Antileukemic activity of the combination of ionizing radiation with valproic acid in promyelocytic leukemia cells HL-60

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Valproic acid (VA) possesses anticonvulsant as well as anticancer properties of histondeacetylases inhibitor. Incubation of human promyelocytic leukemia cells HL-60 with VA leads to acetylation of nuclear histones H3 and H4. Using 2 mmol/l concentration we proved the expression of protein p21, which relates to the arrest of cell proliferation and decrease in number of cells in S phase of cell cycle. Treatment of HL-60 cells with VA causes their differentiation, proved as increase in CD11b expression. The most widely used method in cancer treatment is radiotherapy. 24 hours after irradiation by the therapeutical dose of 2 Gy, 56% of HL-60 cells are accumulated in G2 phase of cell cycle. VA had no influence on this accumulation, but 24 h-long pretreatment of cells with 1 mmol/l VA provoked higher decrease in cell number in S phase (18%) comparing with only irradiated cells (25%). The results of our work show that VA posseses radiosensitizing properties when applied 24 hours prior to irradiation and that during parallel long-term action of VA and IR the cells undergo differentiation and faster apoptosis induction. Radiosensitizing effect of VA is not caused by abrogation of G2/M cell cycle arrest, but VA induces p21 and leads to differentiation of HL-60 cells.

Key words: apoptosis, ionizing radiation, p21, radiosensibilisation, valproic acid

Histone acetylation is a posttranslational modification of the core histones that affects chromatin structure and gene expression. Defects in both histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities have been described in a variety of cancers. Inhibitors of HDAC activity (HDACi) induce differentiation, growth arrest and apoptosis of transformed cells in culture and inhibit tumor growth in animals [1,2].

Radiotherapy is frequently used method in the treatement of cancer. Therapeutical potential of radiotherapy can be increased by application of radiosensitizing agents before irradiation. Despite intensive research of these agents, such as iodo-2-pyrimidinone-2-deoxiuridine [3] or misonidazole [4] the radiosensitizers are not used in clinic. Radiation-induced damage of DNA is related to chromatin structure. One of the processes involved in chromatin remodeling is histone acetylation, regulated by HDAC; therefore HDAC inhibition might have radiosensitizing effect. As soon as in 1985 [5] radiosensitizing effect of sodium butyrate (NaB), short-chain fatty acid, has been proved on colon cancer cells. Biade et al. [6] during studies of trichostatin A (TSA) linked its radiosensitizing effect with hyperacetylation of histones. Karagiannis et al. [7] stated, that 24 hours long incubation of human chronic myelogenous leukemia cells K562 with trichostatin A increased radiation sensitivity. Dose modification factor ranged from 1.1 when cells were incubated with 0.1 umol/l TSA to 2.3 at 1 umol/l TSA. Camphausen et al. [8] observed maximal acetylation of nuclear histones H3 and H4 after 24 and 48 hours long incubation of prostate carcinoma (DU145) and glioma (U251) cells with HDAC inhibitor MS-275. The cultivation of cells with MS-275 after irradiation had only weak effect, whereas the best results were obtained with a 48 hours long incubation prior and continuously following the irradiation. Munshi et al. [9] compared radiosensitizing

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DSB – double strand break; EC50 – the concentration, after which 50% of cells retain their clonogenic capacity and form colonies; EC70 – the concentration, after which 30% of cells retain their clonogenic capacity and form colonies; HAT – histone acetyl transferase; HDAC – histone deacetylase; HDACi – histone deacetylase inhibitor; IR – ionizing radiation; NaB – sodium butyrate; TSA – trichostatin A; VA – valproic acid

effect of NaB in melanoma cells with normal fibroblasts. They proved NaB to reduce significantly the expression of reparation proteins Ku70 and Ku86, but only in cancer cells, capacity of reparation proteins in normal fibroblasts stayed unchanged. It can be concluded that radiosensitizing effect of HDACi in melanoma cells is linked to the inhibition of reparation of DNA damage induced by ionizing radiation (IR).

Chemical structure of valproic acid (2-propylpentanoic acid, VA) is similar to NaB. However, NaB has a very short half-life (30 min), which makes it inconvenient for clinical application. On the other hand, VA has well known anti-convulsant properties and it is routinely used in treatment of epilepsy. Moreover, during the past years it has become evident that valproic acid possesses antitumor properties related to inhibition of histone deacetylases [10]. It has been proved by Phiel et al. [11] that antiepileptic activity of VA is not related to inhibition of HDAC.

Three days-long incubation of leukemic cells HL-60 and MOLT-4 with VA causes proliferation arrest and decrease in number of cells in S phase of the cell cycle [12]. Despite the same EC50 value determined by colony-forming assay (1.8 mmol/l) both cell lines react differently to VA. In MOLT-4 cells VA induces primarily apoptosis, while in HL-60 cells it leads to differentiation (increase in CD11b and CD86) and proliferation arrest. Apoptosis in HL-60 cells was observed only after exposure to high concentrations of VA (4 mmol/l). During long cultivation (14 days) of HL-60 cells with VA significant protective effect of cytokines cocktail (IL-3, SCF and G-CSF) has been proved [12].

In our previous studies of VA effects in T-lymphocyte leukemia cells MOTL-4 we proved synergic action of VA and IR. Continual exposure of MOLT-4 cells to VA increases apoptosis and decreases colony-forming capacity of the cells irradiated with small dose of radiation. EC70 value dropped from 0.97 to 0.38 mmol/l when the cells were irradiated with a dose of 1 Gy before the continual cultivation with VA. Also 3 days-long preincubation of MOLT-4 cells with VA before irradiation has radiosensitizing effect [13].

HL-60 cells do not express functional protein p53 and they react to irradiation by long cell cycle arrest in G2/M phase. During this cell cycle arrest the HL-60 cells repair radiation-induced damage, which grants them relatively high radioresistance. Abrogation of G2/M phase arrest by application of caffeine (inhibitor of ATM kinase) has significant radiosensitizing effect [14]. Inhibition of cellular proliferation is result of increased expression of protein p21.

In this study we evaluated radiosensitizing effect of VA on human promyelocyte leukemia cells HL-60 and the effect of irradiation combined with continuous presence of low concentrations of VA.

Material and methods

Cell cultures and culture conditions. The human promyelocyte leukemia cells HL-60 from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK) have been cultured in Iscove's modified Dulbecco's medium (Sigma) supplemented with a 20% fetal calf serum in a humidified incubator at 37°C and a controlled 5% CO_2 atmosphere. The cultures have been divided every 2nd day by a dilution to a concentration of $2x10^5$ cells/ml. The cell counts have been performed with a hemocytometer, the cell membrane integrity has been determined by using the Trypan blue exclusion technique. The cell lines in the maximal range of up to 20 passages have been used for this study.

Gamma irradiation. The exponentially growing HL-60 cells have been suspended at a concentration of $2x10^5$ cells/ml in a complete medium. Aliquots of 10 ml have been plated into 25 cm^2 flasks (Nunc) and irradiated using a ^{60}Co gamma-ray source with a dose-rate of 0.4 Gy/min. After the irradiation the flasks have been placed in a 37^o C incubator with 5% CO_2 and the aliquots of the cells have been removed at various times after irradiation for analysis.

Valproic acid (VA). VA (Sigma-Aldrich) has been added into the cultivation flask for various time (1 – 12 days) in a final concentration of 0.5 – 4 mmol/l. The VA (sodium salt, Sigma) was dissolved in PBS to a stock concentration of 100 mM and stored at -20 °C.

In vitro clonogenic survival assay. The survival curves have been generated using an in vitro clonogenic assay. The untreated control (10^2 cells/ml) and the irradiated and treated HL-60 cells (10^2–10^5 cells/ml) were plated in Iscove's medium containing 0.9% methylcellulose and 30% FBS. HL-60 cells were stimulated by 10% conditioned medium of human cell line 5637 (urine vessel carcinoma) per 1 ml of medium. 1 ml of the plating mixture has been dispersed into 35 mm tissue culture Petri dishes. The colonies (containing 40 or more cells) have been counted after 14 days of the incubation in 5% CO_2 at 37°C and the curves have been generated. All semi-solid cultures have been performed in duplicates. Two independent experiments (4 measurements) have been performed.

*Cell cycle analysis.*Following the incubation, the cells were washed with cold PBS, fixed by 70% ethanol and stained with propidium iodide (PI) in Vindelov's solution for 30 minutes at 37°C. 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.

CD11b antibody, apoptosis detection. For apoptosis detection we used APOPTEST-FITC (DakoCytomation, Brno, Czech Rep.). For detection of cell surface markers in HL-60 cells we used PE-conjugated anti-human CD11b (Bear1, IgG1-IM2581) – obtained from Immunotech, Marseille, France. Flow cytometric analysis was performed on a Coulter Epics XL flow cytometer equipped with a 15mW argon-ion laser with excitation capabilities at 488 nm (Coulter Electronic, Hialeah, FL, USA). A minimum of 10,000 cells was collected

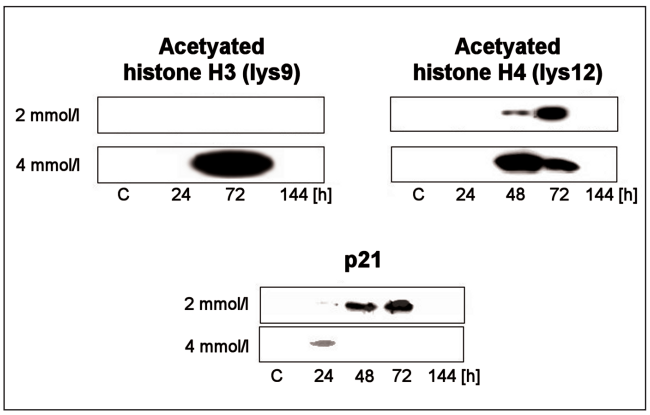

Figure 1 Changes in protein expression and acetylation of histones after treatment with valproic acid in concentration of 2 and 4 mmol/l in HL-60 cells.

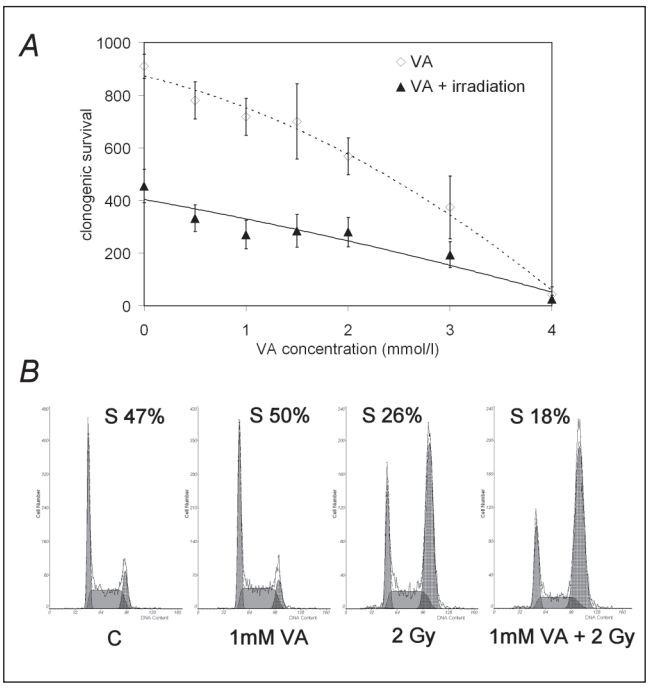

Figure 2A Comparison of colony forming capacity of HL-60 cells only treated with valproic acid (EC70=3.4 mmol/l) with cells pretreated with VA for 24 h and then irradiated with the dose of 2 Gy (EC70=1.4 mmol/l). Irradiation by the dose of 2 Gy itself decreases clonogenic survival to 50%. Figure 2B Flow-cytometric analysis of DNA content of HL-60 cells 24 hours after irradiation with or without 24 h-long preincubation with 1 mmol/l VA. Percentage represents number of cells in S-phase of cell cycle.

for each 2-colour sample in a list mode file format. List mode data were analyzed using Epics XL System II software (Coulter Electronic, Hialeah, FL, USA).

Western Blotting. At various time after VA treatment and irradiation the HL-60 cells have been washed with a PBS. The lysates containing an equal amount of protein (30 μg) have been loaded into each lane of a polyacrylamide gel. After electrophoresis, the proteins were transferred to a PVDF membrane. The membranes were blocked in Tris-buffered saline containing 0.05 % Tween 20 and 5% non-fat dry milk and then incubated with primary antibody (p21 – Sigma; acetylated histone H3 and H4 – Cell Signaling Technologies) at 4°C overnight. After washing, the blots were incubated with secondary antibody (Dako) and the signal was developed with a chemiluminiscence (ECL) detection kit (Boehringer).

Results

Acetylation of histones H3 a H4 and expression of p21. To characterize response of HL-60 cells to VA we studied changes in protein expression and acetylation after treatment with 2 and 4 mmol/l VA. We proved increased acetylation of histone H4 on lysine 12 at HL-60 cells exposed to both concentrations of VA 48-72 h after the beginning of incubation. Acetylation of histone H3 on lysine 9 was detected only after exposure to 4 mmol/l VA, 72 h after the beginning of cultivation. Incubation of the cells with 4 mmol/l VA caused increase in inhibitor of cyclin dependant kinases p21 as soon as after 24 h, while after the lower concentration of VA (2 mmol/l) p21 was detected later, 48-72 h after the beginning of the incubation. Maximal increase in p21 thus precedes maximal acetylation of histones H3 and H4 (Fig1).

Radiosensitizing effect of VA . To evaluate radiosensitizing effect of VA the HL-60 cells were cultivated 24 h in presence of VA and then irradiated with gamma rays. The cells were exposed to increasing concentration of VA, irradiated, and colony-forming assay was performed. 24 h-long preincubation with VA decreases clonogenic survival of HL-60 cells only in high concentrations. EC70 value calculated for VA alone was 3.4 mmol/l. Irradiation by the dose of 2 Gy itself decreases clonogenic survival to 50%. When the cells were incubated with VA 24 h before the irradiation by the dose of 2 Gy, EC70 decreased to 1.4 mmol/l VA (Fig. 2A). Figure 2B compares DNA content analysis of HL-60 cells irradiated with or without 24 h-long preincubation with 1 mmol/l VA. VA itself in concentration 1 mmol/l does not significantly change distribution of cells throughout the cell cycle. Irradiation of HL-60 cells by the dose of 2 Gy causes pronounced accumulation of the cells in G2/M phase (56%) and decrease in percentage of cells in S phase (26%) 24 h after the irradiation. When the cells were incubated with VA 24 h before the irradiation by the dose of 2 Gy, G2/M phase arrest was not abrogated (65% of cells in G2/M), but the percentage of the cells in S phase (18%) was more decreased then in only irradiated cells. Radiosensitizing effect of preincubation with VA thus cannot be explained by abrogation of G2/M phase arrest.

Concomitant effect of irradiation and VA. We studied the effect of simultaneous application of VA and irradiation by gamma rays. HL-60 cells were irradiated and continuously

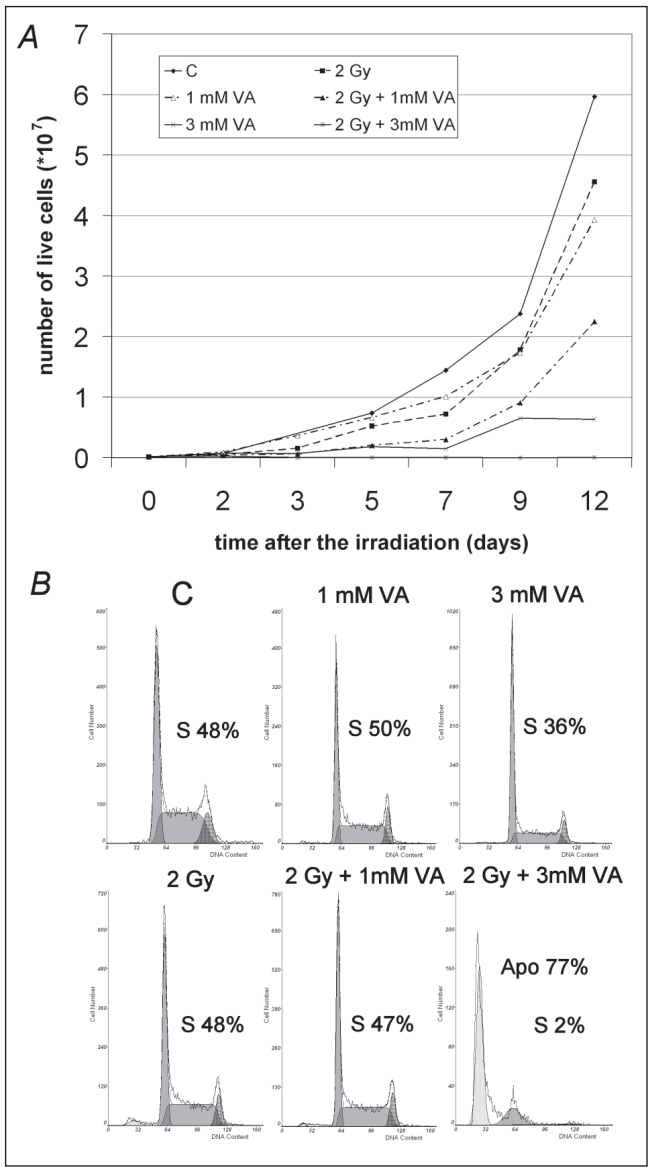

Figure 3A Decrease in proliferation during 12 days of continous cultivation of HL-60 cells with valproic acid in concentration of 1 and 3 mmol/l compared to HL-60 cells irradiated by the dose of 2 Gy and consequently cultivated for 12 days with valproic acid in concentrations mentioned above.

Figure 3B Changes in cell cycle distribution 12 days after treatment with valproic acid in concetration of 1 and 3 mmol/l compared to cells irradiated by the dose of 2 Gy and subsequently cultivated with valproic acid in the same concentrations. Percentage represents number of cells in S-phase of cell cycle.

exposed to VA in concentration of 1 and 3 mmol/l. Figure 3A shows decrease in proliferation during 12 days of continuous incubation with VA, which causes decrease in number of viable cells to 65% (1 mmol/l) or 10% (3 mmol/l) of control sham treated cells after 12 days. Irradiation with the dose of 2 Gy decreased proliferation of the cells to 76% of control. When irradiation was applied together with VA, the effect was cumulative: in the case of 1 mmol/l VA + 2 Gy the number of viable cells decreased to 37%, in the case of 3 mmol/l VA + 2 Gy viable cells were almost erradicated.

Changes in cell cycle distribution after 12 days are shown in Fig. 3B. At this interval the cells, which were only irradiated already overcome G2/M arrest and the passage of surviving cells through the cell cycle is regenerated. VA in concentration 1 mmol/l does not significantly alter cell cycle, but VA in concentration 3 mmol/l causes significant decrease in number of cells in S phase, which corresponds with limited proliferation as mentioned above. Cell cycle distribution was not significantly altered after combination of 1 mmol/l VA + 2 Gy, but the combination of 3 mmol/l VA + 2 Gy induced massive apoptosis in majority of the cells.

Differentiation and apoptosis induction after VA and IR. Figure 4 shows changes in expression of CD11b antigen and Annexin V binding in HL-60 cells, three days after irradiation with the dose of 2 Gy and incubation with VA. VA in concentration of 1 mmol/l leads to cell differentiation and increase in CD11b expression (17.75% resp. 0.9%-control) without apoptosis induction, whereas concentration of 3 mmol/l increased CD11b to 78.3% and apoptosis is induced in 64.9% cells (4.6% in control cells). Three days after irradiation of cells with the dose of 2 Gy a slight increase of CD11b to 9% as well as apoptosis induction (11.3% A+ cells) can be seen. Low concentration of VA (1 mmol/l) acting concomitantly with irradiation provoked mainly increase of differentiation measured as CD11b positivity (36.3%), effect on apoptosis induction was only additive (15.4% A+ cells). The effect of VA in concentration 3 mmol/l applied concomitantly with 2 Gy IR was different: The differentiation was comparable with effect of VA (3 mmol/l) alone, but percentage of apoptotic cells raised to 76.2%. Study of flow-cytometric record (Fig.4A) shows that highly positive CD11b cells (well differentiated) are alive without Annexin V positivity. Once the cells are Annexin V positive, the CD11b expression declines untill this differentiation marker is completely lost.

Discussion

Based on the presented results as well as on our previous studies [12,13,15] it is evident that both IR and VA posses antileukemic effect in leukemia cell lines HL-60 and MOLT-4. MOLT-4 are lymphoblasts expressing CD4 and CD7 markers, HL-60 represent human promyelocytic leukemia cells and are poorly differentiated – CD34-/CD33+/CD15+.

Mentioned cell lines differ in their p53 status – MOLT-4 have functional wild type p53, which is phosphorylated promptly within hours following irradiation [16]. VA in concentration of 2 mmol/l likewise causes phosphorylation of p53 after 2 hours long incubation. Looking at posttranslational modifications after VA treatment, we proved acetylation

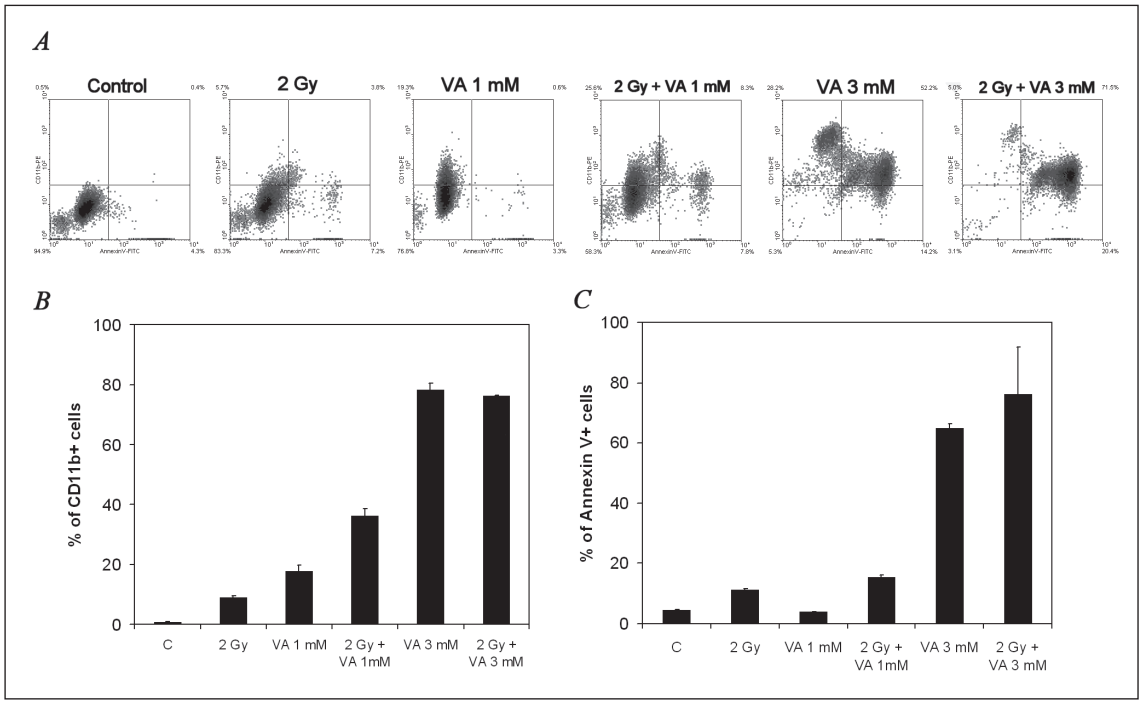

Figure 4 Flow-cytometric analysis of changes in expression of CD11b antigen and annexin V in HL-60 cells, three days after irradiation with the dose of 2 Gy and incubation with valproic acid in concentrations of 1 and 3 mmol/l.

of histone H4 48 and 72 hours after beginning of cultivation. Histone H3 acetylation occurred as far as after 72 hours long incubation with 4 mmol/l VA. In MOLT-4 T-lymphocytic leukemia cells acetylation of both nuclear histones H3 and H4 occurs already after 24 h-long incubation with 2 mmol/l VA [13].

In contrast to IR, VA in 2 mmol/l concentration does not implicate accumulation of cells in G2 phase. The arrest of proliferation provoked at HL-60 cells by VA was accompanied by increased expression of protein p21, an inhibitor of cyclin-dependent kinases. Since HL-60 cells are p53 negative, increase in p21 induced by VA must be p53 independent. Deubzer et al. [17] stated that differentiation and inhibition of HL-60 cells growth is related to unregulated gene p21. Cyklin/dependent kinase inhibitor p57KIP2 is frequently dowregulated in leukemia cells by methylation of appropriate promoters. In parallel to p21 upregulation, Yang et al. [18] proved elevated expression of p57 protein after VA treatment in MOLT-4 and HL-60 cells.

VA acts as a differentiation agens in HL-60 cells in contrary to the effect on MOLT-4. Three day-long incubation with 1 mmol/l VA resulted in elevated expression of antigen CD11b. Irradiation also led to pseudo-differentiation of HL-60 cells, 48 hours after the irradiation with dose of 6 Gy granularity raises, cells are alive and CD11b positive. A part of cells with low granularity and small size is CD11b negative. Those cells express APO2.7 and are apoptotic.

Both the noxes mentioned above provoke rapid apoptosis induction in MOLT-4 cells and their concomitant effect can be observed [13]. Induction of apoptosis is significantly higher in MOLT-4 cells irradiated with 2 Gy and subsequently cultivated for 14 days with VA (0.5 mmol/l) compared to only irradiated cell population.

Yang et al. [18] proved synergistic effect of 5-aza-2-deoxycitidine and VA in MOLT-4 cells in term of inhibition of proliferation, apoptosis induction and rise of antiproliferative proteins p21 and p57. Comparing efficacy of the noxes on different cell lines, they have proved that MOLT-4 cells are more sensitive to VA and HL-60 cells to the action of 5-aza-2-deoxycitidine. Irrespective to upregulation of p21 and p57 they have not proved arrest in any phase of cell cycle neither after VA alone nor after its combination with IR. Combination treatment allows application of relatively low doses of 5-aza-2-deoxycitidine. The common effect of noxes was application schema-independent, they can be applied concomitantly.

We proved that VA has radiosensitizing as well as synergistic effect in long-term cultivation with 2 mmol/l VA after irradiation by 2 Gy. Similarly 24 hours long preicubation of HL-60 cells with VA preceding 2 Gy irradiation caused diminution of EC70 value from 3.3 to 1.35 mmol/l. The number of cells in G2 arrest remained unchanged during this precultivation, while the number of cells in S phase decreased. We proved also radiosensitizing as well as concomitant effect of VA and IR in term of apoptosis induction.

While radiosensitizing effect of HDACi has been reported also by others, the mechanism of it remains to be elucidated. Camphausen et al. [19] demonstrated radiosensitizing effect of valproic acid on brain tumor lines SF539 and U251. They relate this fact to hyperacetylation of H3 and H4 histones, chromatin release and consequently lowered capacity of tumor cells to repair the radiation injury. Munshi et al. [20] showed that Vorinostat (HDACi) reduced the expression of the repair-related genes Ku70, Ku80, and Rad50 in A375 cells as detected by Western blot analysis. Another important phenomenon linked to reparation of radiation caused injury represents the phosphorylation of H2A.X histone, which occurs very early after irradiation in location of DSB and co-localizes with range of other proteins [21]. 24 h-long incubation of melanoma cells with NaB followed by 2 Gy irradiation led to increased phosphorylation of H2AX (30 minutes post radiation) and prolonged period of H2AX phosphorylation [20]. Both observations indicate lowered capacity to DSB repair.

Goh et al. [22] showed that elevated apoptosis induction in tumor cells caused by 24 hours long pretreatment with phenylbutyrate prior radiation is linked to downregulation of antiapoptotic protein bcl-xL and DNA-PK. Cells lacking DNA-PK catalytic activity are defective in DNA DSB repair and extremely sensitive to the effect of ionizing radiation. On the other hand Sutheesophon et al. [23] proved, that HDACi depsipeptide (FK228) led to down-regulation of proapoptic bcl-2 and apoptosis induction through caspase 9 activation and cytochrom c release from mitochondria in HL-60 cells.

In parallel to radiosensitizing effect of VA we studied the importance of long-term incubation of HL-60 cells with VA and proved mainly the differentiation and inhibition of proliferation of cells after 12 days incubation with 1 mmol/l VA and 2 Gy irradiation. Differentiation plays a role in decelerating the proliferation and in ultimate phase induces apoptosis. Despite the radiosensitizing effect of VA, the joint action of VA and IR was not so pronounced as in MOLT-4, where VA promoted apoptosis directly without differentiation [13].

The results of our work have shown that VA posseses radiosensitizing properties when applied 24 hours prior irradiation and that during parallel long-term action of VA and IR the cells undergo differentiation and faster apoptosis induction. Radiosensitizing effect of VA is not caused by abrogation of G2/M cell cycle arrest, but VA induces p21 and leads to differentiation of HL-60 cells.

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