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Neutrophilic differentiation modulates the apoptotic response of HL-60 cells to sodium butyrate and sodium valproate

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Differentiation of myeloid leukemic cells may result in less sensitivity to various apoptotic stimuli. We examined whether human leukemia HL-60 cells differentiating by all-*trans* retinoic acid (ATRA) acquired resistance to the apoptogenic activity of two histone deacetylase (HDAC) inhibitors, butyrate and valproate. In undifferentiated cells, the cytotoxicity of both butyrate and valproate was associated with activation of the intrinsic apoptotic pathway since we observed dissipation of mitochondrial membrane potential, induction of caspase-9 and caspase-3 activities, appearance of sub-G1 DNA and loss of plasma membrane asymmetry and/or integrity. Both HDAC inhibitors were also able to induce accumulation of undifferentiated cells in the G0/G1 phase of the cell cycle. ATRA was found to enhance the apoptotic effect of both butyrate and valproate in undifferentiated cells. This aside, ATRA appeared to synergize with butyrate in the induction of the G0/G1 cell cycle arrest. In cells pretreated for 72 h with ATRA, butyrate and valproate in combination with ATRA induced lower dissipation of mitochondrial membrane potential and weaker apoptotic and/or necrotic changes in plasma membrane, whereas DNA fragmentation was not diminished compared to undifferentiated cells. Similar results were also obtained when butyrate or valproate were combined with another neutrophilic differentiation inducer, dimethyl sulfoxide. We conclude that neutrophilic differentiation modulates but does not abrogate the apoptotic response of HL-60 cells to butyrate and valproate, and nuclei are preferentially affected during apoptosis in differentiated cells.

Key words. All-trans retinoic acid, apoptosis, butyrate, cell cycle, differentiation, valproate

Histone deacetylases (HDACs) play an important role in regulation of gene expression. Since aberrant upregulation of their activity has been linked to various cancers including leukemic disorders, inhibition of HDACs has emerged as a potential strategy in anticancer therapy. The short chain fatty acids, butyrate and valproate (2-propylvalerate) are effective as HDAC inhibitors at millimolar concentrations [1]. They have been shown to induce, depending on their concentration, monocytic differentiation [2–4] and/or apoptosis in several types of human myeloid leukemic cells [3, 5–7]. HDACs have been also identified as molecular targets of all-*trans* retinoic acid (ATRA) in the treatment of acute promyelocytic leukemia (APL) [8]. Neutrophilic differentiation of normal myeloid cells by ATRA is mediated through the nuclear retinoic acid receptor α (RARα). In the absence of ligand, RARα interacts with nuclear corepressor proteins to form a complex including HDAC1 or HDAC2, resulting in transcriptional repression or silencing. Physiological concentrations of ATRA induce dissociation of the nuclear repressor complex and recruitment of coactivators with histone acetyltransferase activity. This permits the transcriptional activation of differentiation-related genes. In APL cells, the gene encoding RARα appears to be involved in chromosomal translocations, producing several types of RAR-fusion proteins [8, 9]. Approximately 98% of APL cases have the t(15;17) translocation leading to the PML-RARα (promyelocytic leukemia protein/RARα) fusion protein. Although ATRA binds to PML-RARα with an affinity comparable to normal RARα, the dissociation of the nuclear repressor complex from PML-RARα and subsequent differentiation occur only at pharmacological doses of ATRA. In contrast, a less common variant of APL with the t(11;17) translocation and the resultant PLZF-RARα (promyelocytic zinc finger protein/RARα) fusion protein has been found resistant to ATRA. This resistance arises from the presence of additional binding site for the nuclear repressor complex in the PLZF moiety of the fusion protein which is insensitive to

ATRA [9]. In accord with the crucial role of HDACs in APL, treatment with ATRA in combination with HDAC inhibitors such as trichostatin A has been found to overcome the block in differentiation of t(11;17)-positive leukemic cells [10].

The therapeutic effect of HDAC inhibitors administered along with ATRA may include both differentiation and apoptosis of leukemic cells. Terminally differentiated myeloid cells are known to undergo spontaneous apoptosis [11–14]. On the other hand, it has been shown that myeloid cells differentiating by ATRA [15–18], dimethyl sulfoxide (DMSO) [19, 20], 1,25-dihydroxyvitamin D_3 [15–17, 21] or phorbol 12-myristate 13-acetate [22–25] become less sensitive to various stimuli initiating apoptosis mediated through both intrinsic/mitochondrial and extrinsic/death receptor pathway. In this study, we examined whether human leukemia HL-60 cells differentiating by ATRA into a neutrophil-like phenotype acquired resistance to the apoptogenic activity of butyrate and valproate. We show that neutrophilic differentiation modulates but does not abrogate the apoptotic response of HL-60 cells to butyrate and valproate, and nuclei are preferentially affected during apoptosis in differentiated cells.

Materials and methods

Chemicals. DMSO (D2650), ATRA (R2625), sodium butyrate (B5887), sodium valproate (P4543), and valinomycin (V0627) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Butyrate and valproate were dissolved in water for molecular biology to a stock concentration of 1 M. ATRA and valinomycin were dissolved in ethanol to a stock concentration of 1 mM. Stock solutions of butyrate, valproate, and valinomycin were stored at -20°C. Stock solution of ATRA was prepared freshly before use with all manipulations performed in subdued light.

Cell culture, differentiation, and treatment. The human promyelocytic leukemia cell line HL-60 (No. 98070106, ECACC, Salisbury, UK) was cultured at 37°C in RPMI-1640 medium containing 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma), and 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) in a humidified atmosphere containing 5% CO_2 and maintained between 1-9 × 10^5 cells/ml. To induce neutrophilic differentiation, 2 × 10^6 of exponentially growing cells in 30 ml of fresh medium were seeded in a 75 cm^2 flask and incubated for up to 5 days with 1 μM ATRA or 1.25% (*v/v*) DMSO. To prevent cell starvation, 20 ml of medium with ATRA or DMSO was added on day 2. During differentiation, cell cycle profile, mitochondrial membrane potential, and p67phox, gp91phox and cytochrome *c* protein levels were analyzed (see below). The cytotoxicity experiments were conducted using cells of passages 10-30. Undifferentiated cells or cells under differentiation for 72 h with ATRA or DMSO were seeded at a density of 4 × 10^5 cells/ml and treated for the appropriate time with butyrate, valproate, or valinomycin in the absence or presence of 1 μM ATRA or 1.25% DMSO. The final concentration of ethanol was 0.1% in cultures treated with ATRA or 0.2% in cultures treated with a combination of ATRA and valinomycin. These ethanol concentrations had negligible effect on the measured parameters, including mitochondrial membrane potential.

Western blot analysis. An appropriate number of undifferentiated or differentiated cells were collected by centrifugation and lysed in lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, protease inhibitors Complete (Roche Diagnostics, Mannheim, Germany), 0.2% sodium dodecyl sulfate (SDS), 1% Nonidet-P40, 1% Triton X-100, pH 7.4). After incubation on ice for 20 min, the cell lysates were centrifuged for 10 min at 16,000×*g* and 4°C and proteins in supernatants were quantified by the BCA protein assay reagents (Thermo Scientific, Rockford, IL, USA). Aliquots containing 30 μg of protein were subjected to electrophoresis through 10% or 12.5% SDS-polyacrylamide gel and proteins were transferred to polyvinylidene difluoride membrane by electroblotting. Cytochrome *c*, gp91phox and p67phox were detected with goat polyclonal cytochrome *c* (C-20) antibody, rabbit polyclonal gp91-phox (H-60) antibody or rabbit polyclonal p67-phox (H-300) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Primary antibodies were detected with anti-goat immunoglobulin G-horseradish peroxidase (IgG-HRP) (Santa Cruz) or anti-rabbit IgG-HRP (Cell Signaling Technology, Danvers, MA, USA) conjugated secondary antibodies using the chemiluminescent reaction.

Flow cytometric evaluation of apoptosis. After treatment, cells were washed with PBS and resuspended in HEPES buffer pH 7.4 containing 140 mM NaCl and 2.5 mM $CaCl_2$. Cell suspension was incubated with annexin V-FITC (Molecular Probes, Eugene, OR, USA) and propidium iodide (PI; Sigma) for 10 min at room temperature and immediately analyzed by flow cytometry on Cytomics FC 500 (Beckman Coulter) as described [26], counting 20,000 events per sample. Dual parametric dot plots were used for calculation of the percentage of nonapoptotic living cells in the lower left quadrant (annexin V-negative/PI-negative), early apoptotic cells in the lower right quadrant (annexin V-positive/PI-negative), and late apoptotic or necrotic cells in the upper right quadrant (annexin V-positive/PI-positive).

Determination of mitochondrial membrane potential by flow cytometry. After treatment, cells were resuspended in fresh medium without inducers of differentiation and tested substances, incubated with 0.5 μM tetramethylrhodamine ethyl ester (TMRE; Sigma) for 20 min at 37°C, and immediately analyzed for the inner mitochondrial membrane potential on a flow cytometer Cytomics FC 500 (Beckman Coulter) as described [26], counting 15,000 events per sample. The percentage of cells with decreased mitochondrial membrane potential was calculated.

Caspase activity assay. After treatment, fold changes in caspase activities were determined as described previously [26]. Specific fluorogenic substrates, Ac-DEVD-AMC (Merck, Darmstadt, Germany) and Ac-LEHD-AFC (Bachem, Buben-

dorf, Switzerland), and specific inhibitors, Ac-DEVD-al (Merck) and Ac-LEHD-al (Bachem), were used to assess the activity of caspase-3 and -9, respectively. The fluorescence of caspase-released AMC or AFC was read on a microplate reader Infinite M200 (Tecan, Salzburg, Austria). The fluorescence differences in the absence and presence of caspase inhibitors were standardised against the protein content and used for calculation of fold changes versus untreated undifferentiated HL-60 cells.

Cell cycle analysis by flow cytometry. After treatment, cells were fixed in 70% ethanol and cellular DNA content was analyzed after cell staining with PI (Sigma) by flow cytometry on Cytomics FC 500 (Beckman Coulter, Fullerton, CA, USA) as described [26], counting 20,000 events per sample. The percentage of cells in G0/G1, S, and G2/M phase of cell cycle and the percentage of cells in sub-G1 peak were calculated using MultiCycle AV Software (Phoenix Flow Systems, San Diego, CA, USA) which eliminated the debris effect.

To examine nuclear morphology, fixed cells were centrifuged for 3 min at 300×*g* and 4°C and stained using PBS pH 7.4 containing 25% glycerol and 2 μg/ml PI. After 5 min of incubation, cells were placed on slides and the nuclear morphology was examined by fluorescence microscopy.

Statistical analysis. Results were expressed as means ± S.D. of three independent experiments. The differences in mean values were analyzed by Student's *t*-test. A *p* value of less than 0.05 was considered as statistically significant.

Results

Differentiation of HL-60 cells by ATRA and DMSO. ATRA and DMSO have been previously shown to induce cell growth arrest and differentiation of HL-60 cells into neutrophil-like phenotype [27]. In our study, undifferentiated HL-60 cells required less than 24 h to double their number (data not shown) and exhibited a normal cell cycle profile, as shown by flow cytometric analysis of DNA content (Fig. 1A). After 72 h of incubation with 1 μM ATRA or 1.25% DMSO, we observed a complete inhibition of cell growth (data not shown) accompanied by an increase in the cell population in the G0/G1 phase of the cell cycle with a parallel decrease in the S phase. DMSO, unlike ATRA, reduced the proportion of cells in the G2/M phase as well (Fig. 1A). To confirm the induction of differentiation-related proteins, expression of two subunits of the phagocyte NADPH oxidase, gp91phox and p67phox, was analyzed by western blotting (Fig. 1B). The levels of both proteins were low in undifferentiated cells and substantially increased in response to both differentiating agents. In contrast, we observed no changes in the expression of cytochrome *c* (Fig. 1B), a proapoptotic protein whose content in neutrophils is very low [28]. Neutrophils contain relatively few mitochondria that maintain their inner mitochondrial membrane potential and play a role in the regulation of apoptosis [29]. Using flow cytometry, we observed that differentiation decreased the stainability of HL-60 cells with a mitochondria-specific dye TMRE (Fig. 1C). DMSO induced a stronger decrease in the TMRE fluorescence than ATRA with a marked shift of the fluorescence found as early as 24 h after the start of the differentiation. However, differentiation by ATRA or DMSO induced only a gentle increase in the cell population with dissipated mitochondrial potential (Fig. 1C). After 5 days of incubation with either differentiation inducer, the loss of mitochondrial potential was detected in 11-12% of cells (data not shown). This suggests that the lower stainability of differentiating cells with TMRE results from structural or functional changes in mitochondria rather than from their damage.

ATRA- and DMSO-induced differentiation attenuates plasma membrane damage induced by cotreatment with differentiating agent and butyrate or valproate. Flow cytometric analysis of cells stained with annexin V and PI enables discrimination of early apoptotic cells which externalize phosphatidylserine on their surface, and late apoptotic or necrotic cells characterized by loss of plasma membrane integrity [30]. Our results showed that 13% of control undifferentiated HL-60 cells were early apoptotic and 2% of cells were late apoptotic or necrotic. Both apoptotic cell populations significantly increased after 24 h exposure to 1 μM valinomycin, a potassium ionophore [31] used as a positive control (Fig. 2A). A significant induction of apoptosis by butyrate was found at concentrations from 2 mM (Fig. 2B). Valproate showed less cytotoxicity than butyrate with significant cell damage observed only at 5 mM concentration (Fig. 2C). Treatment with 1 μM ATRA or 1.25% DMSO for 24 h significantly inhibited the spontaneous apoptosis of undifferentiated cells with the population of early apoptotic cells reduced to 5% and 3%, respectively (Fig. 2B, D). However, ATRA synergistically potentiated apoptosis of undifferentiated cells induced by 1-5 mM butyrate or 3 and 5 mM valproate (Fig. 2B, C). A synergistic induction of apoptosis was also found in cells cotreated with DMSO and 1 mM butyrate (Fig. 2D) or 3 mM valproate (Fig. 2E). On the other hand, with the exception of 1 mM valproate and ATRA, differentiation of HL-60 cells for 72 h with ATRA or DMSO attenuated the apoptosis induced by 24 h exposure to either HDAC inhibitor in the presence of respective inducer of differentiation. Pretreatment with ATRA almost completely abrogated the appearance of late apoptotitc/necrotic cells (Fig. 2B, C). DMSO showed a greater cytoprotective effect, reducing the population of early apoptotic cells as well (Fig. 2D, E). For example, differentiation with ATRA or DMSO decreased the total percentage of apoptotic cells induced by 2 mM butyrate and respective differentiating agent from 67% and 36% to 29% and 8% (Fig. 2B, D). Similarly, differentiating cells were also found to be less sensitive to valinomycin (Fig. 2A). The prolongation of the cotreatments to 48 h showed that the apoptotic response of differentiating cells was time-dependent (Fig. 2A, B, C, D, E).

Neutrophilic differentiation reduces dissipation of mitochondrial membrane potential. Induction of apoptosis by valinomycin is associated with the collapse of the mitochondrial membrane potential ($\Delta\Psi_m$) [31]. In undifferentiated

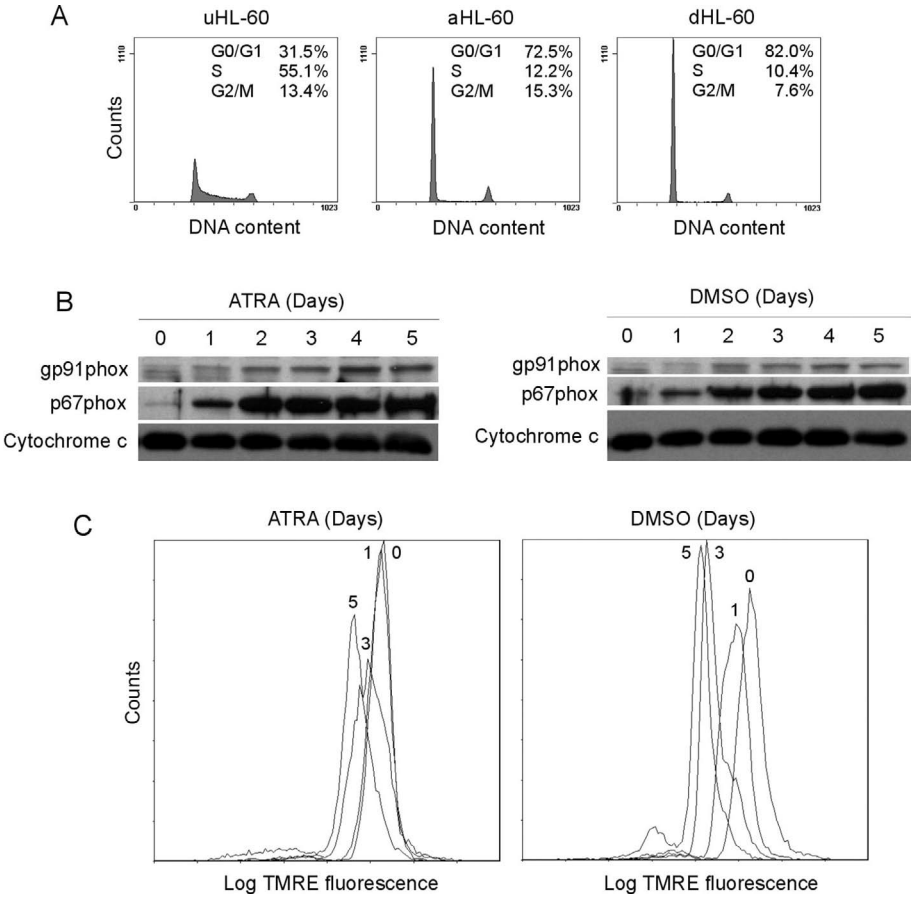

Figure 1. Effect of ATRA- and DMSO-induced differentiation on cell cycle distribution, protein expression, and mitochondrial membrane potential ($\Delta\Psi_m$) in HL-60 cells. Cells were incubated for up to 5 days in the absence or presence of 1 μM ATRA or 1.25% DMSO. (A) Cell cycle profiles of undifferentiated cells (uHL-60) and cells differentiated for 72 h with ATRA (aHL-60) or DMSO (dHL-60) were analyzed by flow cytometry of PI-stained fixed cells. (B) Proteins in the whole cell lysates (30 μg/lane) were analyzed by western blotting and gp91phox, p67phox and cytochrome *c* were visualized by chemiluminescent detection. (C) At the indicated time points, cells were stained with TMRE and analyzed for $\Delta\Psi_m$ by flow cytometry. Data are means and the figures are representative of three experiments.

HL-60 cells exposed for 24 h to 1 μM valinomycin either alone or in combination with 1 μM ATRA or 1.25% DMSO, the proportions of cells with low $\Delta\Psi_m$ increased from 3-5% (controls) to 60-71% (Fig. 3A). In contrast, substantially less dissipation of $\Delta\Psi_m$ induced by valinomycin in the presence of ATRA or DMSO was found in cells differentiated for 72 h with respective inducer while the loss of $\Delta\Psi_m$ was detected only in 13-14% of cells (Fig. 3A). After 24 h treatment of undifferentiated cells, both butyrate and valproate induced a significant dose-dependent dissipation of $\Delta\Psi_m$. The effect of both HDAC inhibitors was synergistically enhanced by ATRA (Fig. 3B, C). DMSO showed a weak synergism only with 1 mM butyrate and 3 mM valproate (Fig. 3D, E). In ATRA- and DMSO-differentiated cells, a dissipation of $\Delta\Psi_m$ induced by the exposure to either HDAC inhibitor in combination with respective differentiating agent appeared to be markedly reduced in comparison with undifferentiated cells (Fig. 3B, C, D, E). For instance, after 24 h cotreatment with ATRA and 5 mM butyrate or 5 mM valproate, the loss of $\Delta\Psi_m$ was found in 77% and 69% of undifferentiated cells, and in 27% and 25% of ATRA-differentiated cells, respectively (Fig. 3B, C).

Butyrate and valproate induce activation of caspase-9 and caspase-3 in differentiating HL-60 cells. HDAC inhibitors are known to activate the intrinsic apoptotic pathway [32, 33]. We examined whether butyrate and valproate stimulated activation of the initiator caspase-9 and the effector caspase-3 in HL-60 cells under differentiation by ATRA. Using specific fluorogenic substrates, we observed a dramatic increase in the caspase-9 and -3 activities in undifferentiated HL-60 cells after 12 h exposure to 1 μM valinomycin and after 24 h exposure to 5 mM butyrate or 5 mM valproate (data not shown). When HL-60 cells were incubated with 1 μM ATRA, no changes in the caspase activities were found after 24 h, but after 4 days of incubation the levels of caspase-9 and -3 activities reached 3.4-fold and 3.2-fold values, respectively, compared to control undifferentiated cells (Fig. 4A). The combined treatment with

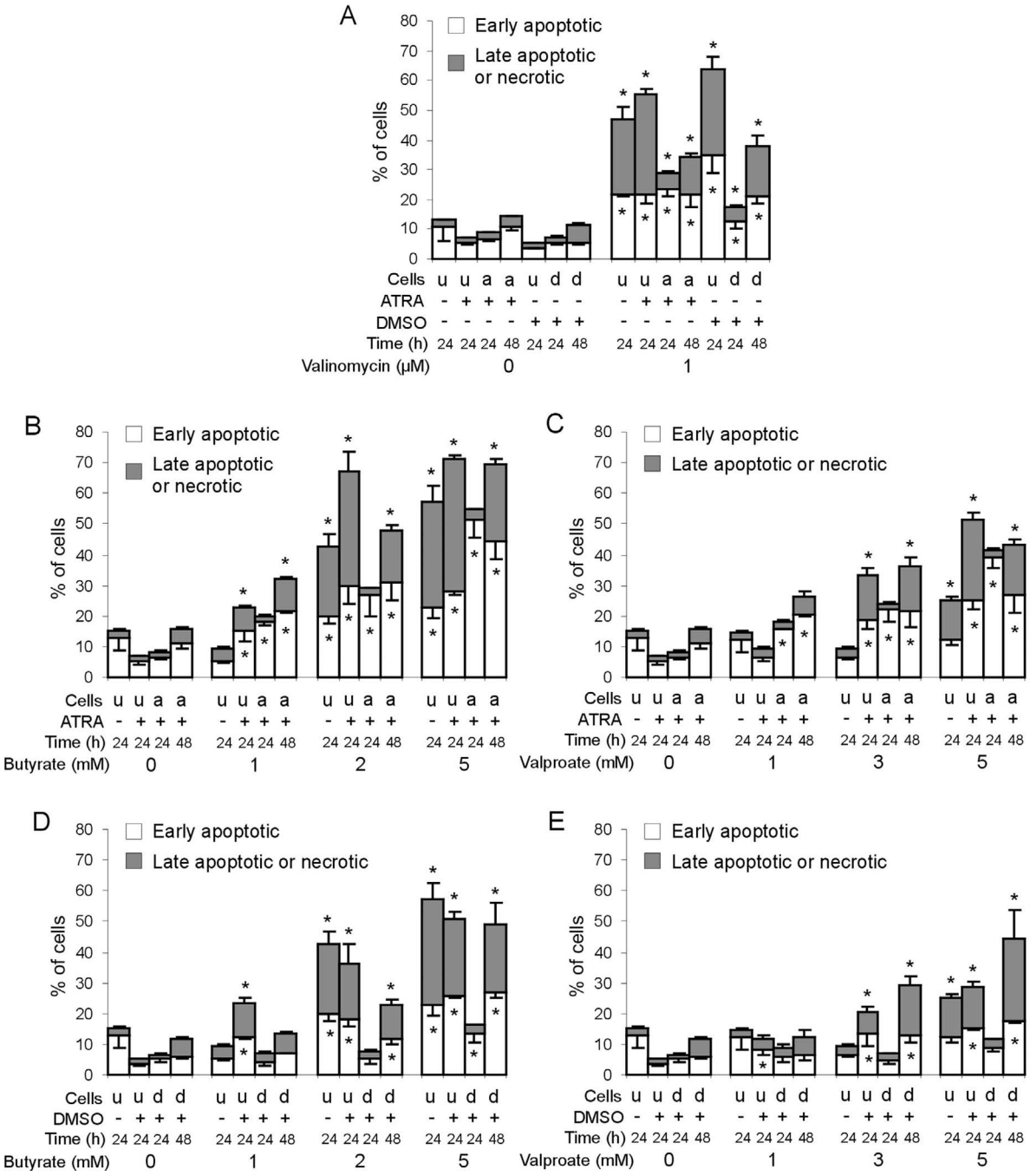

Figure 2. Induction of apoptosis by butyrate, valproate, and valinomycin in HL-60 cells induced to differentiate by ATRA or DMSO. Undifferentiated cells (u) or cells differentiated for 72 h with 1 μM ATRA (a) or 1.25% DMSO (d) were treated for 24 or 48 h with (A) valinomycin, (B, D) butyrate, or (C, E) valproate in the absence or presence of respective inducer of differentiation (as indicated), stained with annexin V-FITC and PI, and analyzed by flow cytometry. The percentages of early apoptotic cells (annexin V^+/PI^-) and late apoptotic or necrotic cells (annexin V^+/PI^+) are expressed as means ± SD of three experiments. * $p < 0.05$, significantly increased versus respective control.

ATRA and butyrate or valproate resulted in a dose-dependent elevation of the caspase activities in both undifferentiated cells and cells pretreated for 72 h with ATRA. At higher doses of the HDAC inhibitors, caspase activities reached comparable values regardless of the differentiation status of HL-60 cells (Fig. 4A, B). Maximum caspase-9 and -3 activity induced by ATRA in

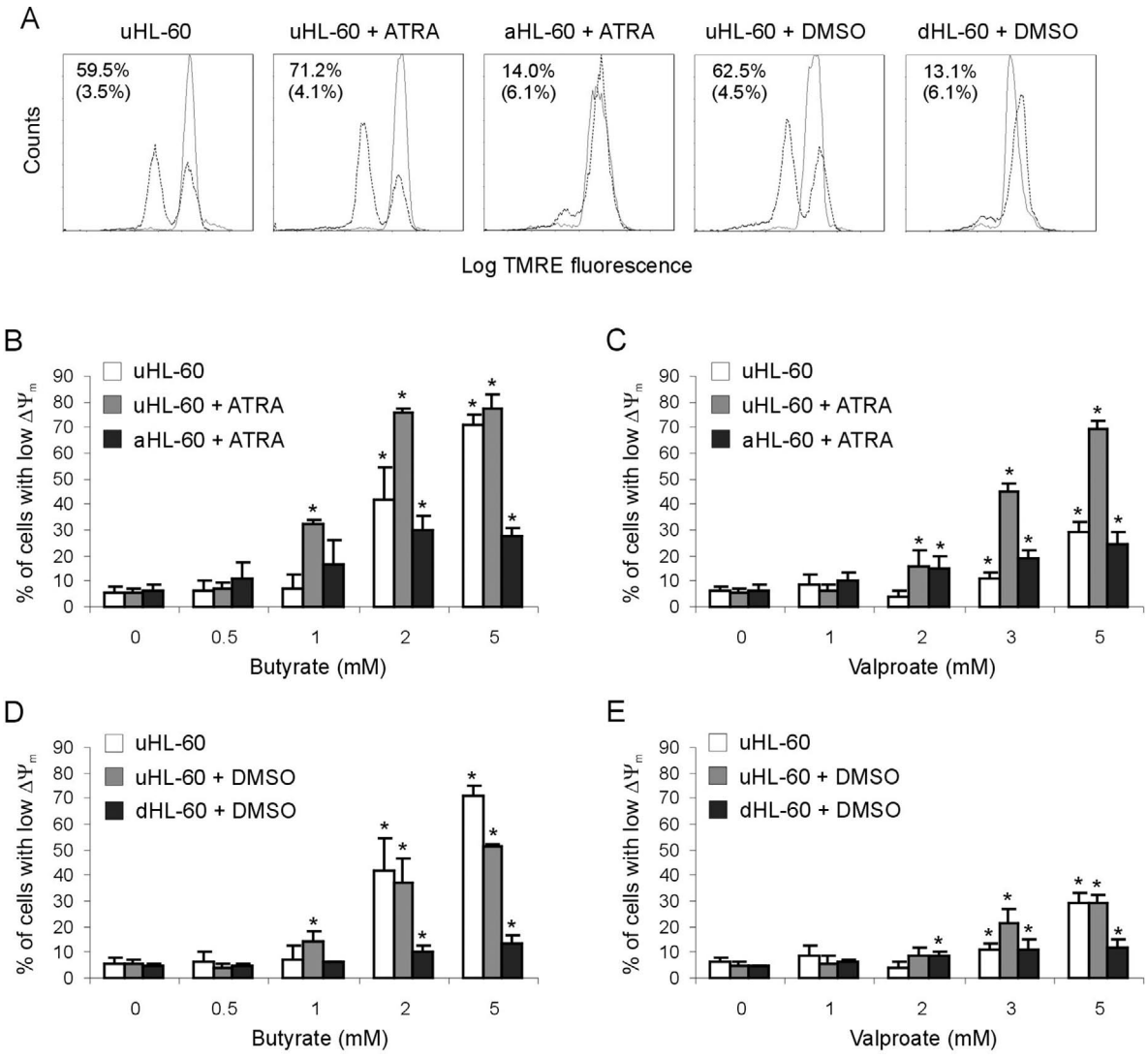

Figure 3. Dissipation of $\Delta\Psi_m$ induced by butyrate, valproate, and valinomycin in HL-60 cells differentiating by ATRA or DMSO. Undifferentiated cells (uHL-60) or cells differentiated for 72 h with 1 μM ATRA (aHL-60) or 1.25% DMSO (dHL-60) were treated for 24 h with (A) valinomycin, (B, D) butyrate, or (C, E) valproate in the absence or presence of respective inducer of differentiation (as indicated), stained with TMRE, and the percentage of cells with low $\Delta\Psi_m$ was determined by flow cytometry. (A) Representative histograms showing $\Delta\Psi_m$ of control cells (solid lines) and cells treated with 1 μM valinomycin (dashed lines). Data are means of three experiments with the control values quoted in brackets. (B, C, D, E) Data are means ± SD of three experiments. * $p < 0.05$, significantly increased versus respective control.

combination with 1 μM valinomycin was found after 24 h of incubation and reached respectively 20.5-fold and 19.7-fold values in undifferentiated cells, and 11.2-fold and 14.0-fold values in differentiated cells in comparison with control undifferentiated cells (data not shown).

Neutrophilic differentiation does not prevent DNA fragmentation induced by cotreatment with differentiating agent and butyrate or valproate. Flow cytometric analysis of the DNA profiles of PI-stained fixed cells is widely used to detect both cell cycle changes and an appearance of cells with DNA content less than G1 (sub-G1), characteristic of apoptotic cells. We observed that untreated undifferentiated HL-60 cells showed a normal cell cycle profile without any sub-G1 peak (Fig. 1A). A 24 h exposure of undifferentiated cells to butyrate (0.5-2 mM) or valproate (2-5 mM) induced a significant cell accumulation in the G0/G1 phase of the cell cycle with a parallel decrease in the S phase. For instance, 1 mM butyrate and 3 mM valproate elevated the population of cells in the G0/G1 phase from 31% to 67% and 69%, respectively (data not shown). The G0/G1 arrest of HL-60 cells was also observed after 24 h of

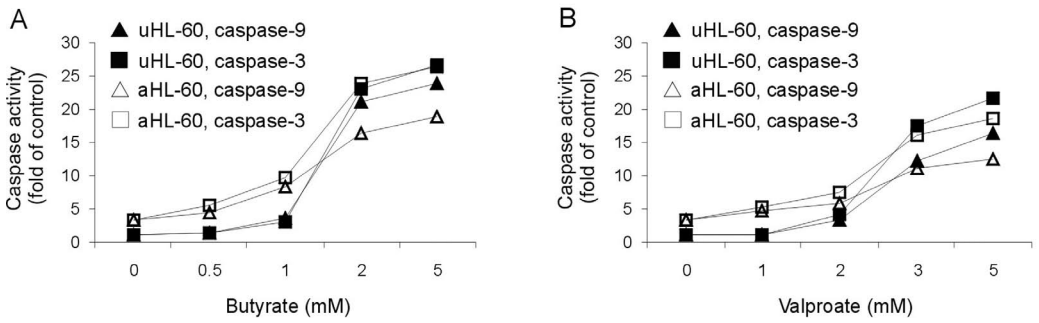

Figure 4. Activation of caspase-9 and -3 by butyrate and valproate in HL-60 cells induced to differentiate by ATRA. Undifferentiated cells (uHL-60) or cells differentiated for 72 h with 1 μM ATRA (aHL-60) were treated for 24 h with (A) butyrate or (B) valproate in the presence of 1 μM ATRA. The levels of caspase-9 and -3 activities versus untreated undifferentiated HL-60 cells were evaluated using specific fluorogenic substrates. Data are means of three experiments.

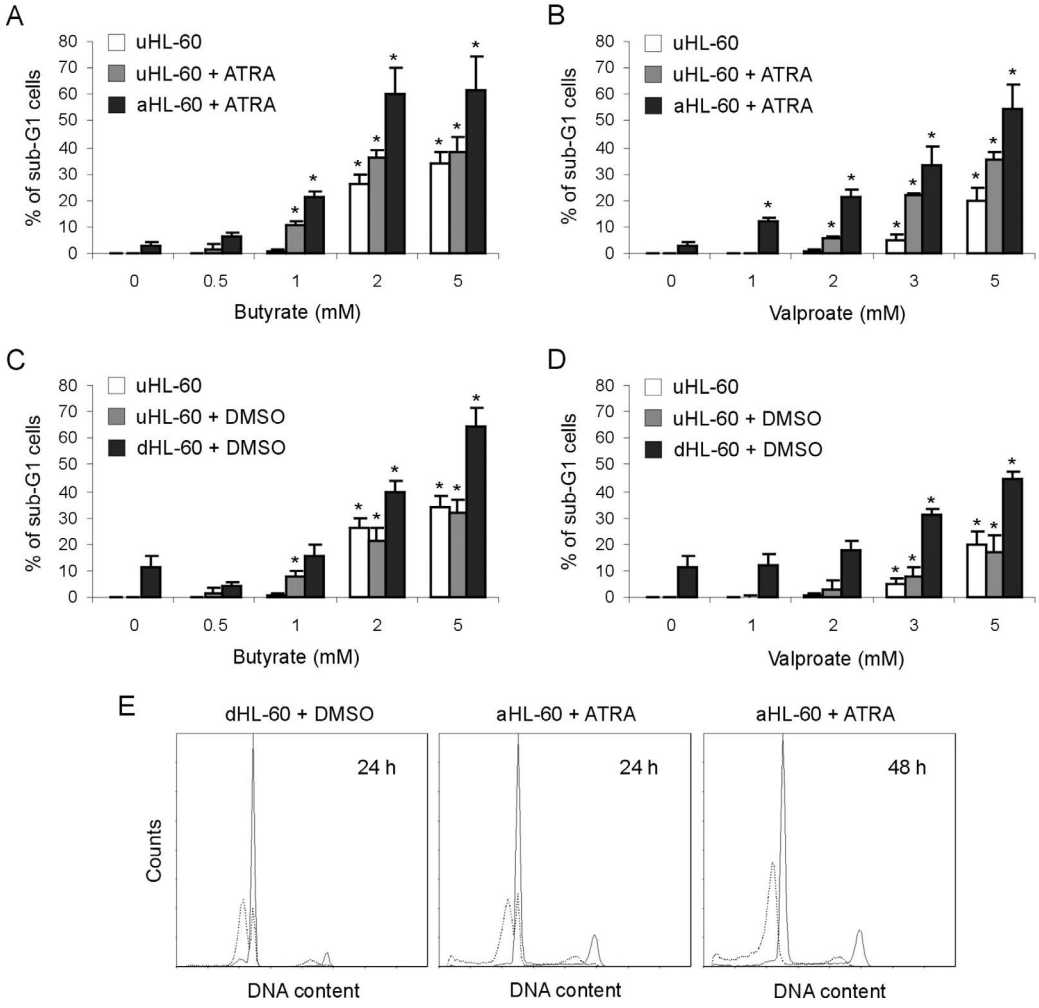

Figure 5. Induction of apoptotic DNA fragmentation by butyrate and valproate in HL-60 cells differentiating by ATRA or DMSO. Undifferentiated cells (uHL-60) or cells differentiated for 72 h with 1 μM ATRA (aHL-60) or 1.25% DMSO (dHL-60) were treated for 24 h with (A, C) butyrate or (B, D) valproate in the absence or presence of respective inducer of differentiation (as indicated). Cells were fixed in ethanol, stained with PI, and the percentage of cells in the apoptotic sub-G1 peak was determined by flow cytometry. Data are means ± SD of three experiments. * $p < 0.05$, significantly increased versus respective control. (E) Representative histograms showing cell cycle profiles of aHL-60 or dHL-60 treated respectively with ATRA or DMSO either alone (solid lines) or in combination with 5 mM butyrate (dotted lines). Similar results were obtained for valproate.

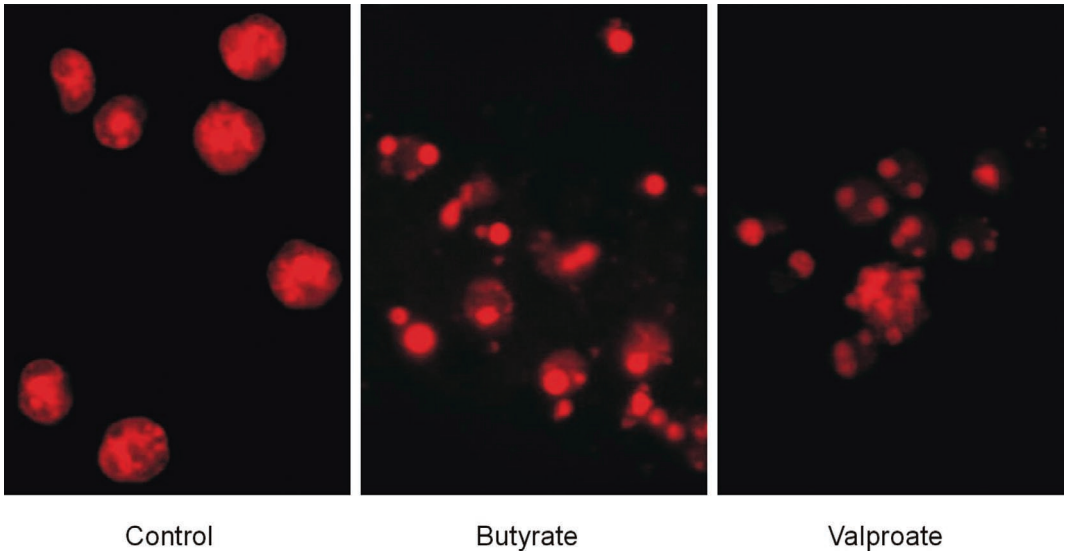

Figure 6. Induction of nuclear condensation and fragmentation by butyrate and valproate in HL-60 cells differentiating by ATRA. HL-60 cells were differentiated for 72 h with 1 μM ATRA and then treated for 48 h with 1 μM ATRA either alone (control) or in combination with 5 mM butyrate or 5 mM valproate. Cells were fixed in ethanol, stained with PI, and examined by fluorescence microscopy (original magnification, ×400). Representative results are shown.

incubation with 1 μM ATRA or 1.25% DMSO with the proportion of G0/G1 cells significantly increased to 41% and 48%, respectively (data not shown). When the undifferentiated cells were treated with HDAC inhibitors in combination with the differentiating agents, we found a synergistic or additive effect on the induction of the G0/G1 cell cycle arrest only in cells cotreated with ATRA and 0.5 or 1 mM butyrate, respectively (data not shown). In addition to the G0/G1 phase arrest, the treatment of undifferentiated HL-60 cells with butyrate or valproate also caused a dose-dependent appearance of cells with sub-G1 DNA while a significant sub-G1 cell population was found at concentrations from 2 mM and 3 mM, respectively (Fig. 5A, B). The formation of sub-G1 cells induced by both butyrate and valproate appeared to be potentiated by ATRA (Fig. 5A, B). In contrast, DMSO synergistically increased only the effect of 1 mM butyrate (Fig. 5C, D).

HL-60 cells induced to differentiate for 72 h with ATRA or DMSO showed 73% and 82% of cells, respectively, arrested in the G0/G1 phase (Fig. 1A). Further 24 h incubation with differentiation inducers resulted in negligible changes in the cell cycle (data not shown) together with an appearance of small populations of sub-G1 cells (Fig. 5A, C). When ATRA- or DMSO-differentiated cells were treated for 24 h with either HDAC inhibitor and respective differentiating agent, we observed a significant dose-dependent induction of sub-G1 cell formation by both butyrate (Fig. 5A, C) and valproate (Fig. 5B, D), with the exception of 0.5 mM butyrate which decreased the effect of DMSO alone (Fig. 5C). In undifferentiated cells, the appearance of sub-G1 cells was accompanied by formation of cell debris with randomly fragmented DNA (data not shown), whereas the dying differentiated cells maintained DNA content corresponding to the sub-G1 peak (Fig. 5E). Thus, the populations of sub-G1 cells found in differentiated cells appeared to be larger than those in undifferentiated cells. Furthermore, in differentiated cells both butyrate and valproate induced an obvious appearance of sub-G2 peak with a parallel dissipation of the G2/M cell population. As shown in ATRA-differentiated cells exposed to ATRA and 5 mM butyrate or 5 mM valproate, almost the whole cell population showed either sub-G1 or sub-G2 DNA after 48 h of incubation (Fig. 5E). The induction of DNA fragmentation by HDAC inhibitors in ATRA-differentiated cells was confirmed by fluorescence microscopy. After 48 h, we observed intact nuclei in cells treated with ATRA alone but in combination with 5 mM butyrate or 5mM valproate the cells exhibited condensed and fragmented nuclei indicative of apoptotic cell death (Fig. 6). In contrast to butyrate and valproate, ATRA-differentiated cells showed lower sensitivity to DNA fragmentation induced by valinomycin. After 12 h exposure to 1μM valinomycin in combination with ATRA or DMSO, sub-G1 DNA was detected respectively in 35% and 34% of undifferentiated cells, and in 14% and 31% of cells pretreated for 72 h with respective differentiating agent (data not shown).

Discussion

HDACs are recognized as molecular targets of ATRA in the differentiation therapy of APL. Combination of ATRA and HDAC inhibitors has been found to synergize in differentiation of t(15;17)-positive APL cell line NB4 [34] and to overcome

the differentiation block in ATRA-resistant t(11;17)-positive leukemic cells [10]. Besides differentiation, the combined treatment with ATRA and HDAC inhibitors may induce cell death as well. Since differentiation of myeloid leukemic cells may result in resistance to various apoptotic stimuli, we examined whether human leukemia HL-60 cells differentiating by ATRA into neutrophil-like phenotype become resistant to the apoptogenic activity of two HDAC inhibitors, butyrate and valproate. Antiproliferative and/or cytotoxic effects of these HDAC inhibitors have been previously demonstrated in several myeloid cell lines [3, 5, 35] including HL-60 cells [3, 6, 7]. Our results confirm the ability of butyrate and valproate to induce the G0/G1 cell cycle arrest and apoptosis in undifferentiated HL-60 cells, with the cell death being associated with the activation of the intrinsic apoptotic pathway. This aside, the apoptotic effect of butyrate and valproate in undifferentiated HL-60 cells is potentiated by ATRA and, at certain concentrations of the HDAC inhibitors, by another neutrophilic differentiation inducer, DMSO. In cells pretreated with ATRA or DMSO, butyrate and valproate in combination with the differentiating agents induce lower dissipation of $\Delta\Psi_m$ and weaker apoptotic and/or necrotic changes in the plasma membrane in comparison with undifferentiated cells. In contrast, DNA fragmentation induced by the same cotreatment is not diminished in differentiated cells. These results indicate that neutrophilic differentiation modulates but does not abrogate the apoptotic response of HL-60 cells to butyrate and valproate, and nuclei are preferentially affected during apoptosis in differentiated cells.

ATRA and DMSO induce neutrophilic differentiation via different mechanisms [36]. In HL-60 cells, the differentiation activity of ATRA is mediated through RARα [37], while the mechanism of DMSO action is not clear [36]. However, both inducers cause the G0/G1 cell cycle arrest [13, 38] and continuous cell treatment with ATRA or DMSO eventually leads to apoptosis. Both ATRA and DMSO are known to alter the expression of various apoptosis-related genes [12, 14, 39]. The main role in mediating the apoptosis associated with the terminal differentiation of HL-60 cells is attributed to downregulation of the anti-apoptotic protein Bcl-2 [39, 40]. Despite the susceptibility to spontaneous apoptosis, differentiation of myeloid leukemic cells along the neutrophilic pathway reduces cell sensitivity to various apoptosis-inducing anticancer drugs with diverse mechanisms of action. For instance, pretreatment with ATRA has been shown to attenuate the apoptotic response of HL-60 cells to DNA intercalator idarubicin, topoisomerase II inhibitor etoposide, or tubulin polymerization inhibitor vinblastine [16]. The molecular mechanisms that modulate the sensitivity of differentiating cells to chemotherapeutics remain unclear. In this study, the inhibition of apoptosis induced by butyrate or valproate in differentiated cells correlated with the reduced dissipation of $\Delta\Psi_m$. Similar results were also found in cells exposed to the potassium ionophore valinomycin. Mitochondria play an important role in triggering the intrinsic apoptotic pathway. The loss of $\Delta\Psi_m$ promotes the release of cytochrome *c* from mitochondria, resulting in activation of the initiator caspase-9 which subsequently activates the effector caspase-3 [41]. Neutrophilic differentiation is associated with changes in the abundance and function of mitochondria. Neutrophils contain few mitochondria which do not produce energy by respiration, but still maintain their $\Delta\Psi_m$ and regulate the apoptotic processes [29, 42]. Neutrophils also have a lowered requirement for cytochrome *c* for the engagement of the intrinsic pathway [28]. We confirmed that neutrophilic differentiation of HL-60 cells is accompanied by decrease in cell stainability with mitochondria-specific dyes [38]. In contrast, we did not observe the previously reported downregulation of cytochrome *c* [42]. Valinomycin is a potassium ionophore which induces apoptosis primarily through the collapse of $\Delta\Psi_m$ [31]. Consistent with this, the reduced loss of $\Delta\Psi_m$ found in ATRA-differentiated cells exposed to valinomycin corresponded with somewhat lower induction of the caspase activities and reduced fragmentation of DNA in comparison with undifferentiated cells. In contrast, ATRA- or DMSO-differentiated cells treated with butyrate or valproate remained susceptible to nuclear apoptosis. Without identifying the molecular mechanisms that underlie the susceptibility of nuclei of differentiated cells to apoptotic degradation, we observed similar induction of the caspase-3 activity by butyrate and valproate regardless of the differentiation status of HL-60 cells. The proteolytic activity of caspase-3 is required for the activation of endonucleases involved in apoptosis [43] and it has been demonstrated that DNA associated with hyperacetylated histones is preferentially digested by apoptotic endonucleases, presumably due to easier accessibility of the DNA [44].

In summary, we have shown that ATRA enhances the apoptotic effect of butyrate and valproate in undifferentiated HL-60 cells. Differentiation of HL-60 cells along the neutrophilic pathway by ATRA modulates the cell response to the HDAC inhibitors, however, the cotreatment with ATRA and butyrate or valproate induces apoptosis in ATRA-differentiated cells. These findings might be taken into consideration in the development of anticancer strategies combining differentiating agents and HDAC inhibitors.

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