Effect of Valproic Acid and Antiapoptotic Cytokines on Differentiation and Apoptosis Induction of Human Leukemia Cells

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Abstract. This work compares effect of histondeacetylase inhibitor, valproic acid (VA), on proliferation, differentiation and apoptosis induction in two human leukemic cell lines: HL-60 (human promyleocytic leukemia, p53 negative) and MOLT-4 (human T-lymphocyte leukemia, p53 wild type). Incubation with VA caused decrease in percentage of cells in S phase of cell cycle. The decrease was more intensive in HL-60 cells, where the cells in S phase were absent 6 days after the beginning of incubation with VA (4 mmol/l). 3-day-long incubation of HL-60 cells with 4 mmol/l VA caused differentiation of these cells, marked by increase in CD11b and co-stimulatory/adhesion molecule CD86, and induction of a significant apoptosis. Annexin V positive cells lost the CD11b antigen. 3-day-long incubation of MOLT-4 cells with VA (1–2 mmol/l) inhibited proliferation and decreased percentage of cells in S phase of the cell cycle. 90 % of MOLT-4 cells are CD7 positive. This CD7 positivity is not changed during apoptosis induction (detected as Annexin V positivity). On the other hand, CD4 marker expression decreases after incubation with 1–2 mmol/l VA, but during apoptosis induction by 4 mmol/l VA, most of the apoptotic Annexin V positive cells were also CD4 positive. Using a clonogenic survival assay EC_{50} for 3-day-long incubation with VA was determined. For HL-60 cells, the established EC_{50} was 1.84 mmol/l, for MOLT-4 cells it was 1.76 mmol/l. Ability of VA to induce differentiation in HL-60 cells thus does not affect final cell killing. However, the elimination of the cells was considerably affected by presence of hematopoietic growth factors. 14-day-long incubation of HL-60 cells with VA in conditioned medium (source of IL-3, SCF, G-CSF) caused increase in EC_{50} to 4 mmol/l, while in MOLT-4 cells (cultivation without conditioned medium), the EC_{50} decreased to 0.63 mmol/l.

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Introduction

It has been found that defects of status of histone acetylation are related to an expression of many cellular oncogens and tumor-suppressor genes (Gottlicher 2004; Rosato and Grant 2004; Vávrová et al. 2005). These findings have initiated an extensive search for substances capable of histondeacetylases (HDAC) inhibition. Among these compounds, differing greatly in their chemical structure, is a group of short chain carboxylic acids such as butyrate or valproic acid (VA). VA or sodium valproate is a commonly used anticonvulsant in the management of epilepsy with good response rates and an acceptable toxicity. Aberrant recruitment of HDAC by several acute myeloid leukemia (AML) fusion proteins is required for their capacity to block myeloid differentiation (Minucci et al. 2001). Gottlicher et al. (2001) proved that VA at concentrations of 0.3–1 mmol/l, which are achieved in a patient serum during the therapy of epilepsy with a daily dose of 20–30 mg/kg, acts as a potent inhibitor of HDAC activity. They observed that myeloid differentiation, induced when leukemic blasts of AML patients are cultured for 5 days in the presence of VA (1 mmol/l), is comparable to that induced by all-trans retinoic acid (ATRA) and the best results were obtained by the combination of VA and ATRA. Remarkably, the combination of retinoic acid and VA treatment induces the appearance of cells with metamyelocyte or neutrophil like morphology.

Trus et al. (2005) described that VA could mimic or enhance retinoid sensitivity in the AML cell line, OCI/AML-2, and in the clinical samples derived from patients with AML. Using GeneChip experiment they demonstrated that VA modulated the expression of numerous genes, including p21, that were not affected by ATRA. Expression of p21 is related with cell cycle arrest. Kawagoe et al. (2002) studied the effect of VA on apoptosis induction in the leukemic line MV411 and they showed that VA induced apoptosis by mitochondrial pathway through cytochrome c release from mitochondrias and activation of caspases 8, 9 a 3. A caspase inhibitor, zVAD-FMK, inhibited the DNA fragmentation caused by VA, but not the cell death.

HL-60 cells of human promyelocytic leukemia induced to undergo maturation by retinoic acid ultimately die by an apoptotic death, although as a relatively late event. It becomes apparent that terminal differentiation of leukemic cells and apoptosis are closely related processes. Rather than a differentiation, probably a dysregulation of maturation of U937 human leukemia cells leading to apoptosis would be induced after treatment with phorbol myristate acetate (De Vente et al. 1995). Their data suggest that the decision of a cell to undergo death or differentiation in response to phorbol esters may, in part, be modulated by alterations within the protein kinase C signal transduction pathway. Vrana et al. (1999) proved that HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) induces maturation and apoptosis at HL-60 and U937 cells. SAHA-mediated apoptosis is associated with decreased mitochondrial membrane potential, caspase 3 activation and poly

(ADP-ribose) polymerase degradation and is p53 independent, but at least partially regulated by Bcl-2/Bcl-XL, p21 signaling cascade. Tang et al. (2004) found that VA induces apoptosis in AML patient cell expressing of both P-glycoprotein and/or MDR-associated protein 1 (MRP1).

In our work we compared the effect of VA in p53 negative HL-60 cells, which are capable of terminal differentiation after exposure to retinoic acid, and p53 wild type cells MOLT-4 (human T-lymphocyte leukemia). We found that VA induces apoptosis not only through terminal differentiation of cells (HL-60), but also directly in p53 wild type MOLT-4 cells, indicating that the process of apoptosis induction caused by HDAC inhibitors is not directly dependent on cell differentiation.

Materials and Methods

Cell culture and culture conditions

HL-60 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK) and MOLT-4 cells were obtained from American Type Culture Collection (University Blvd., Manassas, USA). The cells were cultured in Iscove's modified Dulbecco's medium (Sigma) supplemented with 20 % fetal calf serum in humidified incubator at 37 ℃ and controlled 5 % CO_2 atmosphere. The cultures were divided every 2nd 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. Cells in the maximal range of 20 passages were used for this study.

Incubation of cells with VA

VA (Sigma) was dissolved in phosphate buffered saline (PBS) to achieve stock solution of 1 mol/l, it was stored at −20 ℃ until required. Two ways of exposure of cells to VA were used:

1. Cells in exponential growth were seeded at 2–4 $\times$ 10^5/ml in 25 cm^2 culture flasks and supplemented with 0.1–10 mmol/l VA. Cells were harvested after 1–6 days of incubation, washed and used for other assay.

2. VA was added to Iscove's medium containing 0.9 % methylcellulose and 30 % fetal bovine serum (FBS) (MOLT-4 cells). For HL-60 cells, 10 % conditioned medium (source of interleukine 3 (IL-3), stem cell factor (SCF) and granulocyte colony stimulating factor (G-CSF)) from human cell line 5637 (urine vessel carcinoma) were added to higher specified Iscove's medium. Both cell lines were cultured in this medium continuously for 14 days.

Cell cycle analysis

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

CD11b, CD86, CD4 and CD7 antibody, apoptosis detection

For apoptosis detection we used Apoptest-FITC kit (DakoCytomation, Brno, Czech Republic). During apoptosis, cells expose phosphatidylserine at the cell surface. Annexin V is a phospholipid binding protein which, in the presence of calcium ions, binds selectively and with high affinity to phosphatidylserine. For detection of cell surface markers in HL-60 cells we used PE-conjugated anti-human CD11b (Bear1, IgG1-IM2581) and anti-human CD86 (HA5.2B7, IgG2b-IM2729) – obtained from Immunotech (Marseille, France). For detection of cell surface markers in MOLT-4 cells we used PE-conjugated anti-human CD7-PE (8H8.1, IgG2b-IM1429) and anti-human CD4 (13B8.2, IgG1-IM0449) – obtained from Immunotech (Marseille, France).

Flow cytometric analysis

The ability of the cells to scatter light in a forward direction (FS) correlates with cell volume, while their ability to scatter light in side direction (SS) correlates with cell granularity. We analyzed VA-treated cells for changes in the intensity of FS and SS as compared to untreated control cells. 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 2-colour sample in a list mode file format. List mode data was analyzed using Epics XL System II software (Coulter Electronic).

In vitro clonogenic survival assay

10^2–10^5 cells 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) (used only for HL-60 cells) *per* 1 ml of medium. Duplicate dishes were plated for each experiment. The cells were incubated for 14 days at 37 ℃ 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 from four measurements from two experiments.

Statistical analysis

The results were statistically evaluated with Student's *t*-test. The values represent mean ± SD (standard deviation of the mean) of three independent experiments. Statistical significance of the differences of means between comparable sets is indicated.

Results

Cell growth and viability

Fig. 1 shows the effects of VA on the proliferation rate of the HL-60 and MOLT-4 cell lines. As can be seen from this figure, cultivation of HL-60 cells with 2 mmol/l VA induced a slight decrease in proliferation in comparison with control cells. Cultivation with 4 mmol/l VA induced high inhibition of the HL-60 cell growth rate. The decrease in the proliferation rate observed in HL-60 cells after addition of 10 mmol/l VA was caused by the cytotoxic effect of VA. After 6 days, all cells treated with 10 mmol/l VA were dead.

As can be seen from the Fig. 1B, cultivation of MOLT-4 cells with 1 mmol/l VA induced a slight decrease in proliferation in comparison with control cells. Cultivation with 2 mmol/l VA induced high inhibition of the MOLT-4 cell growth rate. The decrease in the proliferation rate observed in MOLT-4 cells after addition of 4 mmol/l VA was due to the cytotoxic effect of VA. After 6 days, all cells treated with 4 mmol/l VA were dead.

Clonogenity

Using colony assay we established EC_{50} value (the concentration, after which 50 % of cells retain their clonogenic capacity and form colonies) of 3-day-long incubation with VA for HL-60 and MOLT-4 cells. The detected EC_{50} value for HL-60 cells was 1.84 mmol/l VA and for MOLT-4 cells 1.76 mmol/l VA. We also established EC_{50} value for continuous exposure to VA, where VA was added to clonogenity cultivation dishes (14-day-long incubation). In the case of MOLT-4 cells, the EC_{50} value decreased to 0.63 mmol/l (Fig. 2B). HL-60 cells require a 10 % conditioned medium as a source of cytokines (IL-3, SCF, G-CSF) for colony formation. For HL-60 cells cultured 14 days in presence of VA and conditioned medium, the EC_{50} value increased to 4 mmol/l (Fig. 2A).

Analysis of apoptosis

Fig. 3 shows changes in MOLT-4 cells after 3-day-long incubation with VA. Induction of early apoptosis was monitored mainly by Annexin V positivity, which binds selectively to phosphatidylserine. We also evaluated changes of forward and side light scattering, where the intensity of light scattered in forward direction correlates with cell size, and side scatter correlates with granularity. Further we observed expression of CD7 and CD4 markers and changes in distribution of the cells in cell cycle phases.

A cell triggered to undergo apoptosis activates a cascade of molecular events, which leads to its total disintegration. One of the early events is condensation of the cytoplasm followed by a change in cell size, shape and granularity. Therefore, we analyzed VA-treated cells for changes in the intensity of FS and SS as compared to untreated control. Population of cells with low FS (decrease in size) and high SS (increase in granularity) appears after incubation with VA in concentration-dependent

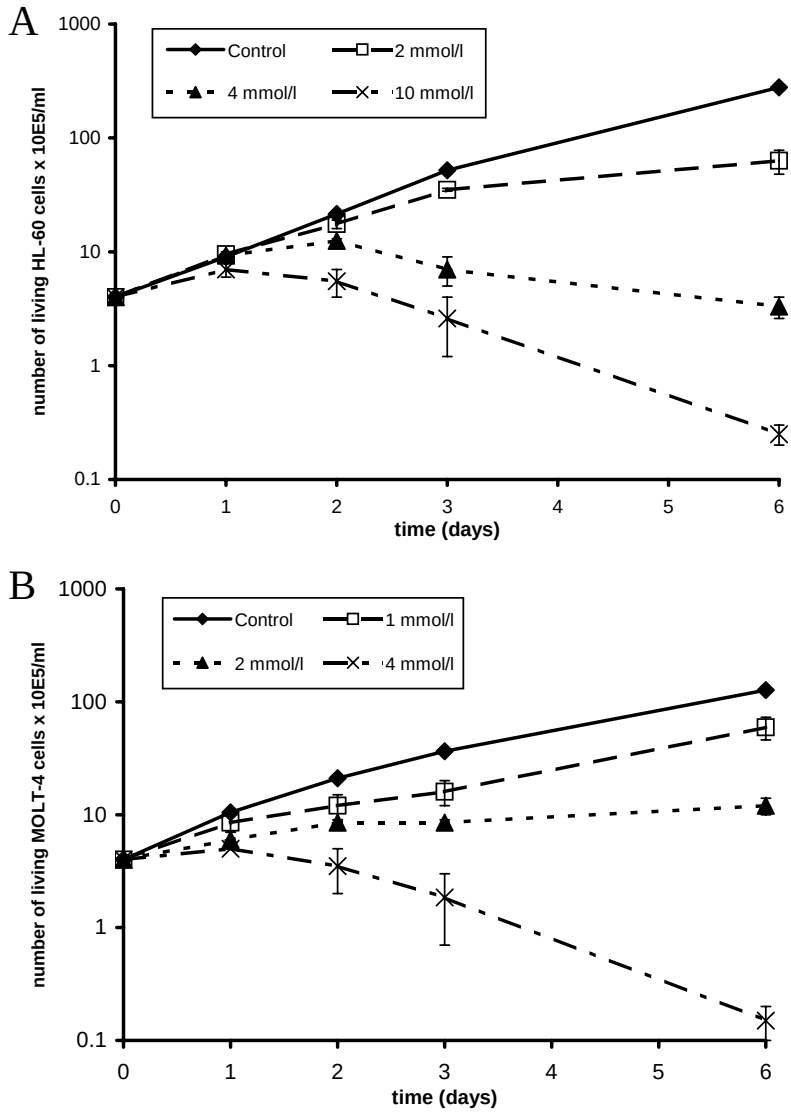

Figure 1. Kinetics of VA effect on the proliferative rate of the HL-60 and MOLT-4 cell line. The cells were exposed to various VA concentrations (HL-60 1–10 mmol/l, part A; MOLT-4 1–4 mmol/l, part B). Numbers of viable cells were determined by Trypan blue staining. Each point represents mean value from at least 3 experiments ± SEM.

manners. After incubation with 4 mmol/l VA, this subpopulation represents 85 % of cells. Induction of apoptosis was confirmed by Annexin V positivity. 90 % of control MOLT-4 cells are $CD7^+$ and Annexin V^-. The percentage of $CD7^+$ and

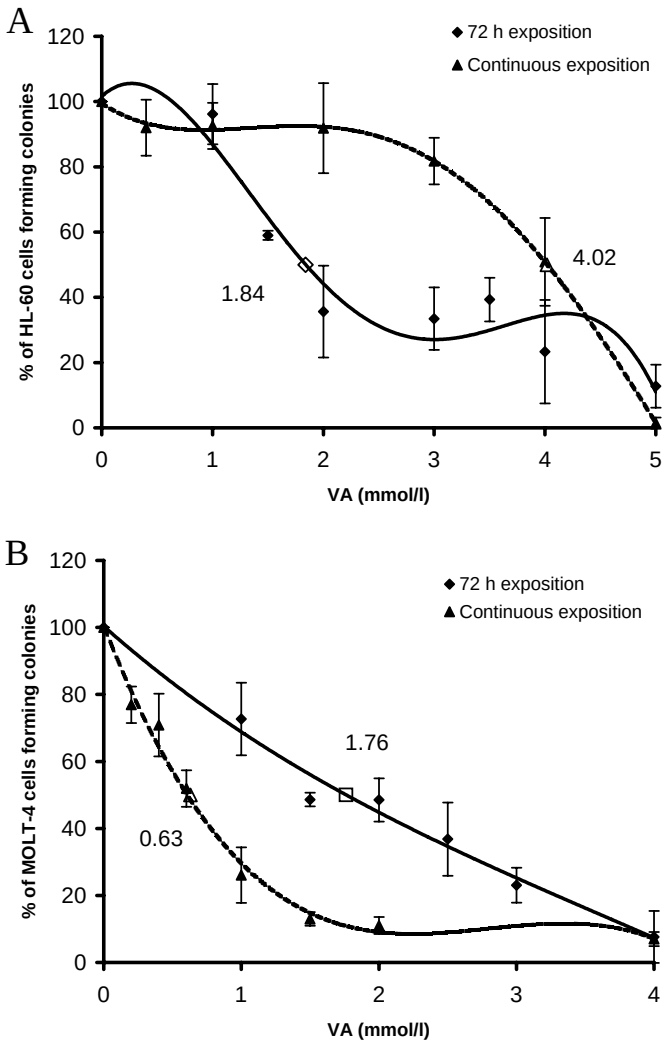

Figure 2. Effect of 72 h-long and continuous treatment of HL-60 and MOLT-4 cells with VA on colony-forming properties. In the case of HL-60 cells, conditioned medium providing cytokines (IL-3, SCF, G-CSF) was used. The presence of cytokines protected the cells from apoptosis during continuous exposure to VA (part A). MOLT-4 cells colonies were grown in standard cultivation medium (part B). Each point represents mean value from at least 4 measurements ± SEM.

Annexin V^+ cells increase in concentration-dependent manners (from 8 % in control cells up to 87 % in cells exposed to 4 mmol/l VA). 48 % of control MOLT-4 cells are $CD4^+$ and Annexin V^-. After incubation with VA, this subpopulation signif-

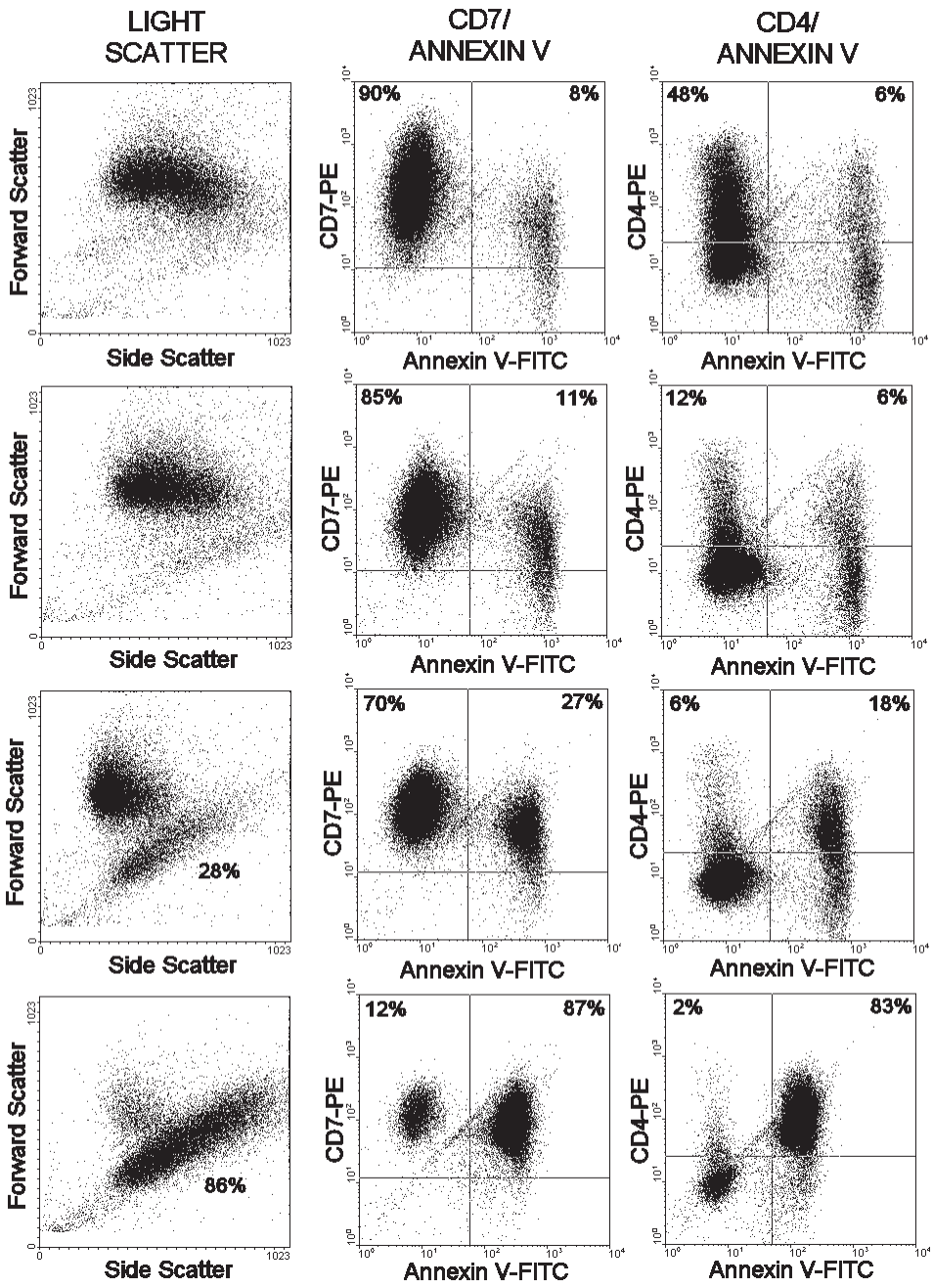

Figure 3. Changes in MOLT-4 cells after 3-day-long incubation with VA. The cells were treated with increasing concentrations of VA (from top row to bottom: control, 1 mmol/l VA, 2 mmol/l VA, 4 mmol/l VA) for 72 h, harvested and analyzed by flow cytometry.

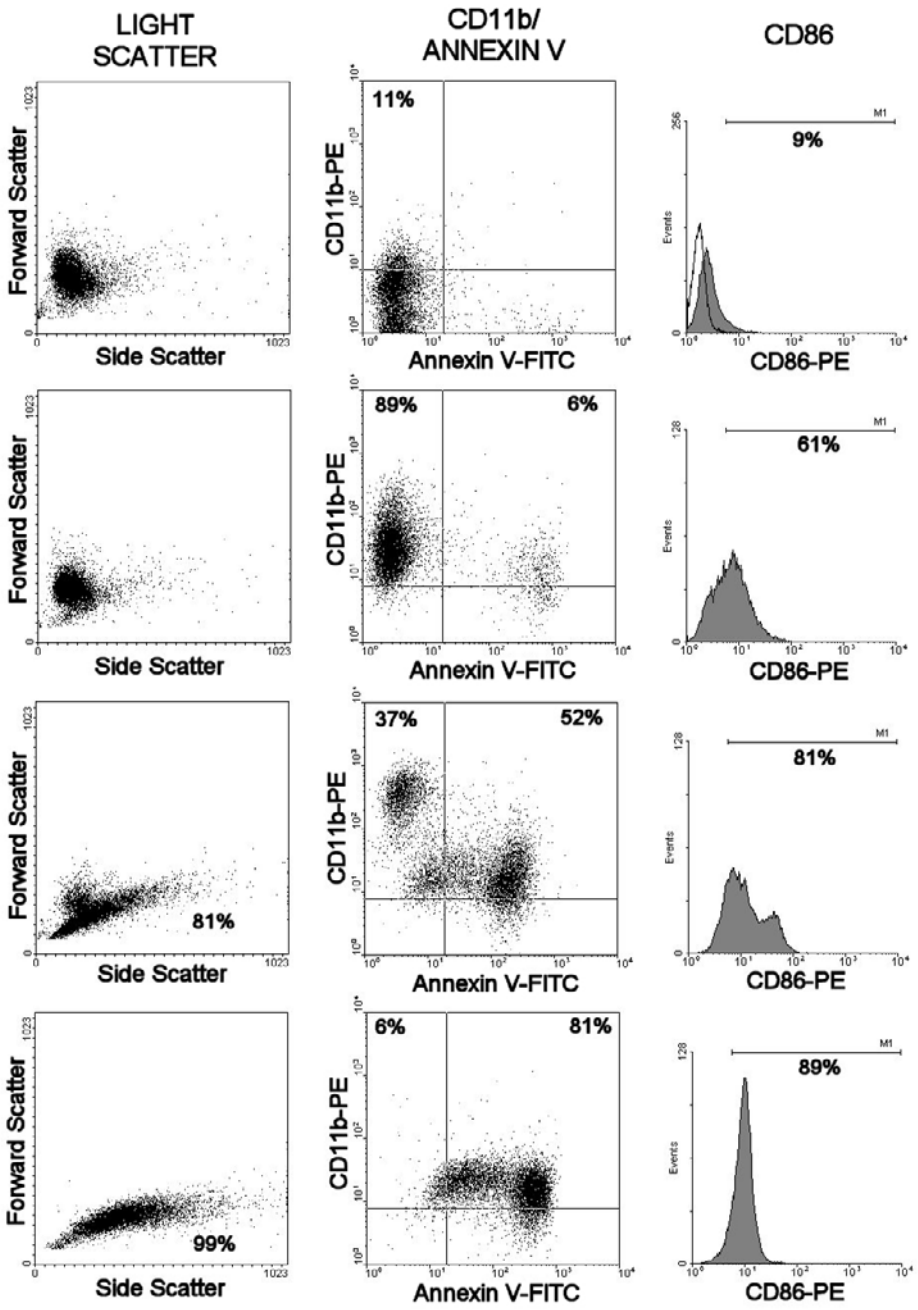

Figure 4. Changes in HL-60 cells after 3-day-long incubation with VA. The cells were treated with increasing concentrations of VA (from top row to bottom: control, 2 mmol/l VA, 4 mmol/l VA, 10 mmol/l VA) for 72 h, harvested and analyzed by flow cytometry.

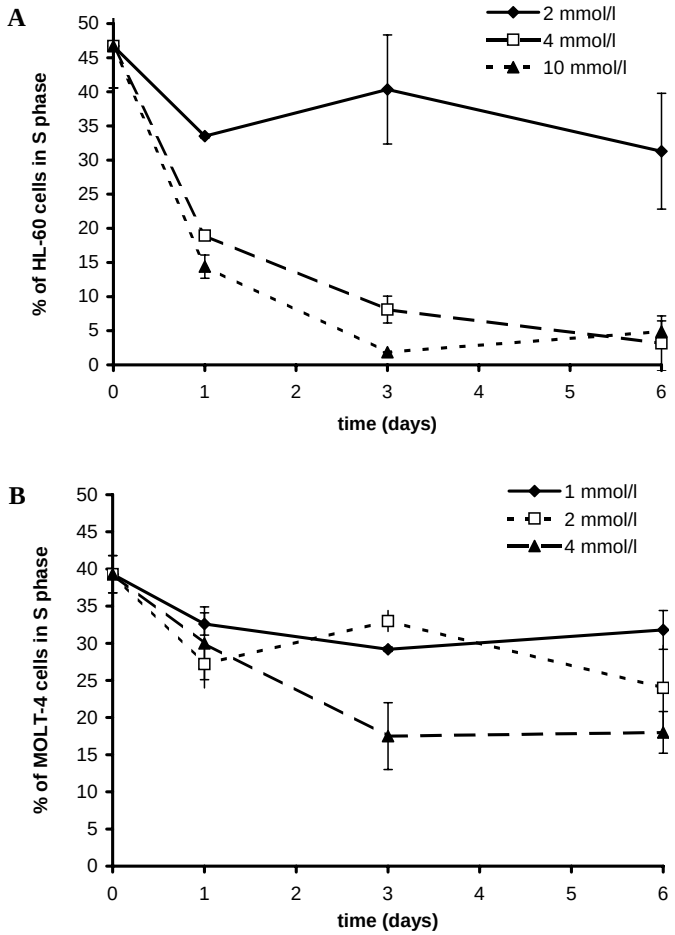

Figure 5. Kinetics of distribution of cells in S phase of the cell cycle after VA treatment. The cells were exposed to various VA concentrations (HL-60 1–10 mmol/l, part A; MOLT-4 1–4 mmol/l, part B). Numbers of viable cells is S phase were determined using flow cytometric DNA analysis. The graph shows percentage of cells in S phase from viable cells only. Each point represents mean value from at least 3 experiments ± SEM.

icantly decreases (only 12 % of cells after incubation with 1 mmol/l VA). This is accompanied by increase in $CD4^+$/Annexin V^+ subpopulation (from 8 % in control cells up to 83 % in cells exposed to 4 mmol/l VA). It is interesting that the cells maintain CD7 and CD4 markers even during apoptosis.

Similar analysis for changes in HL-60 cells after 3-day-long incubation with VA is shown on Fig. 4. We evaluated changes of FS and SS, positivity of Annexin V staining and expression of CD11b and CD86 markers. Population of cells with low FS and high SS appears after incubation with VA in concentration-dependent

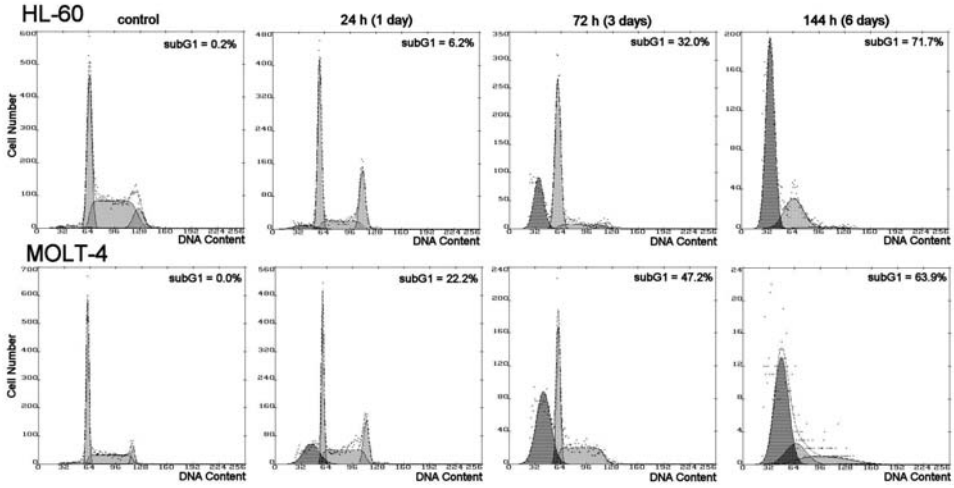

Figure 6. Apoptosis and cell cycle changes after treatment by VA. The MOLT-4 and HL-60 cells were treated with 4 mmol/l VA for 1 to 6 days, harvested and analyzed by flow cytometric detection of DNA content. Apoptotic cells are detected as subG1 peak. Sample results of 1 of 3 experiments.

manners. After incubation with 4 mmol/l VA, this subpopulation represents 81 % of cells. 6,8 % of control cells are $CD11b^+$ and Annexin V^-. After incubation with the lowest tested concentration – 2 mmol/l VA, nearly all cells are CD11b positive, which proves ability of VA to induce differentiation of HL-60 cells. 89 % of cells were $CD11b^+$/Annexin V^- and 6 % $CD11b^+$/Annexin V^+. After exposure to higher concentrations of VA, $CD11b^+$/Annexin V^+ subpopulation prevails. After 3-day-long incubation with 10 mmol/l VA, nearly all cells are apoptotic. Also expression of CD86 increases after 3-day-long incubation with 2 mmol/l VA (61 %) and further increases with increasing concentration of VA (10 mmol/l VA, 89 %).

Analysis of cell cycle

In next part of our work we analyzed distribution of the cells in cell cycle phases using flow cytometric DNA content analysis. Most significant changes were observed in percentage of cells in S phase. The results are shown on Fig. 5. The percentage of cells in S phase of cell cycle decreased in concentration dependent manners and intensified with the time of incubation. The decrease in S phase in HL-60 cells was significant already after 24 h-long incubation with 4 and 10 mmol/l VA. The decrease in S phase in MOLT-4 cells is significant, but less pronounced in comparison to HL-60 cells. Fig. 6 shows the time course of cell cycle changes and apoptosis detected by subG1 peak after the treatment of HL-60 and MOLT-4 cells by VA during 6 days. Significant changes in distribution of cells in cell cycle phases – progressive decrease in S phase cells (see also Fig. 5) from 24 to 144 h of VA

treatment and accumulation of cells in G2/M phase after 24 h of VA treatment could be seen in both cell lines. During first 3 days, the increase in apoptotic subpopulation is more intensive in MOLT-4 cells, as VA in HL-60 cells induces mainly differentiation in the beginning of the treatment.

Discussion

The results obtained in this work prove that VA stimulates differentiation of HL-60 cells and acts as highly potent inducer of apoptosis in human leukemia cells of myeloid (HL-60) and T lymphocyte (MOLT-4) origin. Our data corresponds with findings of Gottlicher et al. (2001), who proved induction of apoptosis by VA in leukemic blasts of AML of patients after 5-day-long incubation with 1 mmol/l VA.

It is generally accepted that there is a close relation between differentiation and apoptosis. Almost the same EC_{50} value (1.8 mmol/l) was established for both MOLT-4 and HL-60 cells after 3-day-long incubation with VA. Slow apoptosis is also induced in the case of continuous incubation of MOLT-4 cells (on the clonogenity dishes, where VA is added directly to cultivation medium with methylcelulose) and therefore the EC_{50} value decreases to 0.63 mmol/l. HL-60 cells are cultured for clonogenity determination in presence of conditioned medium providing cytokines (IL-3, SCF, G-CSF), which have antiapoptotic effect. During continuous exposure to VA in this model the cells were resistant to apoptosis induction and EC_{50} value paradoxically increased to 4 mmol/l. The curve exhibits a long plateau up to VA concentration 3 mmol/l (where the loss of clonogenity was not considerable) followed by a linear part of the curve. After exposure to concentrations 5 mmol/l and higher all cells lost colony-forming ability. Antiapoptic effect of cytokines (IL-3, SCF and FLT3-ligand) on hematopoietic stem cells after *in vitro* irradiation was described by Vávrová et al. (2002). In their work they proved that *in vitro* irradiation of $AC133^+$ cells isolated from peripheral blood of healthy donors after mobilization induces apoptosis in 80 % of these cells 72 h after irradiation by 2.5 Gy. However, in presence of cytokine combination IL-3+SCF+FLT3-ligand, surviving 20 % of cells are able to divide and on 7^{th} day after irradiation, 35 % of cells are in S phase of cell cycle. Results of our work also emphasize importance of culture medium, as in the presence of antiapoptic cytokines, the cells treated by VA only differentiate and apoptosis is minimal. From the clinical point of view an important finding is (Bug et al. 2005; De Felice et al. 2005), that VA, known to induce differentiation or apoptosis in leukemic blasts, stimulates the proliferation of normal $CD34^+$ hematopoietic stem cells in presence of cytokine coctails (IL-3+SCF+FLT3-ligand+trombopoetin) and therefore increases expansion potential of these cells.

We detected that incubation of HL-60 cells with VA after causes differentiation, as after 3-day-long exposure to 2 mmol/l VA nearly all cells gained CD11b positivity. In humans, integrin CD11b is strongly expressed on myeloid cells, and weakly expressed on NK cells and some activated lymphocytes. In our previous

work (Mareková et al. 2003) we have described differentiation and apoptosis induction in HL-60 cells after incubation with ATRA. It seems that VA, similarly as ATRA, triggers in these cells an aberrant differentiation program, a phenomenon known to lead to apoptosis. Similar results were obtained in HL-60 cells after 3-day-long incubation with other HDAC inhibitors, such as SAHA (Vrana et al. 1999) or butyrate (Maeda et al. 2000). Maeda et al. (2000) proved in AML cell lines that some of HDAC inhibitors (i.e. butyrate and trichostatin A) increase expression of CD86 and ICAM-1 molecules. They also proved that in 30 clinical AML samples, CD86 expression was significantly increased after treatment with butyrate. CD86 (also called B7-2) is capable of binding the receptors CD28 and CTLA-4. Its binding to CD28 provides critical co-stimulatory signal enabling activation of T-lymphocytes. In contrast to the stimulatory effects of CD28 ligation, CTLA-4 acts as an inhibitory receptor that is vital for down-modulation of the immune response. In our experiments VA induced increased expression of CD86 in HL-60 cells. However, the immunomodulatory effects of HDAC inhibitors are poorly understood. These studies show that HDAC inhibitors, including VA, can enhance expression of co-stimulatory/adhesion molecules in AML cells. It was found that during allogenic transplantation of bone marrow, the antileukemic immune response (graft *versus* leukemia) occurs and that donor T-lymphocytes play an important role in residual leukemic cells removal. Expression of CD86 on the surface of leukemic cells is related to T-lymhocytes interactions and killing (Maeda et al. 2000). It is becoming apparent that VA in AML cells can be used as suppressor of cellular growth, inductor of differentiation and apoptosis and also as immunotherapeutic agent.

VA causes quick apoptosis also in MOLT-4 cells, which do not differentiate. Interesting observation is that percentage of $CD4^+$/Annexin V^- dramatically decreases after exposure of MOLT-4 cells to all tested concentrations of VA, but $CD7^+$/Annexin V^- subpopulation is significantly affected only after the highest VA concentration (4 mmol/l). The fact that most of Annexin V^+ MOLT-4 cells probably recruit from CD4 positive subpopulation deserves further elucidation. However, it is clear that apoptosis induced after incubation with VA can be initiated without connection with previous differentiation of cells.

In MOLT-4 cells, the concentrations 1–2 mmol/l of VA had mostly cytostatic effect during 6-day-long incubation. The number of cells in the S phase of cell cycle decreased significantly, as well as the ability of the cells to form colonies. The highest concentration studied (4 mmol/l) had cytotoxic effect. Apoptosis induction by VA in concentration 4 mmol/l in MOLT-4 cells was proved by presence of subG1 peak in DNA content analysis and by Annexin V binding accompanied by retained expression of CD4 and CD7 markers. MOLT-4 cells quickly transform from early apoptotic phase to late apoptotic phase, which is not detectable by subG1 peak, therefore part of late apoptotic MOLT-4 cells are not analyzed by DNA content analysis.

In both cell lines, VA inhibits proliferation of the cells which corresponds to observed decrease in percentage of cells in S phase of cell cycle. MOLT-4 cells enter

apoptosis more quickly. The importance of mitochondrial pathway of apoptosis induction by VA is still in question. Our preliminary results indicate significance of p53 increase and its phosphorylation in MOLT-4 cells. Future studies should further concentrate on molecular mechanisms important in proliferation inhibition and apoptosis induction by HDAC inhibitors.

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References

Bug G., Gul H., Schwarz K., Pfeifer H., Kampfmann M., Zheng X., Beissert T., Boehrer S., Hoelzer D., Ottmann O. G., Ruthardt M. (2005): Valproic acid stimulates proliferation and self-renewal of hematopoietic stem cells. Cancer Res. **65**, 2537—2541

De Felice L., Tatarelli C., Mascolo M. G., Gregorj C., Agostini F., Fiorini R., Gelmetti V., Pascale S., Padula F., Petrucci M. T., Arcese W., Nervi C. (2005): Histone deacetylase inhibitor valproic acid enhances the cytokine-induced expansion of human hematopoietic stem cells. Cancer Res. **65**, 1505—1513

De Vente J., Kiley S., Garris T., Bryant W., Hooker J., Posekany K., Parker P., Cook P., Fletcher D., Ways D. K. (1995): Phorbol ester treatment of U937 cells with altered protein kinase C content and distribution induces cell death rather than differentiation. Cell Growth Differ. **6**, 371—382

Gottlicher M. (2004): Valproic acid: an old drug newly discovered as inhibitor of histone deacetylases. Ann. Hematol. **83** (Suppl. 1), S91—92

Gottlicher M., Minucci S., Zhu P., Kramer O. H., Schimpf A., Giavara S., Sleeman J. P., Lo Coco F., Nervi C., Pelicci P. G., Henzel T. (2001): Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. EMBO J. **20**, 6969—6978

Kawagoe R., Kawagoe H., Sano K. (2002): Valproic acid induces apoptosis in human leukemia cells by stimulating both caspase-dependent and independent apoptotic signaling pathways. Leuk. Res. **26**, 495—502

Maeda T., Towatari M., Kosugi H., Saito H. (2000): Up-regulation of costimulatory/adhesion molecules by histone deacetylase inhibitors in acute myeloid leukemia cells. Blood **96**, 3847—3856

Mareková M., Vávrová J., Vokurková D., Psutka J. (2003): Modulation of ionizing radiation induced apoptosis and cell cycle arrest by all-trans retinoic acid in promyelocytic leukemia cells (HL-60). Physiol. Res. **52**, 599—606

Minucci S., Nervi C., Coco F. L., Pelicci P. G. (2001): Histone deacetylases: a common molecular target for differentiation treatment of acute myeloid leukemia? Oncogene **20**, 3110—3115

Rosato R. R., Grant S. (2004): Histone deacetylase inhibitors in clinical development. Expert Opin. Investig. Drugs **13**, 21—38

Tang R., Faussat A. M., Majdak P., Perrot J. Y., Chaoui D., Legrand O., Marie J. P. (2004): Valproic acid inhibits proliferation and induces apoptosis in acute myeloid leukemia cell expressing P-gp and MRP1. Leukemia **18**, 1246—1251

Trus M. R., Yang L., Suarez S. F., Bordeleau L., Jurisica I., Minden M. D. (2005): The histone deacetylase inhibitor valproic acid alters sensitivity towards all trans retinoic acid in acute myeloblastic leukemia cells. Leukemia **19**, 1161—1168

Vávrová J., Vokurková D., Mareková M., Bláha M., Jebavý L., Filip S. (2002): Antiapoptotic cytokine IL-3+SCF+FLT3L influence on proliferation of gamma-irradiated $AC133^+/CD34^+$ progenitor cells. Folia Biol. (Prague) **48**, 51—57

Vávrová J., Řezáčová M., Osterreicher J. (2005): Inhibitors of histone-deacetylase. J. Appl. Biomed. **3**, 13—24

Vrana J. A., Decker R. H., Johnson C. R., Wang Z., Jarvis W. D., Richon V. M., Ehinger B., Grant S. (1999): Induction of apoptosis in U 937 human leukemia cells by suberoylanilide hydroxamic acid (SAHA) proceeds through pathways that are regulated by Bcl-2/Bcl-XL, c-Jun, and p21CIP1, but independent of p53. 0ncogene **18**, 7016—7025

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