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Modulation of Ionizing Radiation-Induced Apoptosis and Cell Cycle Arrest by All-Trans Retinoic Acid in Promyelocytic Leukemia Cells (HL-60)

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Summary

Acute promyelocytic leukemia is characterized by a block of myeloid differentiation. The incubation of cells with 1 μmol/l all-trans retinoic acid (ATRA) for 72 h induced differentiation of HL-60 cells and increased the number of CD11b positive cells. Morphological and functional changes were accompanied by a loss of proliferative capacity. Differentiation caused by preincubation of leukemic cells HL-60 with ATRA is accompanied by loss of clonogenicity (control cells: 870 colonies/10^3 cells, cells preincubated with ATRA: 150 colonies/10^3 cells). D_0 for undifferentiated cells was 2.35 Gy, for ATRA differentiated cells 2.46 Gy. Statistical comparison of clonogenity curves indicated that in the whole range 0.5-10 Gy the curves are not significantly different, however, in the range 0.5-3 Gy ATRA differentiated cells were significantly more radioresistant than non-differentiated cells. When HL-60 cells preincubated with 1 μmol/l ATRA were irradiated by a sublethal dose of 6 Gy, more marked increase of apoptotic cells number was observed 24 h after irradiation and the surviving cells were mainly in the G_1 phase of the cell cycle, while only irradiated cells were accumulated in G_2 phase. Our results imply that preincubation of cells with ATRA accelerates apoptosis occurrence (24 h) after irradiation by high sublethal dose of 6 Gy. Forty-eight hours after 6 Gy irradiation, late apoptotic cells were found in the group of ATRA pretreated cells, as determined by APO2.7 positivity. This test showed an increased effect (considering cell death induction) in comparison to ATRA or irradiation itself.

Key words

Apoptosis • All-trans retinoic acid • Ionizing radiation • HL-60 cells

Introduction

Exposure of cells to ionizing radiation leads to cellular damage primarily through a spectrum of lesions in DNA, of which double-strand breaks represent the most lethal form of damage. These lesions are recognized by a number of different proteins including DNA-dependent protein-kinase, ataxia telangiectasia mutated

(ATM) protein and the rad3-related protein (Smith and Jackson1999). Recognition of the damage leads to the activation of these proteins. This in turn transfers the signal by activating other proteins such as p53 that induce gene expression to activate repair, cell cycle checkpoints, and apoptosis (Lavin *et al.* 1999).

The TP53 gene is often mutated or missing in cells of human tumors. These cells have some proliferative advantages over normal cells. Some groups (Santana *et al.* 1996, Herr *et al.* 1997) have proved that ionizing radiation and chemotherapeutical drugs initialize an increase of ceramide concentration, which leads the cell to apoptosis. This pathway is initialized by changes on cell membrane. Both molecules - p53 and ceramide - are important in regulation of cell cycle, aging and apoptosis.

Acute myeloid leukemia is caused in 40 % by chromosomal translocation and the TP53 gene is usually mutated or missing. HL-60 cells of human promyelocytic leukemia do not have the TP53 gene, they have normal or slightly increased expression of Bcl-2 and minimal expression of Bcl-x_L (Terui *et al.* 1998).

Many studies have indicated a link between apoptosis and progression of cells through the phases of the cell cycle. Such relation could be an upregulation or activation of the cell cycle regulatory proteins like Jun, Fos, Myc, JNK, Cdc2, and Rb in pre-apoptotic cells (Pandey and Wang 1995). Cells of the human T-cell line Jurkat were separated by centrifugal elutriation into populations enriched in G_1, S and G_2/M phase cells before irradiation. After a dose of 20 Gy, the onset of massive apoptosis occurred about 6 h after irradiation in all populations regardless of the phase of the cell cycle in which they were irradiated. In contrast, cells after 2 Gy died at various times after a pronounced G_2/M phase arrest (Syljuasen and McBride 1999). Apoptosis is dependent on the radiation dose also in TP53 negative leukemic line HL-60. After 20-100 Gy irradiation, HL-60 cells were found to die 6 h after irradiation primarily by apoptosis (Dynlacht *et al.* 1999, Vávrová *et al.* 2001). After doses lower than 10 Gy these cells are preferentially arrested in the G_2 phase and apoptosis occurs later, 48-72 hours after irradiation. However, it seems that ionizing radiation also induces rapid degradation of protein Cdc25A-phosphatase, which is TP53 independent. (Mailand *et al.* 2000).

Retinoic acid is an oxidized form of vitamin A. It has three naturally occurring isomers: all-trans-retinoic acid (ATRA), 13-cis-retinoic acid and 9-cis-retinoic acid. The cellular effect of retinoic acid is transmitted by cytoplasmatic proteins CRAB I and II and by nuclear receptors RAR and RXR. The regulations of proliferation and differentiation during embryogenesis are the main physiological functions of retinoic acid. Defects of cellular pathways regulated by retinoic acid are closely connected to tumorigenesis. Tumors are often associated with inability of cells to differentiate. *In vitro* studies (Breitman *et al.* 1980, Hofmanová *et al.* 1998) show that retinoic acid and DMSO induce differentiation of human promyelocyte leukemic cells accompanied by changes of morphological and functional markers and by loss of proliferative activity. Acute promyelocytic leukemia (APL) is characterized by a block of myeloid differentiation caused by expression of the fusion oncoprotein promyelocytic leukemia-retinoic acid receptor alpha (PML-RARalpha) (Rusiniak *et al.* 2000). The relationship between differentiation of human myeloid cells and apoptosis is still not clear, e.g. what effect has the induction of differentiation on the sensitivity of leukemic cells to undergo apoptosis in response to cytotoxic agents. Furthermore, it remains unclear, whether the use of differentiating agents in combination with chemotherapy is rational. Some studies have shown that induction of differentiation is related to increased resistance to apoptosis-induction after such agents as etoposide or azacytidine (Solary *et al.* 1993, Del Bino *et al.* 1994). Ketley *et al.* (1997) studied the effect of idarubicine on AML cells after incubation with ATRA. They proved that following 72-h incubation with ATRA, HL-60 cells are resistant to idarubicine-induced apoptosis 24 h after idarubicine addition and proportion of cells in G_1 phase increases. However, despite *in vitro* evidence for reduced drug-induced cell death after ATRA (Cotter *et al.* 1994, Ketley *et al.* 1997), there is accumulating clinical evidence from APL trials that a greater remission and reduced relapse are induced with ATRA plus chemotherapy as compared to either modality alone (Fenaux *et al.* 1999).

In our study we investigate the relationship between apoptosis induction, cell-cycle arrest in the G_1 or G_2 phase and differentiation in cells treated by a combination of ATRA and sublethal irradiation.

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) supplemented with 20 % fetal calf serum in humidified incubator at 37 ºC and controlled 5 % CO_2 atmosphere.

The cultures were divided every second 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.

Incubation of cells with ATRA

Cells in exponential growth were seeded at 2×10^5/ml in 25 cm^2 culture flasks and supplemented with 1 μmol/l ATRA. ATRA (Sigma) was dissolved in ethanol to achieve stock solution of 1 mmol/l and stored at –20 ºC until required. The final concentration of ethanol in culture did not exceed 0.1 %. Cells were harvested after 72 h, washed and used for another assay.

Gamma irradiation

Exponentially growing HL-60 cells and cells after incubation with ATRA were suspended at a concentration of 2×10^5 cells/ml in complete medium. Ten ml of aliquots were plated into 25 cm^2 flasks (Nunc) and irradiated at room temperature using ^{60}Co γ-ray source with a dose rate 0.66 Gy/min. After irradiation, flasks were placed in a 37 ºC 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 by the Trypan blue exclusion assay.

Cell morphology

To calculate the percentage of cells showing the 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 (Dade Behring, Switzerland) stained cytospin preparations. Apoptotic cells were identified by the condensed and fragmented state of their nuclei and focal protrusions of the cell surface. Three independent experiments were performed.

Cell cycle analysis

Following incubation, the cells were washed with cold PBS, fixed by 70 % ethanol and stained with propidium iodide (PI) in Vindelov's solution for 30 min at 37 ºC. 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.

APO2.7 antibody

Flow cytometry was used for cell surface antigen analysis and for apoptosis monitoring. Cells were washed twice with PBS containing 5 % FCS. Then, 1×10^5 cells suspended in 0.5 ml PBS with 5 % FCS and 0.02 % Na_3N were incubated with mAbs for 30 min at 4 ºC.

For apoptosis detection the mouse phycoerythrin (PE)-conjugated mAb APO2.7 (clone 2.7 A6A3) (obtained from Immunotech, Prague, CR) for detecting 7A6 antigen expressed by cells undergoing apoptosis was used. The method without cell membrane permeabilization was used.

CD11b antibody

For detection of cell surface markers we used PE-conjugated anti-human CD11b (Bear1), Prague, CR, mAbs (obtained from Immunotech, Prague, CR).

Flow cytometric analysis

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 were analyzed using Epics XL System II software (Coulter Electronic, Hialeah, FL, USA).

Colony assay

10^2-10^5 cells were plated in Iscove's medium containing 0.9 % methylcellulose and 30 % FBS. Duplicate dishes were plated for each experiment, stimulated by 10 % conditioned medium from the 5637 human bladder carcinoma cell line and 4 units of erythropoietin per 1 ml of medium and incubated for 14 days at 37 ºC in humidified atmosphere containing 5 % CO_2 and 5 % O_2. Colonies containing more than 40 cells were scored. For the clonogenic survival data, each point is the mean of four measurements from two experiments. The cells were irradiated with an increasing dose of radiation in the range 0.5-10 Gy.

Statistical analysis

The results were statistically evaluated with Student's t-test. The values represent mean ± S.D. Statistical significance of the difference of means in comparable sets is indicated. Statistical evaluation of clonogenity curves was performed by the bilateral t-test of difference of corresponding (from the polynomial degree point of view statistically optimalized) polynomial regression functions for dense net (1000 points) of

independent variable, i.e. dose in Gy. For testing of difference of polynomial regression functions in whole values of mean integral probability (accordance) through whole observed range of independent variable (i.e. dose in Gy) were used. (Knížek *et al.* 2001).

Results

Cell death and differentiation

Seventy-two hours long incubation of HL-60 cells with 1 μmol/l ATRA leads to increased expression of integrin CD11b, which is known as a marker of differentiation. Expression of CD11b increased from 1.5±0.4 % to 25.9±4.8 %. Increased expression of this antigen lasted for 10 days. Flow cytometric analysis of DNA content revealed that a part of ATRA-treated cells is apoptotic. Apoptosis was detected after 72-h preincubation (24 %) and lasted at a similar level for 7 days (23-29 %) (Fig. 1). The number of living cells in culture did not change from the end of preincubation with ATRA till the 10th day, contrary to the control group, where the cells grew exponentially (data not shown).

In the next part of our work, we observed the influence of 72-h preincubation of cells with ATRA on radiosensitivity of these differentiated cells. Figure 2 shows the results of morphological evaluation of HL-60 cells preincubated 72 h with ATRA and then irradiated by a high sublethal dose of 6 Gy. Morphologic changes of HL-60 cells were evaluated on Diff-Quick stained cytospin preparations. It is apparent that irradiation by a high sublethal dose of 6 Gy itself induces maximum of morphological changes linked with apoptosis 48 h after irradiation. Ninety-six hours after irradiation late apoptotic (necrotic) cells stained by Trypan blue prevailed. Cells preincubated for 72 h with ATRA showed a maximum of apoptosis 24 h after irradiation, 48 h after irradiation of ATRA-preincubated cells 70 % of cells were stained by Trypan blue, and after 96 h all cells were stained by Trypan blue (Fig. 2).

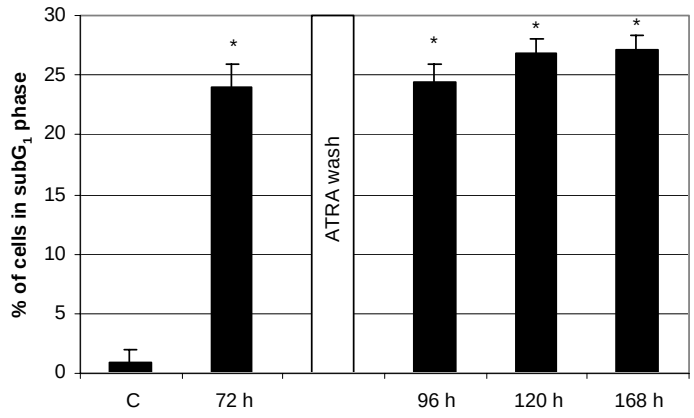

Fig. 1. *Apoptosis of HL-60 cells caused by 1 μmol/l ATRA. Flow cytometric analysis of DNA content and cell-cycle of HL-60 cells treated with 1 μmol/l ATRA, 72-168 h after beginning of incubation. Apoptotic cells were identified as cells with subdiploid DNA content (lower DNA content than cells in G_0/G_1 phase), i.e. $subG_1$ peak. * $p < 0.01$*

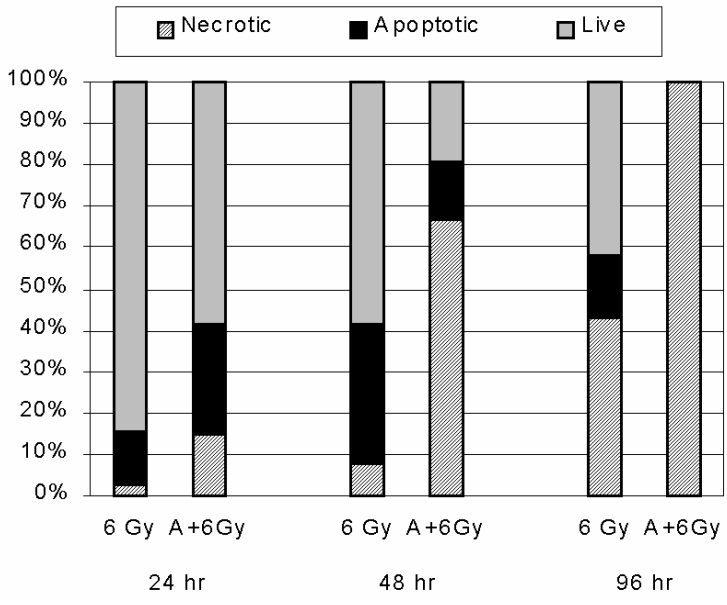

Fig. 2. *Evaluation of percentage of living (non-apoptotic), apoptotic and necrotic cells after irradiation of normal and ATRA preincubated HL-60 cells with the dose of 6 Gy. Number of necrotic cells was determined by Trypan blue staining, number apoptotic cells was determined by cell morphology examined on Diff-Quik stained cytospin preparation.*

It is evident from Figure 3 that 24 h after irradiation of HL-60 cells by a dose of 6 Gy a majority of cells (79 %) is arrested in G_2 phase of cell-cycle and 48 h after irradiation, 49 % of cells are apoptotic (subG_1 peak). This arrest was not observed in cells irradiated after previous 72-h preincubation with ATRA, but apoptosis (10 %) was detected 24 h after irradiation; 20 % of cells was apoptotic 48 h after irradiation and remaining cells were mainly accumulated in G_1 phase of cell cycle.

Monoclonal antibody APO2.7 was used for detection of 38 kDa mitochondrial membrane protein 7A6. Since the 7A6 antigen is selectively expressed on the mitochondrial membrane in cells undergoing apoptosis, this staining could be used for detection of apoptotic cells. After 72-hincubation with ATRA, 16 % of cells were APO2.7 positive. Irradiation by dose of 6 Gy induced apoptosis in 10, 18 and 35 % of cells at 24, 48 and 72 h after irradiation. Percentage of APO2.7 positive cells increased after incubation of cells with ATRA plus irradiation by 6 Gy to 25, 52 and 69 %, at 24, 48 and 72 h after irradiation, respectively (Fig. 4).

Colony assay

We compared effect of a combination of 72-h incubation with ATRA plus γ-irradiation with γ-irradiation itself on colony forming efficiency. Incubation with ATRA alone caused six-fold decrease of formed colonies. Control cells formed 870±94 colonies/10^3 cells; cells preincubated with ATRA formed only 150±49 colonies/10^3 cells. D_0 for undifferentiated cells was 2.35 Gy, for ATRA differentiated cells 2.46 Gy (Fig. 5). Dose-response curves were statistically evaluated by bilateral t-test of polynomial regression functions difference. Both curves on the whole were not different from the point of view of integral mean value. However, these curves could be considered significantly different in the range of doses 0.5-3 Gy, when ATRA differentiated cells are more radioresistant than non-differentiated cells.

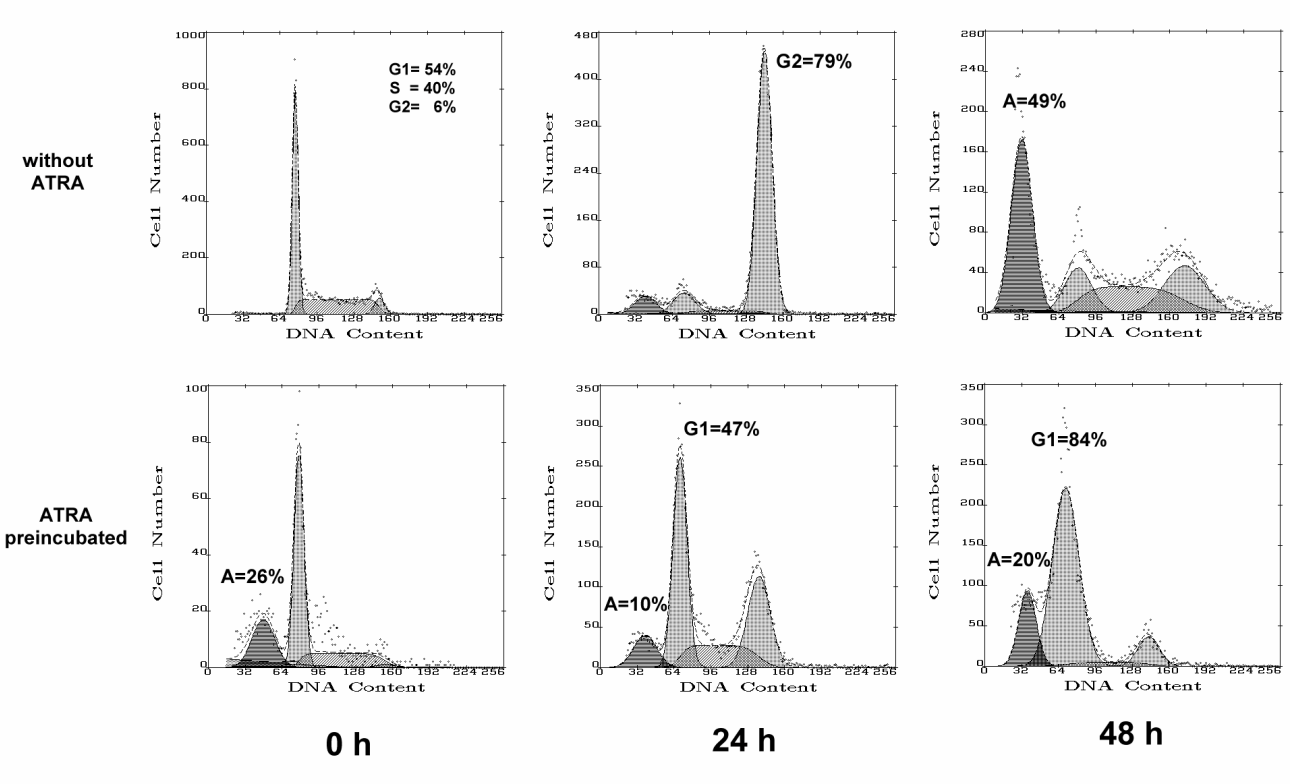

Fig. 3. *Flow cytometric analysis of DNA content and cell-cycle after irradiation of normal and ATRA preincubated HL-60 cells with the dose of 6 Gy. Apoptotic cells were identified as cells with subdiploid DNA content (lower DNA content than cells in G0/G1 phase), i.e. subG_1 peak. Representative results of one experiment are shown.*

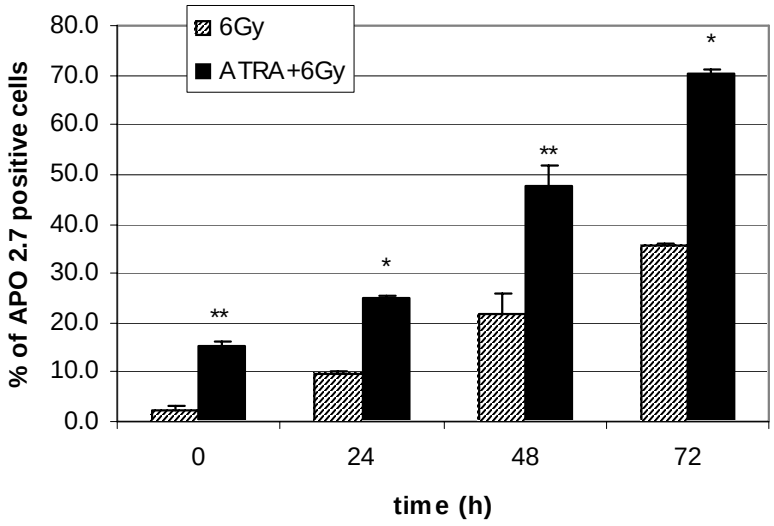

Fig. 4. *Flow cytometric detection of antigen APO2.7 after irradiation of normal and ATRA preincubated HL-60 cells with the dose of 6 Gy without permeabilization. * p<0.01; ** p<0.05*

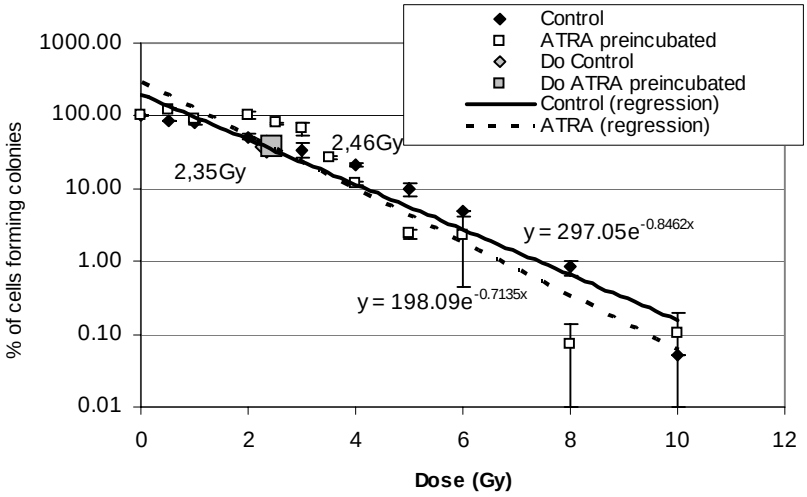

Fig. 5. *Dose-response curve for loss of clonogenicity of normal and ATRA preincubated HL-60 cells exposed to gamma rays. For the clonogenic survival data, each point is the mean of four measurements from two experiments.*

Discussion

Tumors are often connected with inability of cells to differentiate. *In vitro* studies (Breitman *et al.* 1980) showed that all-trans retinoic acid and DMSO induce differentiation of human promyelocyte leukemia cells with morphological and functional changes and loss of proliferative activity.

In our study, we proved that 72-h incubation of HL-60 cells in medium containing 1 μmol/l ATRA causes growth inhibition, induces differentiation (CD11b antigen increase) and apoptosis. However, cells preincubated for 72 h with 1 μmol/l ATRA restored proliferation after 14 days of subsequent incubation in a medium without ATRA (Mareková, data not published). The concentration 1 μmol/l is comparable to the dose used clinically, where plasma concentration of ATRA during therapy of patients is 347 ng/ml (Klener 1996). We found that from the day 3 of incubation of HL-60 cells with ATRA the number of cells in the S phase of the cell cycle decreases and cells in the subG$_1$ phase appear, which is typical for the apoptotic cells. Ketley *et al.* (1997) noted a decrease of cell proliferation after 72 h of incubation in one third of control cells. In our work, using colony assay we observed that proliferation ability of HL-60 cells after incubation with ATRA was reduced to 17 % of control level.

Radiotherapy itself or in combination with conventional systemic chemotherapy is often used in the therapy of extramedullar myeloid tumors (Byrd *et al.* 1995). Observations of a better response of lymphoid tumors to radiotherapy in comparison to myeloid leukemia lead us to the question about the radiosensitivity of cells treated with agents inducing granulocyte differentiation (like ATRA) and whether this differentiation is accompanied by a loss of sensitivity to

ionizing radiation. Studies of the proliferative ability of cells treated with ATRA and then irradiated showed that irradiation induces cell death and ATRA leads to cell differentiation, resulting in additive growth inhibition. Neildez-Nguyen *et al.* (1998) proved that even irradiation by doses of 2-5 Gy prior to incubation of HL-60 cells with ATRA does not abolish ATRA-induced differentiation. In our study, we have showed that irradiation by a dose of 6 Gy itself induces an increase of CD11b antigen, but this increase occurs only temporarily and the cells in culture are undifferentiated on the day 4 after irradiation (Mareková, data not published).

For apoptosis detection we used classic morphology (Diff-Quick test), detection of plasma membrane permeability using Trypan blue staining of cells, detection of subG_1 peak during DNA cell cycle analysis and detection of mitochondrial antigen APO2.7. Koester *et al.* (1997) demonstrated that the mitochondrial membrane protein-specific monoclonal antibody APO2.7 identified an early apoptotic response after treatment of Jurkat cells with anti-CD95, if cells were permeabilized prior staining. If the method is used without permeabilization, it detects late apoptotic cells.

Our results imply that preincubation of cells with ATRA accelerates apoptosis occurrence (24 h) after irradiation by high sublethal dose of 6 Gy, and cells with permeable cytoplasmic membrane (late apoptotic cells) also appear sooner. Determination of apoptosis by subG_1 peak detects only early apoptotic cells and when evaluating combined effect of two noxes it could yield imperfect results (Ketley *et al.* 1997). Considering all criteria the best method for apoptosis determination of late apoptotic cells appears to be the determination of mitochondrial antigen APO2.7 without permeabilization, or for all apoptotic cells after digitonin permeabilization. Using APO2.7 without permeabilization we detected the increased effect (considering cell death induction) of ATRA plus irradiation in comparison to ATRA or irradiation itself.

Interesting results were obtained during statistical evaluation of experiments studying the relation between number of formed colonies and radiation dose in the range 0.5-10 Gy. When compared by the bilateral t-test of difference of corresponding polynomial regression functions there was no significant difference between the curves at whole for non-differentiated and differentiated (ATRA-treated) cells. Furthermore, D_0 value of differentiated cells (2.46 Gy) was only moderately higher than D_0 value of non-differentiated cells (2.35 Gy). Evaluation of response to low doses of radiation (up to 3 Gy) showed significantly higher radioresistance of differentiated cells in comparison to non-differentiated cells, which indicates that in the low dose range differentiated cells are less sensitive to ionizing radiation. It is the range of doses, where ionizing radiation leads mainly to a decrease of cells in S phase of cell-cycle and accumulation of cells in G_2 phase, apoptosis induction is relatively low in this range of doses. In our future experiments we will concentrate on combination of these low doses (2 Gy) with ATRA, as fractionated irradiation by doses of 2 Gy is used in radiotherapy.

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