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VALPROIC ACID DECREASES THE REPARATION CAPACITY OF IRRADIATED MOLT-4 CELLS

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The aim of our work was to evaluate mechanisms leading to radiosensitization of MOLT-4 leukemia cells following valproic acid (VA) treatment. Cells were pretreated with 2 mM VA for 24 h followed by irradiation with a dose of 0.5 or 1 Gy. The effect of both noxae, alone and combined, was detected 1 and 24 hours after the irradiation. Induction of apoptosis was evaluated by a flow cytometry. The extent of DNA damage was further determined by phosphorylation of histone H2AX using confocal microscopy. Changes in protein expression were identified by SDS-PAGE/immunoblotting. Two-millimolar VA increased apoptosis induction after irradiation as well as phosphorylation of H2AX and provokes an increase in the level of p53 and its phosphorylation at Ser392 in 4 h post-irradiation. Likewise, p21 protein reached its maximal expression in 4 h after the irradiation of VA-treated cells. Twenty four hours later, only the p53 phosphorylated at Ser15 was detected. At the same time, the protein mdm2 (negative regulator of p53) was maximally activated. The 24-hour treatment of MOLT-4 leukemia cells with 2 mM VA results in radiosensitizing, increases apoptosis induction, H2AX phosphorylation, and also p53 and p21 activation.

Keywords: **valproic acid, histon deacetylases inhibitors, ionizing radiation, γH2AX, p53.**

INTRODUCTION

Valproic acid (VA) is an established drug used in the treatment of epileptic seizures and mania in bipolar disorders [1]. In addition, it has recently been found that VA possesses an anti-tumor activity of histon deacetylase inhibitors-HDACi [2]. Generally, HDACi induce the accumulation of hyperacetylated histones and cause transcriptional activation of genes. Following HDACi treatment, chromatin becomes more relaxed and accessible for transcription factors [3]. VA inhibits co-repressor-associated HDACs at therapeutically employed concentrations [4]. VA is a class I HDACi. Such activity might be explained by the ability of the compound to fit into the enzyme pocket of class I HDACs and form a complex with a Zn^{2+} ion [5]. Krämer et al. [6] discovered that VA triggers the proteasome-mediated degradation of HDAC2. Thus, VA acts as an isoenzyme-selective down-modulator of HDAC2.

HDACi may modulate the cell cycle, the induction of growth arrest and differentiation and the inhibition of proliferation or it may promote apoptosis. HDACi alter the expression of approximately 2–10% of genes, mainly those involved in the events mentioned above [7]. Many studies have shown that HDACi exhibit selective cytotoxicity against a wide range of cancer cells and are relatively nontoxic for normal cells [8].

Modulation of tertiary chromatin structure represents one mechanism for HDACi activity. Another possible activity is deacetylation and alteration of nonhistone proteins, such as the transcription factor p53. Protein p53 influences the expression of more than 150 genes that mediate the arrest of cells in the cell cycle checkpoints or that induce apoptosis [9]. Acetylation of p53 increases its DNA binding as well as its transcriptional activity at the p21 promotor [10]. HDACi induce p21 in a p53-dependent, but more importantly in a p53-independent manner, which occurs via the Sp1/Sp3 pathway [11]. However attempts are made to combine p53 therapy with HDAC inhibitors treatment [12].

Radiotherapy is a frequently used technique in the treatment of cancer. Ionizing radiation (IR) causes one of the most severe types of damage to DNA – double strand breaks (DSB) [13]. The first response to DNA damage is represented by ATM (ataxia telangiectasia mutated) kinase. ATM kinase transmits the

Abbreviations: VA – valproic acid; HDAC– histondeacetylase; HDACi – histondeacetylases inhibitors; IOD – integral optical density; IR – ionizing radiation; DSB – double strand break.

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signal to down-stream targets through a transduction cascade to activate the signaling mechanisms for cell-cycle arrest and induction of repair, or cell death if the damage is irreparable [14]. Histone H2AX is rapidly phosphorylated in the chromatin micro-environment surrounding DSB. Phosphorylated H2AX (γH2AX) is required for the accumulation of numerous essential proteins responsible for DNA reparation into irradiation induced foci-IRIF [15]. The number of these foci can be used as a marker of DNA damage and its repair. Valproic acid at a concentration of 2 mM also provoked the phosphorylation of histone H2AX after 4 h or 24 h of the treatment [16].

A therapeutic potential of radiotherapy can be increased by the application of radiosensitizing agents before irradiation. HDACi can modulate the cellular response to ionizing radiation by enhancing the radiation sensitivity of cells. Trichostatin A (TSA) is a potent radiation sensitizer in K562 cells. Exposure of cells to TSA prior irradiation results in decreased survival [17]. Similarly, suberoylanilid hydroxamic acid (SAHA) reduces clonogenic survival and enhances the radiation-induced apoptosis in human prostate and glioma cancer cell lines [18].

In our previous work, we proved the combination of VA and IR decreasing clonogenical survival of MOLT-4 leukemia cells when VA is applied before as well as after the irradiation [19]. A combination of irradiation at a dose of 1 Gy and 0.5 mM VA treatment had a synergic effect in respect to the apoptosis induction. In HL-60, the radiosensitizing effect of VA was caused by induction of p21 leading to the differentiation of HL-60 cells, but not by the abrogation of G2/M cell cycle arrest [20]. In this work, properties of 2 mM VA as a radio-enhancer were studied in MOLT-4 leukemia cells. An increased apoptosis induction, change in histone H2AX phosphorylation at Ser139, and the effect on ATM down-stream targets such as p53 and its related proteins have been detected.

EXPERIMENTAL

Cell cultures and culture conditions. Human T-lymphocytic MOLT-4 leukemia cells from the American Type Culture Collection ("ATCC-LGC Standards", USA) were cultured in Iscove's modified Dulbecco's medium ("Sigma") supplemented with 20% fetal calf serum in a humidified incubator at 37°C in a 5% CO_2 atmosphere. The culture was divided by a dilution in a concentration of 2×10^5 cells/mL every 2nd day. The cell counts were performed with a hemocytometer, whereas a cell membrane integrity was determined using the trypan blue exclusion technique. The cell lines in a maximal range of up to 20 passages were used for this study. MOLT-4 p53 has a G→A point mutation at codon 248 (not leading to a substition in the amino acid sequence of p53 protein), thus its function is not compromised.

Gamma irradiation. The exponentially growing MOLT-4 cells were suspended at a concentration of 2×10^5 cells/mL in the complete medium. Aliquots of 10 mL were plated into the 25-cm^2 flasks ("Nunc") and irradiated using the [^{60}Co] gamma-ray source with a dose-rate of 0.4 Gy/min. Afterwards, the flasks were placed in a 37°C incubator with 5% CO_2 and aliquots of the cells were removed for analysis at various times after the irradiation.

Valproic acid (VA). VA ("Sigma-Aldrich") was added into the cultivation flask for a specific amount of time at a final concentration of 2 mM. The VA sodium salt was dissolved in PBS at stock concentration of 100 mM and stored at –20°C.

Flow cytometric analysis of apoptosis induction. The apoptest-FITC kit ("DakoCytomation", Czech Republic) was used for apoptosis detection. During apoptosis, cells expose phosphatidylserine at the cell surface. Annexin V, a phospholipid binding protein, binds selectively and with high affinity for phosphatidylserine in the presence of calcium ions. 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", USA). A minimum of 10000 cells was collected for each sample in a list mode file format.

Activity of caspases 3/7, 8, and 9. The activity of caspases was measured using the Caspase-Glo® luminescent assay ("Promega", USA).

Immunocytochemistry. The cells were fixed with the 4% freshly prepared paraformaldehyde for 10 min at room temperature (RT), washed in PBS, permeabilized in 0.2% Triton X-100/PBS and washed. Before incubating with primary antibodies (overnight at 4°C), the cells were blocked with 7% inactivated FCS + 2% bovine serum albumin in PBS for 30 min at RT. The mouse monoclonal anti-phospho-histone H2A.X ("Upstate", USA) was used for detecting γH2AX. The affinity pure donkey anti-mouse-FITC-conjugated antibody was purchased from Jackson Laboratory (Bar Harbor, USA). The images were obtained by a high-resolution confocal cytometry with use of a completely automated Leica DM RXA fluorescence microscope equipped with a CSU-10a confocal unit ("Leica", Japan) and CoolSnap HQ charged-coupled device camera ("Photometrix", Australia). An integral optical density (IOD) was measured using the image analysis Software ImagePro 4.11 ("MediaCybernetics," USA).

Western blotting. Following the VA treatment and irradiation, the MOLT-4 cells were used for the whole cell lysate preparation. Lysates containing an equal amount of protein (20 μg) were loaded into every 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 the primary antibody against p53,

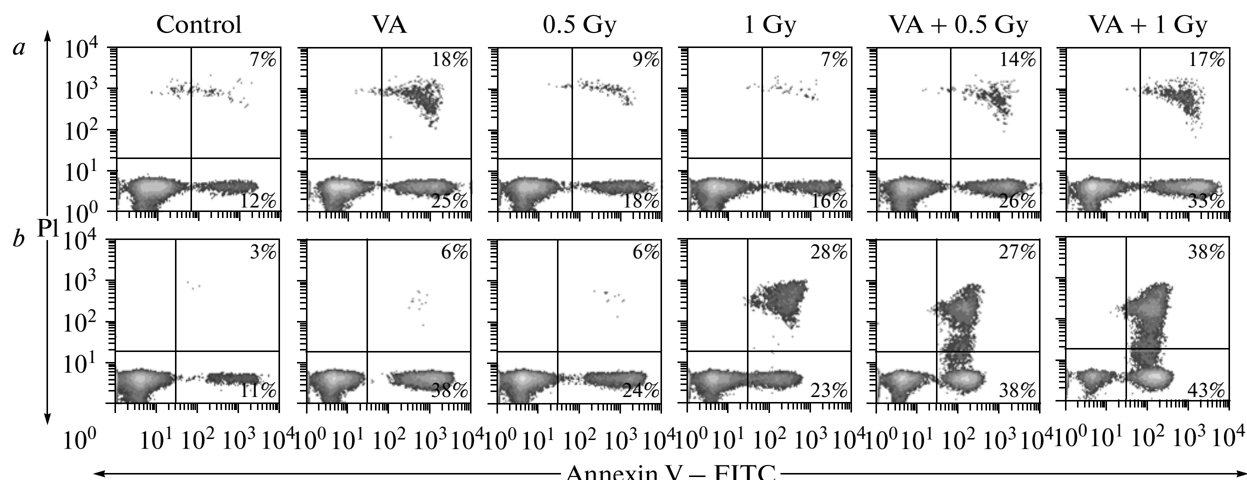

Fig. 1. Flow-cytometric analysis of apoptotic cells. MOLT-4 cells were treated with 2 mM valproic acid (VA) for 24 h and/or irradiated with a dose of 0.5 or 1 Gy. Cells were stained with propidium iodide and annexin V and analysed 1 h (*a*) and 24 h (*b*) after the irradiation. Early apoptotic cells (annexin V positive/propidium iodide negative) are presented in the right bottom corner, late apoptotic or necrotic cells (annexin V positive/propidium iodide positive) are in the right upper one.

p53_S392-Exbio; β-actin, p21 ("Sigma"); lamin B, p53_S15-Calbiochem, mdm2_S166 ("Cell Signalling", USA) at 4°C overnight. After washing, the blotts were incubated with the appropriate secondary antibody ("Dako", Denmark) and the signal was developed with a chemiluminiscence (ECL) detection kit ("Boehringer", Germany). For detecting lowly abundant proteins in the whole cell lysate (eg. phosphorylated p53), we used Imobilon Western ("Milipore", USA) or SuperSignal West Femto ("Pierce", USA) chemiluminiscence substrate and longer exposition times. Thus, a discrepancy can be observed when the level of the phosphorylated protein seems to exceed the level of the total protein.

Statistical analysis. Descriptive statistics of the results were calculated and charts were made in Microsoft® Office Excel 2003 ("Microsoft, Inc.", USA); a detailed statistical analysis (dot plots) was performed in NCSS 6.0.21 ("NCSS", USA).

Obtained values of integral optical density did not correspond to a normal distribution; therefore they are presented as medians and ranges of the values. The Mann–Whitney U-test was used to represent the differences in results between the controls and various experimental groups. The test was rejected at a level of significance of $\alpha = 5\%$. The results are shown as the median with the first and third quartile indicated.

To analyze data on caspase activity F-test and two-sample t-test were used. A value of $p < 0.05$ was considered statistically significant.

RESULTS

We evaluated the effect of VA as a radiosensitizer in MOLT-4 cells. Such parameters as an apoptosis induction, DNA damage and expression of proteins responsible for cell cycle arrest and apoptosis induction were compared at three different experimental conditions, namely: i) cells were pretreated with 2 mM VA for 24 h and then irradiated with a dose of 0.5 and 1 Gy (i.e. VA present in the medium during the whole interval, 48 h); ii) cells were only irradiated; iii) cells were treated with VA only.

Apoptosis induction

A flow-cytometric analysis of annexin V/propidium iodide stained cells was used for the study on apoptosis induction. The general effect on apoptosis induction regardless of a stage of the process was evaluated.

The treatment of the 2 mM VA induced apoptosis in approximately 40% of the cells (fig. 1). One hour after irradiating at a dose of 0.5 or 1 Gy about 20% of apoptotic cells were detected. The ratio of apoptotic cells in the VA-only treated or the 0.5 Gy-only irradiated cells remained unchanged throughout the entire treatment. On the contrary, the number of apoptotic cells increased in time after combined treatment. One hour after the irradiation of the VA-pretreated cells the apoptosis induction varied from 40% (VA + 0.5 Gy) to 50% (VA + 1 Gy) of the cells. Twenty four hours later, it increased to 65% (VA + 0.5 Gy) and even up to 81% (VA + 0.5 Gy). Altogether, the percentage of dead cells increased in time.

The induction of apoptosis was confirmed by the activation of caspases 3/7, 8, and 9. The combined VA+IR treatment caused a significantly higher activity of caspases in 24 h post-irradiation (fig. 2).

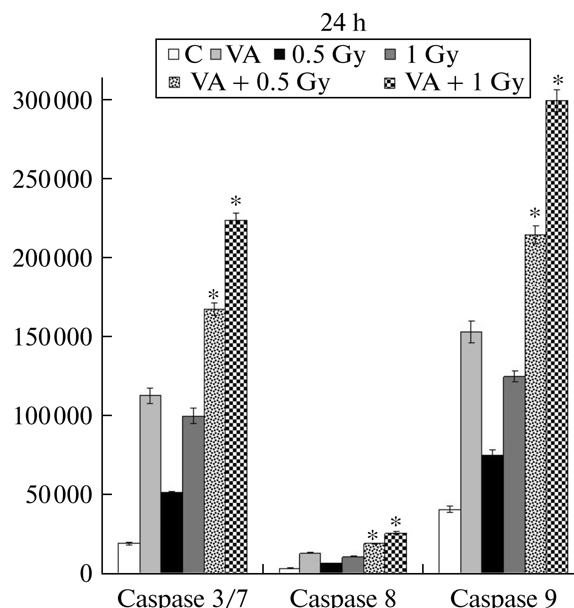

Fig. 2. Activity of caspases 3/7, 8, and 9 after the VA treatment and irradiation. Data are presented as a luminiscence intensity (the mean value ± SEM). Hereinafter, statistically significant results are marked by (*).

Phosphorylation of histone H2AX

The phosphorylation of histone H2AX at Ser139, an important sign of double strand breaks and the corresponding extent of DNA damage, was evaluated by a confocal microscopy. Difference in levels of the H2AX Ser139 phosphorylation at early (1 h) and late (24 h) points in time following IR was studied.

The VA-only treated cells contained homogenously situated foci of phosphorylated histone H2AX. The IR alone is a significantly more potent inductor of H2AX phosphorylation than VA. After the irradiation (doses of both 0.5 and 1 Gy for 1 h) followed the VA treatment phosphorylation of histone H2AX increased significantly compared to the noxae used separately. The rate of H2AX phosphorylation is expressed as an integral optical density measured by the image analysis Software ImagePro 4.11.

In the late interval (24 h after irradiation), the phosphorylation status of H2AX was completely different. The level of phosphorylation was comparable to that of the control in irradiated cells only, indicating that surviving cells repaired DNA damage. Some foci of the phosphorylated histone H2AX could be seen in VA-only treated cells. The most pronounced difference was observed for cells pretreated with VA and then irradiated. 24 h after the IR a few separated large foci of γH2AX could be observed (fig. 3) that shows persisting DNA damage being not successfully repaired. On the contrary, cells possess numerous homogenously situated H2AX foci in the early (1 h) interval.

Changes in phosphorylation and expression of non-histone proteins

For evaluating a level of phosphorylation and expression of proteins SDS-PAGE and Western-blotting assays were used.

The expression of p53 protein slightly increased against control cells and reached its maximum 4 h after IR (fig. 4).

The highest increase in the p53 was observed in the irradiated cells pretreated with VA. Interestingly, a level of p53 protein decreased 24 h after irradiating the VA pretreated cells at a dose of 0.5 Gy, whereas at a dose of 1 Gy p53 protein expression was not detected at all (fig. 5).

Protein p53 underwent numerous post-translation modifications. Phosphorylation of p53 at Ser392 correlated with p53 expression, reaching the maximal level 4 h after irradiating VA pretreated cells. In irradiated-only cells phosphorylation of p53 at Ser392 was observed 24 h after the IR. Phosphorylation of p53 at Ser15 occurred after activation with both noxae throughout the entire length of treatment and was most pronounced after irradiating at a dose of 1 Gy (fig. 5).

Induction of p21 protein followed the activation of p53 protein. The 48-hour-long treatment of cells with VA led to the up-regulation of p21, which is enhanced by irradiation at doses of 0.5 Gy and particularly 1 Gy. The IR alone did not cause an induction of p21 during the whole interval of the experiment (fig. 5).

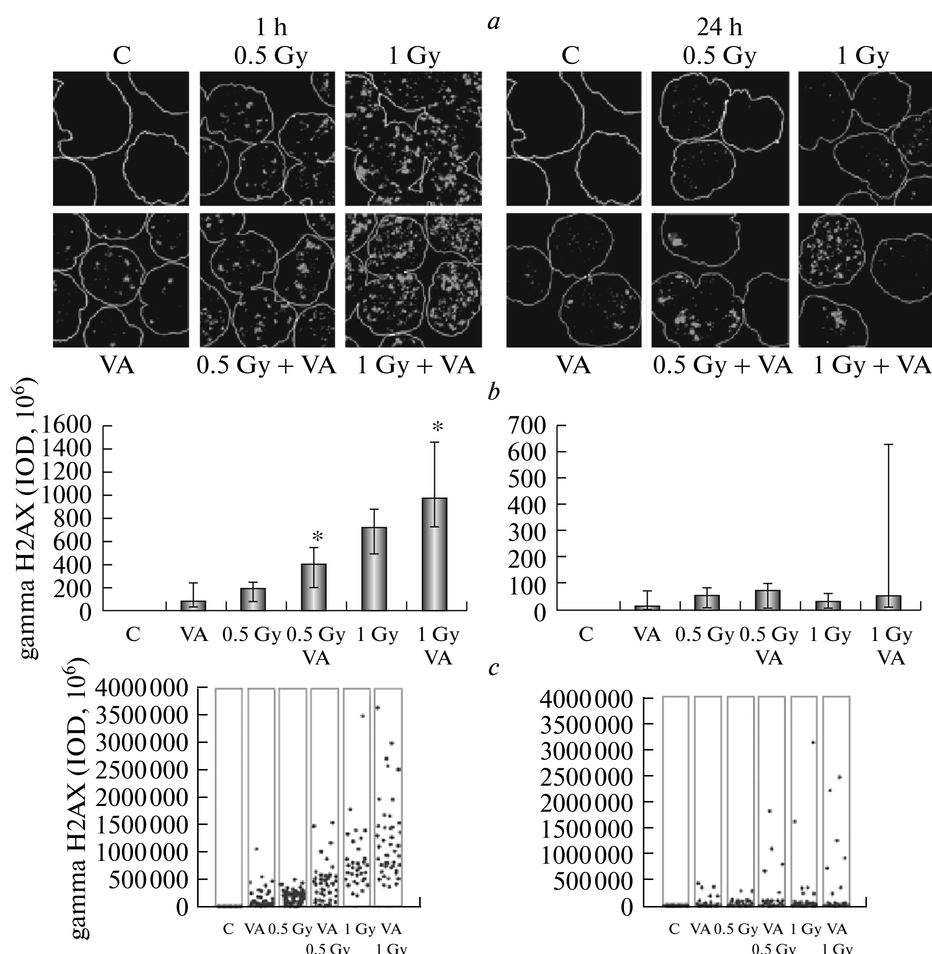

Fig. 3. Phosphorylation of histone H2AX. MOLT-4 cells were treated with 2 mM VA for 24 h and/or irradiated at a dose of 0.5 or 1 Gy. In time intervals 1 h and 24 h after the irradiation, cells were incubated with the primary antibody against Ser139 phosphorylated histone H2AX (gamma H2AX). Images were obtained with a use of a high-resolution microscope with the confocal unit (*a*). Integral optical density (IOD) was measured using the image analysis Software ImagePro 4.11. The data are presented as median $\pm$ 1st and 3rd quartile (*b*) and individual values for each cell (*c*).

The IR caused phosphorylation of mdm2 protein at Ser166 (an activating modification allowing mdm2 to enter a nucleus). A significant increase of phosphorylation was observed 24 h after irradiating VA pretreated cells (fig. 5).

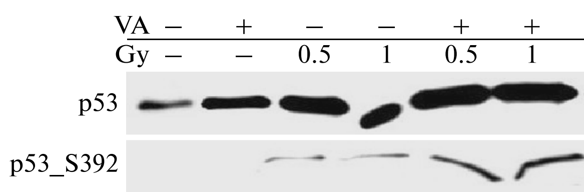

Fig. 4. Expression of p53 and its phosphorylation at Ser392 (p53_S392) 4 h after irradiation at a dose of 0.5 or 1 Gy. Cells were either left untreated or treated with VA for 24 h before the irradiation. Maximal expression is visible for VA + IR (1 Gy dose) treated cells.

DISCUSSION

Ionizing radiation is one of the most frequently used approaches in therapy of malignant diseases. Consequently, the aim of numerous studies is to find a radiosensitizing agent that would allow one to decrease the effective dose of radiation with no side effect on normal cells. No such agent has been approved so far. Although some compounds seem to have promising properties, e.g. the inhibitors of histondeacetylases, the precise mechanism of the synergistic action of HDACi and IR remains to be elucidated.

One of the earliest events in cells exposed to IR is the phosphorylation of histone H2AX at Ser139 (γH2AX) catalyzed by ATM kinase. The γH2AX denotes DSB foci and, thus, enables binding of downstream and repair proteins. The IR is a potent inducer of DSB, at doses of 0.5 and 1 Gy provoking significant phosphorylation of histone H2AX at Ser139. In the same manner, VA, a member of the short-chain fatty

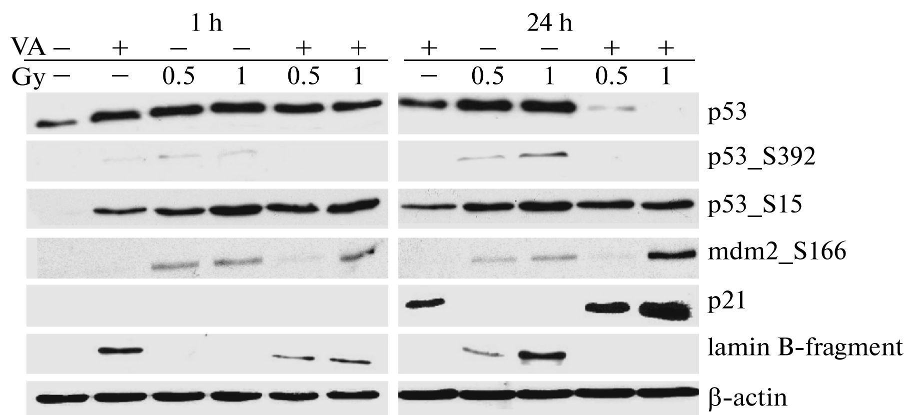

Fig. 5. Expression of p53 and its related proteins in MOLT-4 cells. Cells were untreated or treated with 2 mM VA for 24 h and then irradiated at a dose of 0.5 or 1 Gy. Electrophoresis and Western-blotting analysis of the total cell lysates 1 h and 24 h after irradiation were performed.

acids, at the 2 mM concentration induces phosphorylation of histone H2AX [16]. In this study, we proved that the 24-hour-long preincubation of MOLT-4 cells with VA followed by irradiation at doses of 0.5 or 1 Gy leads to enhanced phosphorylation of H2AX compared to both noxae acting separately. One hour after irradiation, γH2AX forms diffused foci in the whole content of the cell nucleus. Its abundance was significantly higher (evaluated as IOD) compared to the IR-only or VA-only treated cells. On the other hand, foci persisting in the cell were unique 24 h after irradiation, irregular and more pronounced than those for the shorter time interval. Generally, the disappearance of foci is linked with the reparation of injury and the individual strand breaks are usually repaired rapidly, typically within 2 h [21]. Persisting foci are a sign of unrepaired lesions resulting either from initial increased DSB formation or from interactions with the reparation proteins, what leads to prolonged or unsuccessful reparation. Thus, pretreatment with VA can be linked to increased DSB formation after IR that probably results from relaxed chromatin structure. Harikrishnan et al. [22] proved increased γH2AX foci formation in active euchromatine, where acetylation of nuclear histones is also more pronounced compared to heterochromatin.

Histone deacetylases are indispensable in cases requiring DNA repair for the formation of original chromatin structure. This can also be a source of possible post-radiation sensitization. Human glioma cells treated with VA had a lower survival rate after irradiation [21]. In MOLT-4 cells 14 days after irradiation at a dose of 1 Gy, the effective concentration, EC_{50}, of VA decreased from 0.97 to 0.38 mM [19].

A decrease in the repair capacity of cells through the interactions of histondeacetylases with repair proteins can represent another possible mechanism of the radiosensitizing effect. Acetylation of γH2AX that occurs after the VA treatment can affect the repair complex dispersion [21].

A three-day-long incubation of MOLT-4 cells with 2 mM VA decreases the D0 (dose, that reduces number of surviving cells to 37%) value from 0.7 to 0.2 Gy [19]. Karagiannis et al. [23] also observed decreasing the clonogenic survival of K562 cells after 24-hour-long incubation with 10 mM VA followed by IR. Another HDACi, trichostatin A, has a radiosensitizing effect which causes a decrease in the clonogenic survival of cells, an increase in apoptosis induction, and enhances phosphorylation of H2AX [17].

Apoptosis induction is tightly bound to p53 protein. The VA by itself, at a concentration of 2 mM, causes an increase in the level of this nuclear protein after 2 h of treatment and simultaneously its phosphorylation at Ser392 [19]. In this study, we showed that the combination of VA and IR initially enhances the expression of p53 and its phosphorylation at Ser392. Level of the Ser392 phosphorylation reached its maximum 4 h after IR. However, 24 h after IR only Ser15-phosphorylated form of the p53 was detected. This post-translation modification protects p53 from binding to its negative regulator mdm2. Protein mdm2, phosphorylated at Ser166, can penetrate into the nucleus, bind to p53 and provoke its degradation [24]. As it has been shown here, the disappearance of p53 in irradiated cells treated with VA occurred in the same time period as the mdm2 phosphorylation reached maximal level.

HDACi are potent inducers of p21, generally in a p53-independent manner. We proved that the induction of p21 follows an increase in p53-positive cells treated with VA [19]. Also, the maximal expression of p53 (4 h after IR) is followed by the induction of p21 (24 h after IR) in the case of VA pretreated and irradiated cells. As it was described by Szkanderova et al. [25], p53-dependent accumulation of p21 in MOLT-4 appeares after IR alone, when high doses were used.

Apoptosis is mainly provoked by the 24-hour-long pre-incubation with VA 1 h after the IR. On the other hand, 24 h later the effect of the IR itself is already apparent.

Thus, VA has been shown here to possess radiosensitizing properties in terms of increased apoptosis induction. In addition, it affects a range of proteins important in DNA damage repair, such as γH2AX as well as the proteins responsible for apoptosis induction, such as p53, and those responsible for cell cycle arrest, such as p21. Therefore, valproic acid, already well-known in clinics, represents a promising radiosensitizing drug.

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