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INVESTIGATION OF APOPTOSIS IN NORMAL AND LEUKEMIC CELLS INDUCED BY X-RAY IRRADIATION

A. NEVOIE[a*], M. PASCARIU[a], D. JITARU[b], I. IVANOV[b,c],
D. CONSTANTINESCU[b,d], E. CARASEVICI[b,c], T. LUCHIAN[a]
[a]*Faculty of Physics, "Al. I. Cuza" University, Iasi, Romania,*
[b]*Laboratory of Immunology and Genetics, Hospital "St. Spiridon ", Iasi, Romania,*
[c]*University of Medicine and Pharmacy "Gr T. Popa "Iasi, Romania*
[d]*Public Health Institute, Iasi, Romania*

The aim of this work is the stydy of the functional changes of normal and leukemic cells exposed to X radiation. Normal mononuclear cells and two human leukemia cell lines (Jurkat and K562) were used for this study. The cells have been grown in the same medium and same conditions. To irradiate the human cells cultures, different doses were used. The process of programmed cell death, apoptosis, has been analyzed using the flow cytometry technique at 3, 24, 48 and 72 hours from irradiation. After 72 hours after the irradiation, the cells show the characteristic signs of apoptosis.

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1. Introduction

Ionizing radiation has been used to treat human cancer [1]. The objective of radiotherapy is to kill cancer cells and to protect the surrounding normal tissue [1]. The total body irradiation is a external beam radiotherapy used in haematopoietic stem cell or bone marrow transplantation. It involves the irradiation of whole body and it represents radiation sterilization of tumoral cells, especially leukemic cells using high doses which range from 10 to more than 12 Gy. In modern practice, total body irradiation is fractionated. The research in bone marrow transplantation shows that if the total body irradiation dose is fractionated into smaller doses, the toxicity is smaller than in the case of a single dose [2-5]. Leukemic cells are very radiosensitive and they have a little or no capacity to repair the chromosomal abnormalities induced by ionizing radiation. However, the radiosensitivity varies from patient to patient [2]. The *in vitro* cellular systems are very used in radiobiology research, because the cellular and molecular interactions can be precisely controlled and manipulated. Ionizing radiation, induce chromosomal abnormalities including base pair deletion, cross-linking injuries, single and double strand break and multiple lesions appear [6].

Apoptosis plays an important role in tumor response to radiation [7, 8]. Therefore, low doses and repeated action protect normal cells. The ability to cure a tumor with ionizing radiation depends on various factors, most important is tumor cell radiosensitivity [9]. In the present study we proposed to follow the functional changes induced by X-rays with different doses at the cellular level in normal and tumor cell culture. Two human leukemia cell lines (Jurkat and K562) and normal mononuclear cell culture were used for this study.

2. Material and Methods

2.1. Cell line and cell culture

Mononuclear cells were separated from 28,5 mL whole blood using ficoll, which was collected on EDTA. The blood was extracted from a healthy donor. The lymphocytes in the blood samples have been cultured in RPMI 1640 medium, supplemented with 10 % fetal bovine serum (FBS, GIBCO), antibiotics (penicillin G, streptomycin and amphotericin B, Hy Clone) at 37°C in a humidified atmosphere of 5% CO2 [10].

Two human tumor cell lines were used: Jurkat (Disease: acute T cell leukemia; Morphology: lymphoblast) and K562 (Disease: chronic myelogenous leukemia (CML); Morphology: lymphoblast). We used $1x10^6$ cells for culture in RPMI 1640 medium, supplemented with 10 % fetal bovine serum (FBS, GIBCO), antibiotics (Hy Clone) at 37°C in a humidified atmosphere of 5% CO2. Medium was refreshed every 48 hours of culture [11]. All cell lines were grown in same conditions with normal mononuclear cells.

2.2. X-ray irradiation

Each experiment has been repeated five times.

The irradiation equipment was a X-ray SRT100 belonging to the Department of Radiology in "St. Spiridon" Hospital, Iasi. All cell cultures were grown in the same flasks. For the irradiation, a locator with a diameter of 10 cm, 70 kV, 10 mA and a depth of 3 cm was used. The dose rate was 2.27 Gy/min. For example, for an exposure dose of 0.8 Gy, the irradiation time was 21 sec [12].

Normal mononuclear cell cultures used in this experiment were irradiated at 24 hours after the culture of peripheral blood mononuclear cells. The exposure was performed at room temperature once with doses of 0 Gy, 0.8 Gy, 2.5 Gy and 5 Gy. After X irradiation, flasks were placed in an incubator at 37ºC and aliquots of cells were removed at 3, 24, 48 and 72 hours after irradiation for analysis.

Jurkat and K562 cells were irradiated after culturing at 24 hours. Cell cultures were irradiated at room temperature three days with fractionated doses of 0 Gy, 0.8 Gy and 2.5 Gy. Every day after X irradiation, aliquots of cells were removed for analysis and than flasks were placed in incubator at 37ºC. The cell viability was determined by the Trypan blue exclusion assay.

2.3. Assessment of apoptosis by flow cytometry

Ten thousand cells per sample were analyzed using a BD FACSCanto II and BD FACSDiva Software. Apoptosis was evaluated using the Annexin V Binding Buffer, 10X concentrate (BD PharMingen Technical Data Sheet) followed by FACS analysis. Percentage of cell death or apoptosis was assessed flow cytometry at 3, 24, 48 and 72 hours after irradiation. The cells were washed twice in cold TFS (PBS) by centrifugation at 300g, 5 min. Pellet obtained after the second centrifugation was resuspended in binding buffer. We added 5 µL of annexin V and 5 µL of 7AAD to the cells, vortexed, and incubated for 15 minutes in the dark. Finally, 400 µl of Binding Buffer was added, and samples were evaluated by flow cytometry [13-15].

2.4. Statistical analysis

Calculations of mean value, SD, and *p* values were performed on five times experiments using SPSS 13.0 and Microsoft Excel 1997. The Student T-test was used to calculate *p*-values for comparison.

3. Results

We report two series of experiments. In the first experiment, we irradiated normal cells and we used normal mononuclear cells. The process of programmed cell death, apoptosis induced

by X radiation has been analyzed using the flow cytometry technique at 3, 24, 48 and 72 hours from irradiation.

Flow cytometry is a technique for counting and measuring the properties of individual particles [16]. The data generated by flow-cytometers are presented as histograms (figure 1).

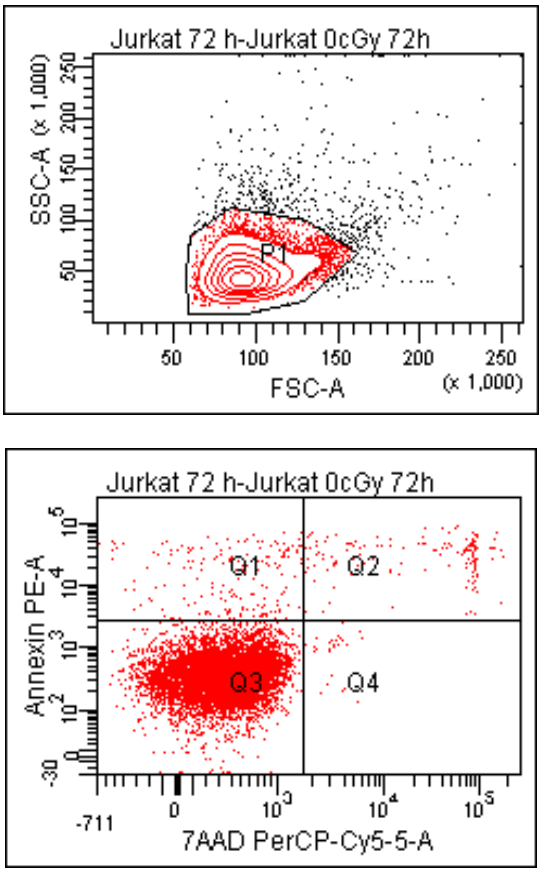

Fig. 1. Two-dimensional histograms (dot displays or contour maps). The first image represented delimitation of interesting cells and the parameters are granularity (SSC) and volume (FSC). In the second image the parameters are fluorescence and in Q1 quadrant are the cells in early apoptosis, in Q2 quadrant are the cells in late apoptosis, in Q3 quadrant are the living cells and in Q4 quadrant are the cells in final apoptosis.

The response of mononuclear cells to ionizing radiation was illustrated in Fig. 2. At 3, 24 and 48 hours the apoptotic process is not detectable. Only 72 hours after the irradiation, cells show the characteristic signs of apoptosis.

Analyzing the results obtained for five determinations that have followed the evolution for cells survive were found statistically significant differences ($p < 0.001$) for the values corresponding to different irradiation doses with assessment by flow cytometry at intervals previously established.

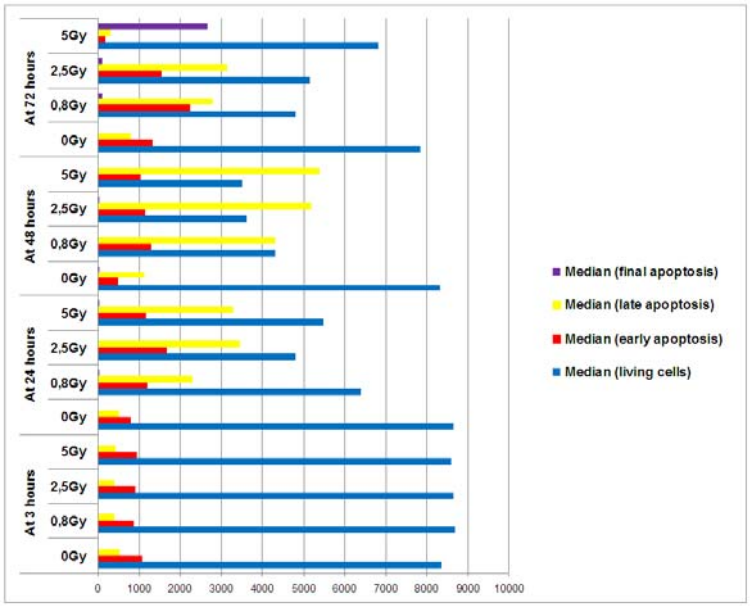

Fig. 2. Analysis of apoptosis in normal mononuclear cells after X irradiation with different doses, 0 Gy, 0.8 Gy, 2.5 Gy and 5 Gy at various time, 3, 24, 48 and 72 hours. The variation of number cells is represented: living cells with blue, early apoptosis with red, late apoptosis with yellow and final apoptosis with purple.

In the second experiment we irradiated tumoral cells. We used two leukemic cell lines: Jurkat and K562. Leukemic cells were exposed at X rays for three day consecutively.
They were irradiated similar with 0 Gy, 0.8 Gy and 2.5 Gy per day.

The response of Jurkat and K562 cells was illustrated in Figure 3, and Figure 4, respectively.

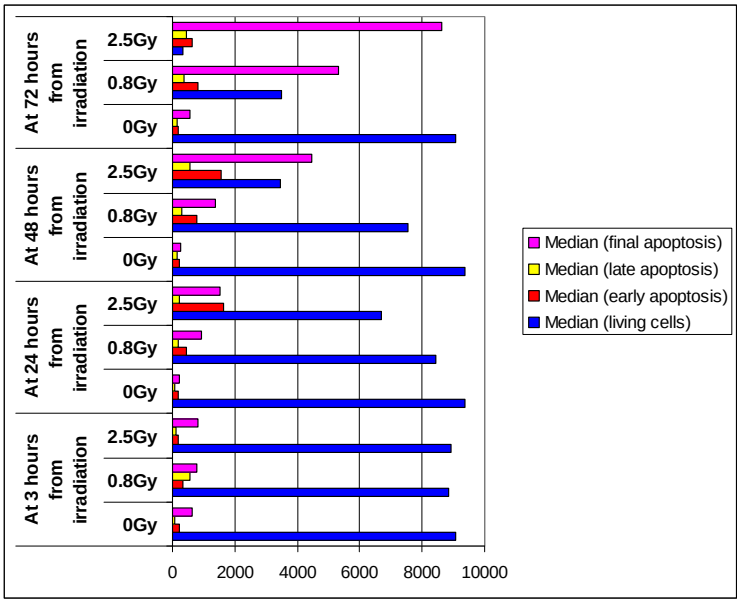

Fig. 3. Analysis of apoptosis in Jurkat cells after X irradiation with different doses, 0 Gy, 0.8 Gy and 2.5 Gy at various time, 3, 24, 48 and 72 hours. The variation of number cells is represented: living cells with blue, early apoptosis with red, late apoptosis with yellow and final apoptosis with purple.

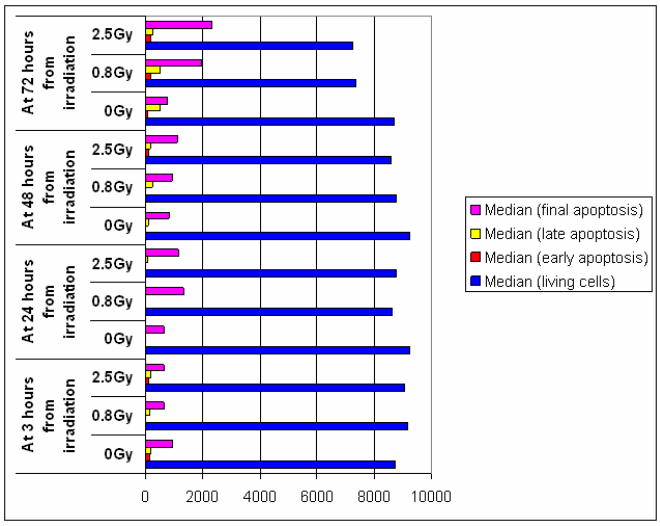

Fig. 4. Analysis of apoptosis in K562 cells after X irradiation with different doses, 0 Gy, 0.8 Gy and 2.5 Gy at various time, 3, 24, 48 and 72 hours. The variation of number cells is represented: living cells with blue, early apoptosis with red, late apoptosis with yellow and final apoptosis with purple.

For the Jurkat and K562 cultures the total doses were 0 Gy, 2.4 Gy and 7.5 Gy.

Analyzing the results obtained for five determinations that have followed the evolution for two leukemic cells statistically significant differences were found ($p<0.001$) between them for values corresponding to different irradiation doses with assessment by flow cytometry at intervals previously established.

3. Discussion

At 3 hours after ionizing irradiation, the changes are not significant for any of the investigated normal and tumoral cell cultures. The number of living cells was bigger than the number of apoptotic cells, which means that a good proliferation of followed cells cultures. At 24 and 48 hours, in normal mononuclear cell cultures number of living cells was decreased significant ($p<0.001$) with the dose of irradiation was more. Cells behavior was similar for 2.5 and 5 Gy for all four quadrants (living cells, early apoptosis, late apoptosis, final apoptosis). The changes become very important for normal mononuclear cells at 72 hours from irradiation. Although flow cytometry showed a similar evolution for cells irradiated with 0.8 Gy and respectively 2.5 Gy. Only cells irradiated with 0.8 Gy also presented normal metaphases (highlighted cytogenetic changes), while the other group has only metaphases abnormal. This means that although cells proliferated similar, at 72 hours after irradiation only in the samples irradiated with small doses (0.8 Gy) were normal cells and in the samples irradiated with 2.5 Gy, the cells were abnormal (with structural and material changes of nucleic acids). At 72 hours from irradiation, the proliferation for cells irradiated with 5 Gy was intense, but the evolution of cells to final apoptosis was extremely fast.

At 24 hours and also at 48 hours, in K562 cell cultures, flow cytometry showed a similar evolution for cells irradiated with 0 Gy, 0.8 Gy and respectively 2.5 Gy. The number of the living cells–increased significantly ($p<0.001$) compared to the number of apoptotic cells. At 72 hours from irradiation, the number of the apoptotic cells increased insignificant compared with other monitoring intervals.

For Jurkat cell cultures number of living cells decreased with increasing doses and time. At 72 hours after irradiation the number of apoptotic cells increased very significant ($p<0.001$),

compared with living cells. At this period, the number of apoptotic cells from Jurkat culture were significant increased ($p<0.001$) compared with the number of K562 cells.

Our study confirms the results obtained from literature according to which normal cells show a higher radioresistance than tumoral cells. Radiosensitivity increases with various doses and different time intervals [1-5].

This experimental model of normal and pathological cell cultures was a good example for the study of cell apoptosis under the influence of various doses of radiation from different intervals of time.

The iInduction of apoptosis, the process of programmed cell death, by ionizing radiation has been studied at many different cell types and it has been found that each cell type behaves differently [7].

4. Conclusions

There were differences in the evolution of cells after irradiation for normal cell cultures and tumoral cell lines. Normal mononuclear cells were more radioresistant than leukemic cells. Between tumoral cells, Jurkat cells were very radiosensitives compared with K562 cells. The results strongly suggest that at low irradiation doses the normal mononuclear cells can recover compared with tumoral cells which had significant functional changes even in small doses from the early hours.

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*Corresponding author: nanisoara@yahoo.com