Monitoring of Premitotic and Postmitotic Apoptosis in Gamma-Irradiated HL-60 Cells by the Mitochondrial Membrane Protein-Specific Monoclonal Antibody APO2.7

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Abstract. Background: Majority of hematopoietic cells die by apoptosis after irradiation with ionizing radiation. In present study it is shown that human promyelocytic leukemia HL-60 cells can undergo two different types of apoptosis, premitotic and postmitotic.

Methods: HL-60 cells were irradiated with doses 8 and 20 Gy. For apoptosis detection APO2.7 antigen (mitochondrial membrane specific protein) expression without and with permeabilization by digitonin was used. This method was compared with flow-cytometric analysis of cell light scattering properties and determination of subG$_1$ DNA.

Result: Cells irradiated with high dose (20 Gy) died rapidly by premitotic apoptosis (interphase death) from all phases of cell cycle. 2 hours after irradiation cells with subdiploid DNA content and cells stained by APO2.7 after digitonin permeabilization appeared. After 6 hours 40 % of cells were apoptotic, nonapoptotic cells were mainly in G$_1$-phase. Lower dose (8 Gy) after 6 hours of irradiation caused accumulation of cells in S-phase. After 24 hours majority of cells was in G$_2$-phase and apoptotic cells appeared (subG$_1$ peak, APO2.7 with permeabilization).

Conclusion: Data presented herein indicate that mitochondrial membrane protein-specific antibody APO2.7 after permeabilization is a useful marker for detection of early apoptotic cells dying by premitotic and postmitotic apoptosis.

Key words: Monoclonal antibody APO2.7 — Apoptosis — HL-60 cells — Ionizing radiation

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Introduction

Induction of apoptosis by ionizing radiation has been studied at many different cell types and it has been found that both mechanism of effect and time dependence are significantly different in particular cell types (Aldridge and Radford 1998). As a result of cell irradiation by gamma- or X-rays two types of cell death were described: interphase death and reproductive or mitotic cell death. Colony forming ability corresponds to the total cell death (interphase death and reproductive death). Interphase death is defined as cell death before reaching the first mitosis after X or gamma irradiation. Results of Shinohara and Nakano (1993) suggest that X-irradiated MOLT-4 cells (the human leukemia T-lymphoid cell line) suffer from interphase death as well as reproductive death. Possibly apoptosis is the mechanism accounting for both modes of death in these cells.

In addition to apoptosis, DNA damage induced by ionizing radiation triggers cell cycle checkpoint activation and subsequent cell cycle arrest, thus augmenting the ability of cells to repair damaged DNA. Accumulating data suggest that ataxia-telangiectasia-mutated (ATM) kinase is a proximal component of DNA damage-induced cell cycle checkpoint pathway (Zhou et al. 2000). The TP53 tumor suppressor gene is a key mediator of the G_1-phase checkpoint (Kuerbitz et al. 1992). Cells without functional TP53 are preferentially arrested in G_2/M phase after DNA damage (Ning and Knox 1999).

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 minimal expression of Bcl-x_L (Terui et al. 1998). HL-60 cells are much more sensitive to radiation-induced loss of clonogenicity, $D_0 = 2.2$ Gy, (D_0 is dose of radiation after which 37 % of cells survive) than to induction of apoptosis at 6 h after irradiation (D_0 for nonapoptotic cells = 32.6 Gy) (Vávrová et al. 2001). Hopcia et al. (1996) described that when HL-60 cells were irradiated with 10 Gy, apoptosis started 2–3 days after irradiation. The findings of Dynlacht et al. (1999) that 10 Gy induces primarily necrosis are in contrast with results of Hopcia et al. (1996) and Vávrová et al. (2001). Shinomiya et al. (2000) have shown that X-ray induced two different types of apoptotic cell death in the human monoblastoid cell line U937, premitotic and postmitotic apoptosis, characterized by the time course and cell cycle specificity.

In present study we succeeded in induction of premitotic and postmitotic apoptosis using HL-60 cells. We investigated correlation among four flow-cytometric methods: flow-cytometric analysis of cell cycle and DNA fragmentation, light scattering measurements and APO2.7 antigen without or with permeabilization used for evaluation of the kinetic of apoptosis induction after irradiation of HL-60 cells with 8 and 20 Gy.

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 modified Dulbecco's medium (Sigma-Aldrich, Prague, Czech Republic) supplemented with 20 % fetal calf serum in a humidified incubator at 37 ℃ and controlled 5 % CO_2 atmosphere. The cultures were divided every 2^{nd} 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

Exponentially 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 (NUNC A/S, Denmark) and irradiated at room temperature using ^{60}Co γ-ray source with a dose rate 3 Gy/min doses 8 and 20 Gy. After irradiation, flasks were placed in a 37 ℃ 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 the Trypan blue exclusion assay.

Cell cycle analysis

Following the incubation, the cells were washed with cold phosphate buffered saline (PBS), fixed by 70 % ethanol. For fixation of low molecular fragments of DNA the cells were incubated 5 min at room temperature in phosphate buffer and then stained with propidium iodide (PI) in Vindelov's solution for 30 minutes at 37 ℃. Fluorescence (DNA content) was measured with Coulter Electronic (Hialeah, FL, USA) apparatus. A minimum of 10,000 cells analyzed in each sample served to determine the percentages of cells in each phase of the cell cycle, using Multicycle AV software. Three independent experiments were performed.

Flow-cytometry light scattering measurements

Ten thousand cells per sample were analyzed for forward and side light scattering using a Coulter Epics XL flow cytometer equipped with a 15 mW argon-ion laser with excitation capabilities at 488 nm (Coulter Electronic, Hialeah, FL, USA). While the intensity of light scattered in forward direction correlates with cell size, side scatter correlates with granularity.

APO2.7 antibody

Cells were washed twice with PBS containing 5 % FCS (fetal calf serum). Then, 1×10^5 cells were suspended in 0.5 ml PBS with 5 % FCS and 0.02 % NaN_3 and incubated with mAbs for 30 min at 4 ℃. For apoptosis detection the mouse phycoerythrin (PE)-conjugated mAb APO2.7 (clone 2.7 A6A3) (obtained from Immunotech, Prague, Czech Republic) for detecting 7A6 antigen expressed by cells

undergoing apoptosis was used. Koester et al. (1997) have demonstrated that the mitochondrial membrane protein-specific monoclonal antibody APO2.7 identifies an early apoptotic response after induction of Jurkat cells with anti-CD95. Non-permeabilized and permeabilized method was used. Cells were permeabilized in 100 μl of 100 μg/ml digitonin solution in PBS and incubated for 20 min on ice. Cells were washed and stained with APO2.7. Flow-cytometric analysis was performed on a Coulter Epics XL flow-cytometer equipped with a 15 mW argon-ion laser with excitation capabilities at 488 nm (Coulter Electronic, Hialeah, FL, USA). A minimum of 10,000 cells was collected for each sample in a list mode file format. List mode data was analyzed using Epics XL System II software (Coulter Electronic, Hialeah, FL, USA).

Results

Induction of apoptosis in HL-60 cells by ionizing radiation was evaluated by different methods during 72 hours after irradiation with 8 and 20 Gy. Apoptosis monitored by determination of subG_1 phase of cell cycle was compared with light scattering properties of cells and with staining of unprocessed (without permeabilization) and processed cells (permeabilization of cells by digitonin before staining) with APO2.7 antibody. Figures 1A,B and 2A,B show changes of all four markers 2, 4, 6, 24, 48 and 72 hours after irradiation with 20 and 8 Gy.

Premitotic apoptosis: From Fig. 1A,B it is apparent that while 4 to 6 hours after irradiation with a dose of 20 Gy changes in light scattering and APO2.7 without permeabilization were not observed, apoptosis was detected using subG_1 phase of cell cycle and APO2.7 after digitonin permeabilization. 6 hours after irradiation the majority of non-apoptotic cells was in G_1 phase of the cell cycle. However, 24 hours after irradiation with 20 Gy the majority of cells was apoptotic – in subG_1 phase and nearly 60 % of cells were stained by APO2.7 after digitonin permeabilization. In this time interval there was a small proportion of cells with low forward scatter (FS) and also the number of cells stained by APO2.7 without permeabilization was small. Their number increased in time and 72 hours after irradiation 90 % of cells were cells with low FS, stained by APO2.7 without permeabilization. Apart from cells in subG_1 phase also late apoptotic (secondary necrotic) cells were present 48 and 72 hours after irradiation. Summary results are shown on Fig. 1B.

Postmitotic apoptosis: As can be seen in Fig. 2A,B, within 2 to 6 hours after irradiation with a dose of 8 Gy no changes in all parameters were observed. 6 hours after irradiation with 8 Gy the majority of cells was in S phase. However, 24 hours after irradiation the majority of cells was arrested in G_2 phase of the cell cycle and early apoptotic cells appeared (stained by APO2.7 after permeabilization and subG_1 cells). Further, 48 hours after irradiation with a dose of 8 Gy 90 % of cells were in subG_1 peak and 60 % of cells were stained by APO2.7 after permeabilization. At this time 40 % of cells already had low FS and were stained by APO2.7 without permeabilization. No difference could be seen between the numbers of cells

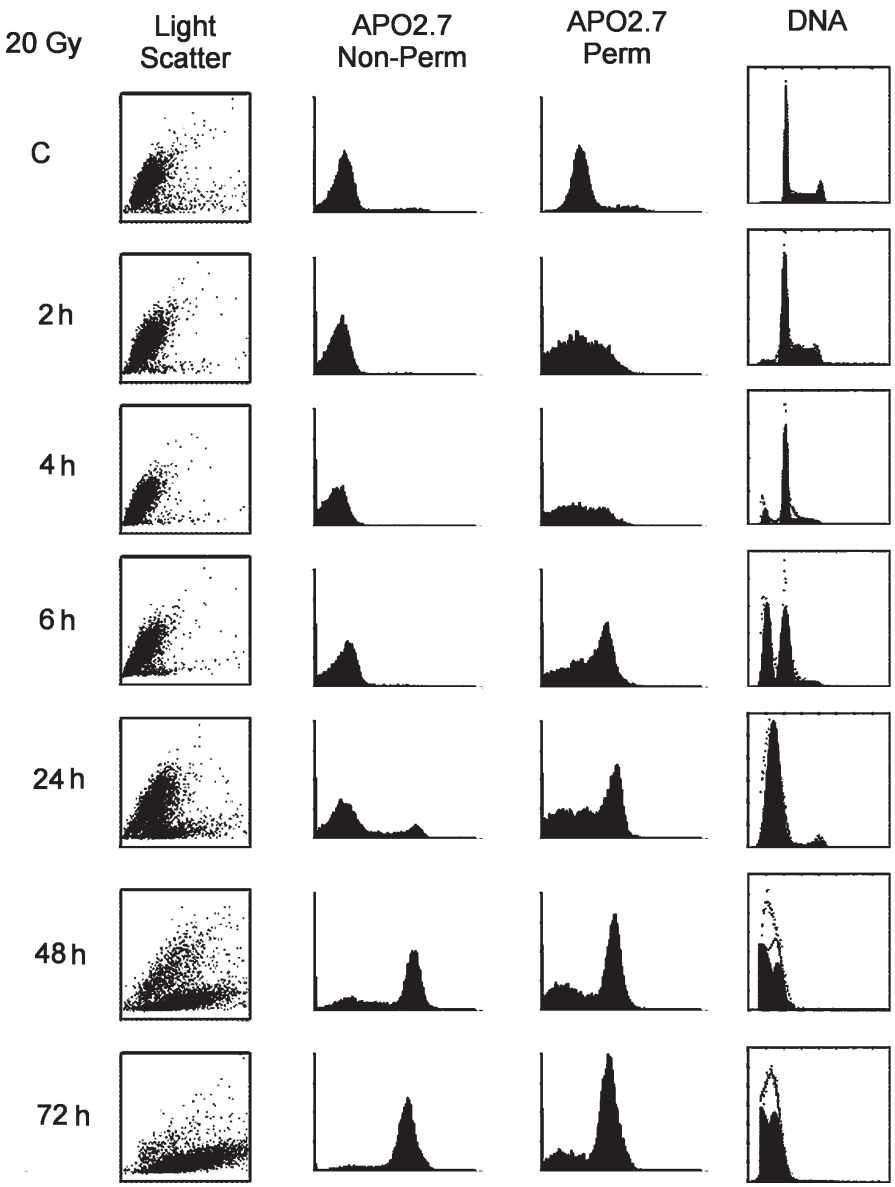

Figure 1A. Data collected during 72 hours to monitor HL-60 cell death induced by irradiation with 20 Gy. Data points represent changes in the forward (y-axis) and side (x-axis) scattering of the light by cells; cells stained with APO2.7-PE (clone 2.7 A6A3) conjugated antibody without digitonin permeabilization (APO2.7 Non-Perm) or after permeabilization (APO2.7 Perm); cells distribution in the phases of the cell cycle, where x-axis represents DNA content and y-axis represents the number of cells (subG_1 phase was calculated).

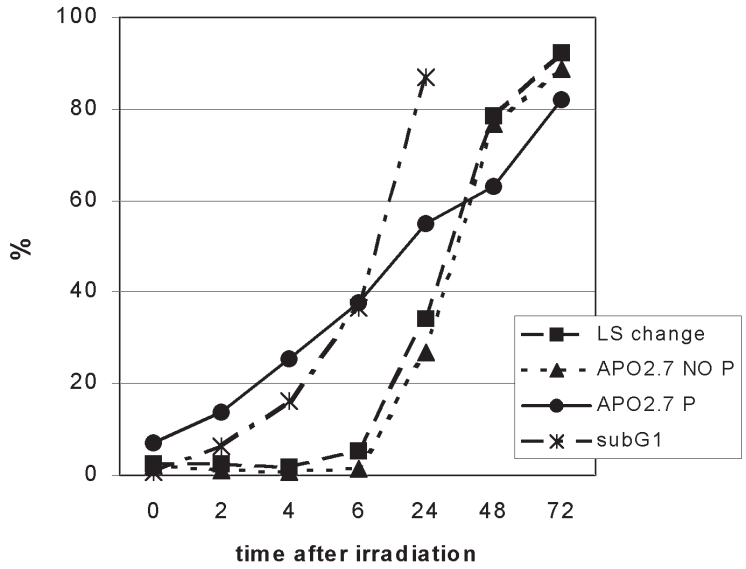

Figure 1B. Time related responses in HL-60 cells to ionizing radiation induced cell death over 72 hours after irradiation with 20 Gy. All data were derived from flow-cytometric analysis. Each point represents the mean from three independent experiments. LS, light scatter; NOP, non permeabilized; P, permeabilized.

stained by APO2.7 with or without permeabilization 72 hours after irradiation. Summary results are shown on Fig. 2B.

Discussion

In this study we proved two different types of apoptotic cell death after exposure of HL-60 cells to ionizing gamma radiation: premitotic and postmitotic apoptosis. A dose of 20 Gy induced early (4–6 hours after irradiation) apoptosis, which was proved by DNA fragmentation and positivity of APO2.7 after permeabilization. In our previous study (Vávrová et al. 2001) we morphologically proved the existence of apoptosis after irradiation with doses of 20–100 Gy 6 hours after irradiation. Established D_0 value for nonapoptotic cells was 32.6 Gy, indicating that this type of apoptosis is related to high doses of radiation. From results acquired in this study it is obvious that the cells die by apoptosis from the phase of cell cycle, in which they were irradiated and that the cells irradiated in G_1 phase are less sensitive to premitotic apoptosis. Similar results have been obtained by Shinomiya et al. (2000) on monoblastoid cell line U937. Syljuasen and McBride (1999) have evaluated apoptosis induction in Jurkat cells irradiated with 20 Gy. Jurkat cells were sorted in different populations by centrifugal elutriation. Regardless of cell

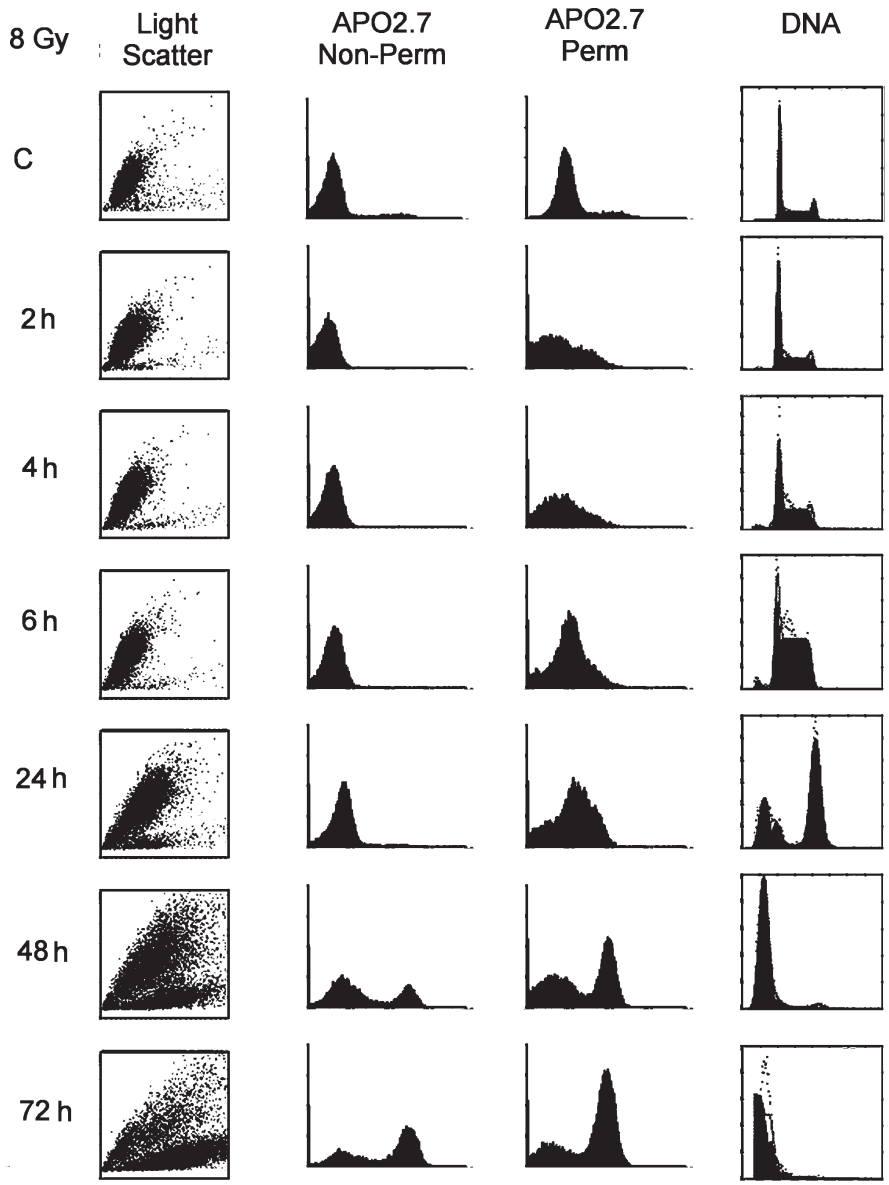

Figure 2A. Data collected during 72 hours to monitor HL-60 cell death induced by irradiation with 8 Gy. Data points represent changes in the forward (y-axis) and side (x-axis) scattering of the light by cells; cells stained with APO2.7-PE (clone 2.7 A6A3) conjugated antibody without digitonin permeabilization (APO2.7 Non-Perm) or after permeabilization (APO2.7 Perm); cells distribution in the phases of the cell cycle, where x-axis represents DNA content and y-axis represents the number of cells (subG_1 phase was calculated).

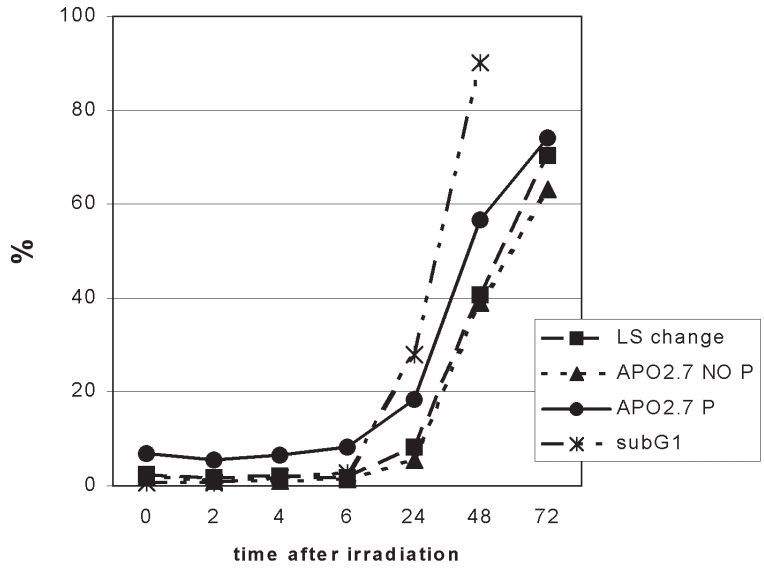

Figure 2B. Time related responses in HL-60 cells to ionizing radiation induced cell death over 72 hours after irradiation with 8 Gy. All data were derived from flow-cytometric analysis. Each point represent mean from three independent experiments.

cycle position at the time of irradiation, cells with sub-G_1-phase DNA content had accumulated in all populations at 6 h after a dose of 20 Gy, indicating that apoptosis is most likely independent of cell cycle arrests. Cells irradiated in G_1 phase underwent apoptosis more slowly than cells irradiated in other phases.

Premitotic apoptosis could be characterized as interphase death. In this study we proved that mitochondrial membrane protein-specific monoclonal antibody, APO2.7 (after permeabilization) identified an early apoptotic response after irradiation with a dose of 20 Gy. Also Koester et al. (1997) have described detection of early apoptosis at Jurkat cells following CD95 induction of apoptosis. We determined that the staining response to APO2.7 antibody had the same time dependence as DNA fragmentation measured as subG_1 phase of cell cycle after irradiation of HL-60 cells. Light scatter changes and staining of APO2.7 without permeabilization appeared much later after irradiation and therefore these methods are not eligible for determination of early apoptosis. The dynamic of the above-mentioned methods was practically concordant.

Irradiation of cells can induce a blockage at checkpoints in different phases of the cell cycle, including TP53 dependent arrest in G_1 and TP53 independent arrests in S and G_2. These arrests are ultimately caused by inhibition of cyclin-dependent kinases that are necessary for cycle progression (Ning and Knox 1999). A dose of 8 Gy induced postmitotic apoptosis of HL-60 cells, apoptosis appeared after progress

of cells through cell cycle and G_2 phase arrest. Syljuasen and Mc Bridge (1999) have detected that after irradiation with 2 Gy the onset of apoptosis appeared earlier in cells irradiated in G_2/M phase than in cells irradiated in the other phase. HL-60 cells are very radiosensitive as results from clonogenicity test, where D_0 value was 2.2 Gy (Vávrová et al. 2001) which corresponds to value $D_0 = 2$ Gy determined by Hopcia et al. (1996). Findings of Dynlacht et al. (1999) that 10 Gy induced primarily necrosis are in contrary to that of Hopcia et al. (1996), who have shown that a significant amount of apoptosis occurred 2–3 days after irradiation with 10 Gy. We proved that 24 hours after irradiation with 8 Gy the cells were accumulated in G_2 phase and part of cells was apoptotic (subG_1 phase, APO2.7 positivity after permeabilization). 72 hours after irradiation a majority of cells was late apoptotic, with low FS and APO2.7 positive without permeabilization. DNA analysis detected these cells as necrotic.

Our work could be concluded by statement that HL-60 cells die after irradiation with high doses (over 20 Gy) early after irradiation by so-called premitotic apoptosis. Lower doses (up to 10 Gy) kill the cells by postmitotic apoptosis after G_2 phase arrest of the cell cycle. During both types of apoptosis (post and premitotic) early and late phases of apoptosis could be distinguished using mitochondrial antigen APO2.7. In early phase the cell membrane is intact and APO2.7 antigen could only be detected after permeabilization of cells by digitonin (2–4 hours after irradiation by 20 Gy and 24 hours after irradiation with 8 Gy). In late phase of apoptosis the cell membrane is permeable and APO2.7 is detected without permeabilization of cells by digitonin.

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