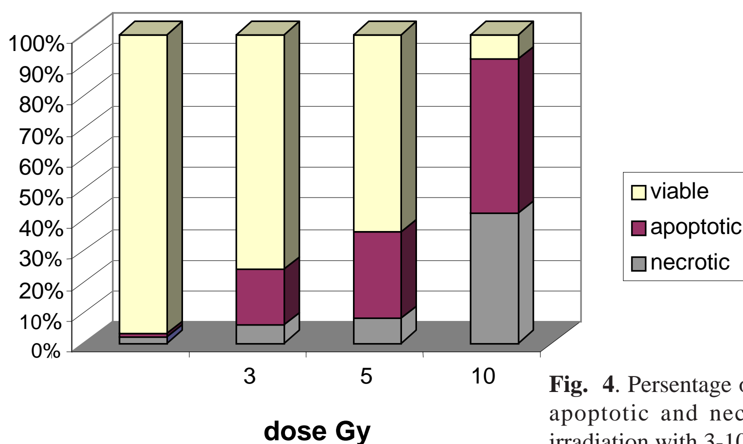

Fig. 4. Persentage of viable (nonapoptotic), apoptotic and necrotic cells 48 h after irradiation with 3-10 Gy.

in many other cell lines after irradiation is dependent on the dose. (1).In our work we proved quick apoptosis induction in HL-60 cells after irradiation with high doses of gamma rays (20-50 Gy). Six hours after irradiation with 20 Gy 49% of cells were apoptotic and after 50 Gy 80 %. Ten Gy dose induced only 11% of apoptotic cells 6 hours after irradiation.

For apoptosis detection have been used morphological evaluation of Diff-Quick stained cytospin preparations. Six hours after irradiation with 20 Gy significant apoptosis occurred-49%. Our conclusions are in good agreement with the data of Hopcia et al (5) and Dynlacht et al (3). Conventional agarose gel electrophoresis related that death of HL-60 cells by apoptosis was accompanied by intranucleosomal DNA fragmentation within 3 h after irradiation with 50 Gy.

HL-60 cells are very radiosensitive as a result from clonogenenicity test, where D_{37} value is 2.2 Gy which corresponds to value D_0 = 2 Gy determined by Hopcia at al (5).High doses (20-50) needed to trigger massive apoptosis within 6 h are therefore supralethal. Mode of death 48 h after irradiation with 10 Gy is not clear. Findings of Dynlacht et al (3) that 10 Gy induced primarily necrosis is in contrast to findings of Hopcia at al (5), who show that a significant amount of apoptosis occurs if the cell population is analyzed 2-3 days after irradiation with 10 Gy. Our study indicates that 48h after irradiation with 5 and 10 Gy 35% and 41 % show apoptosis.

Our result suggest that HL-60 cells are much more sensitive to radiation induced loss of clonogenicity than to induction apoptosis at 6 h after irradiation. Surprisingly, 6 hours after irradiation with 20-50 Gy nonapoptotic live cells were accumulated in G_1 phase of cell cycle.

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