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Research Article

HDAC inhibition is associated to valproic acid induction of early megakaryocytic markers

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ABSTRACT

Valproic acid (VPA), a histone deacetylase inhibitor, causes differentiation in different cell lines and in a cell-specific manner; yet, its effect on megakaryocytic (MK) differentiation has not been studied. We evaluated whether VPA induces MK differentiation in a UT-7 cell line through histone acetylation in the GpIIIa gene region and activation of the ERK pathway. UT-7 cells, derived from megakaryoblastic leukemia, were treated with VPA at various concentrations, and the expression of differentiation markers as well as the gene expression profile was assessed. Flow cytometry, immunoblot analysis, and RT-PCR demonstrated that VPA induced the expression of the early MK markers GpIIIa (CD61) and GpIIb/IIIa (CD41) in a dose-dependent manner. The VPA-treated cells showed hyperacetylation of the histones H3 and H4; in particular, histone acetylation was found to have been associated with CD61 expression, in that the GpIIIa promoter showed H4 hyperacetylation, as demonstrated by the chromatin immunoprecipitation assay. Furthermore, activation of the ERK pathway was involved in VPA-mediated CD61/CD41 expression and in cell adhesion, as demonstrated by using the MEK/ERK inhibitor U0126. In conclusion, the capacity of VPA to commit UT-7 cells to MK differentiation is mediated by its inhibitory action on HDAC and the long-lived activation of ERK1/2.

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Introduction

Histone acetylation [1] by histone acetyltransferase (HAT) promotes transcription, facilitating the binding of transcription factors to gene promoters [2]. Histone deacetylases (HDAC) reverse chromatin acetylation and promote transcriptional repression and silencing [2,3], whereas HDAC inhibitors (HDACis) enhance the transcriptional activity of several genes [4,5]. Currently known HDACis [i.e., sodium butyrate (NaB) and trichostatin A (TSA)] cause changes in the cell cycle, differentiation, and apoptosis, and they stimulate adhesion molecules in different hematopoietic cell lines and in a cell-type specific manner [6–8]. Recent data suggest that valproic acid (VPA), a short chain fatty acid with a known anticonvulsant and mood-stabilizing action, also inhibits HDAC [4,5]. VPA suppresses cell growth in several cell lines

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and reduces tumor growth and metastasis formation in vivo. It also induces differentiation in transformed hematopoietic progenitor cells and in blasts from persons with acute myeloid leukemia [4]. In U937 and K562 cells, VPA induces persistent acetylation of the histones H3 and H4 and increases the transcription of endogenous genes, which is strongly correlated with the induction of differentiation in a myeloid- and erythroid-specific manner [9]. No data on megakaryocyte (MK) differentiation are available.

Gene expression induced by VPA has been reported to involve the activation of the extracellular signal-regulated kinase (ERK) pathway [10]. ERK1 and ERK2 are members of the mitogen-activated protein kinase (MAPK) family, which also comprises the stress-activated protein kinase p38 and N-terminal Jun kinases (JNK) [11]. Phosphorylated MAPKs have been implicated as key regulators of specific responses [12–14]. The activation of ERK1/2 is critical for MK differentiation: the inhibition of the MAPK-activating enzymes (MEK)/ERK pathway has been found to alter polyploidization or to delay MK maturation [15–18], whereas p38 activation is involved in erythroid differentiation [19].

The human UT-7 cell line, which derives from megakaryoblastic leukemia [20], can be induced to differentiate along the MK pathway by phorbol myristic acetate (PMA), with the appearance of mature MK properties and the expression of specific proteins such as platelet factor 4 and β-thromboglobulin [20]. The objective of the present study was to determine whether VPA induces MK gene expression in a UT-7 cell line through the inhibition of HDACs and the activation of the ERK pathway.

Materials and methods

Antibodies and reagents

The following antibodies were used: anti-phospho p38 (New England Biolabs, NEB, Beverly, MA), -CD61 (-integrin β3, -GpIIIa), -ERK2, and -p38 (Santa Cruz, CA) polyclonal antibodies; anti-phospho ERK1/2, -α-tubulin (Santa Cruz, CA), and -gelsolin (Sigma, St. Louis, MO) monoclonal antibodies; PE-conjugated CD41 (Immunotech, Marseille, France); and FITC-conjugated CD61 (Becton Dickinson, San José, CA). Antibodies recognizing the histones H3 and H4 and their anti-acetylated forms were provided by Upstate Biotechnology Inc. (Lake Placid, NY). VPA, NaB, and TSA were provided by Sigma, VPD by Lancaster Synthesis (Morecambe, UK), and the MEK/ERK pathway inhibitor U0126 by Promega (Madison, WI).

Cell culture and treatments

The UT-7 cell line [20] was cultured in liquid culture with Iscove's modified Dulbecco's medium (IMDM; GIBCO Laboratories, Grand Island, NY) containing 10% fetal calf serum (FCS; Hyclone) and 2 ng/ml of recombinant human GM-CSF (Pepro Tech EC, London, UK). The cells were seeded at a density of 5×10^4/ml and cultured for 3 days in the presence or absence of VPA (0.1, 0.25, 0.5, and 1.0 mM). Cultures with one of two HDACis (i.e., 6 ng/ml TSA or 1.0 mM NaB) were also performed for 3 days.

Total and nuclear acid extracts

Floating cells were pelleted and suspended in lysis buffer [2% sodium dodecyl sulphate (SDS) containing 2 mM phenyl-sulphonyl-fluoride (PMSF), 10 μg/ml antipain, leupeptin and trypsin inhibitor; 10 mM sodium fluoride; and 1 mM sodium orthovanadate]. Adherent cells were harvested directly in lysis buffer. The whole-cell lysates were sonicated for 30 s.

For nuclear acid extracts, 2×10^6 cells/sample were suspended in 500 μl of ice-cold lysis buffer (10 mM Tris–HCl pH 6.5, 50 mM sodium bisulfite, 1% Triton X-100, 10 mM $MgCl_2$, 8.6% sucrose) containing the protease and the phosphatase inhibitors. The nuclei were obtained after Dounce homogenization, and the acid-soluble histone fraction was extracted according to the procedure of Yoshida et al. [6].

Immunoblot analysis

Proteins of total lysates and nuclear acid extracts were measured [21], and equal amounts were separated by 10% and 15% SDS-PAGE, respectively. The proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell BioScience GmbH, Germany) by electroblotting. The quantity of total protein levels was confirmed by staining the membranes with Ponceau S.

The antibodies for immunoblots were diluted according to the manufacturer's recommendations, and incubation with primary antibodies was carried out overnight at 4°C. After 1.5 h of incubation with peroxidase-conjugated secondary antibody at room temperature, immunoreactive bands were detected by chemiluminescence combined with peroxidase activity (Product no. 34075, Pierce, Rockford, IL, USA). The immunoblots were quantified by densitometric scanning of the film using the Imaging Densitometer GS-700 (Bio-Rad, Hercules, CA, USA).

Flow cytometric analysis

The UT-7 cells were washed twice in PBS supplemented with 0.1% bovine serum albumin (Sigma) and incubated for 30 min with fluorescent dye-conjugated CD41 and CD61 monoclonal antibodies. After washing with PBS, the cells were analyzed.

The analysis of the UT-7 cell cycle was performed after 3 days of VPA treatment, incubating the cells in 10 μM bromodeoxyuridine (BrdU) for 30 min and then using the in situ proliferation kit FLUOS (Roche, Basel, Switzerland). For the bivariate analysis, the BrdU-labeled cells were counterstained with 1 μg/ml of propidium iodide (PI) (Becton Dickinson).

Viable cells were determined by propidium iodide exclusion test.

FACScan flow cytometer and Cell Quest software (Becton Dickinson) were used.

RT-PCR analysis

Total RNA and reverse-transcribed-RNA were prepared as described elsewhere [22]. cDNAs were equalized by RT-PCR using primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Amplification consisted of denaturation at 95°C for 45 s, annealing for 45 s (at the temperatures specified below),

and extension at 72°C for 45 s. The primers, probes, annealing temperatures, and PCR product lengths were as follows:

GpIIIa: forward 5′-ATCGAGTTCCCAGTGAGT-3′, reverse 5′-TACTGGTGAGCTTTCGCA-3′, probe 5′-GATGATTCGAA-GAATTTCTCCATCCAAGTG- 3′ (54°C, 283 bp);
α-globin: forward 5′-GACAAGACCAACGTCAAGGCCGCC-3′, reverse 5′-CAGGAACTTGTCCAGGGAGGC-3′, probe 5′-GC-AAGAAGGTGGCCGACGCGCTGAC-3′ (62°C, 372 bp);
GAPDH: forward 5′-ACATCAAGAAGGTGGTGAAGCAGG-3′, reverse 5′-CTCTTCCTCTTGTGCTCTTGCTGG-3′, probe 5′-TGACTTCAACAGCGACACCCACTCCTCCAC-3′ (56°C, 282 bp).

PCR was performed using Taq-Gold polymerase (Perkin-Elmer, provided by Roche Molecular System Inc., NJ), following the manufacturer's instructions; 10 μl of each sample was separated in a 2% agarose gel (BRL Life Technologies Ltd., Paisley, UK), transferred to a nitrocellulose filter (Schleicher and Schuell) and hybridized with the specific γ^{32}P-ATP-labeled probe. A mock reaction was used for each RT-PCR experiment. Densitometric analysis was performed using Instant Imager (Packard, Canberra, Australia).

Chromatin Immunoprecipitation (ChIP) Assay

Cells were cultured for 24 h without VPA, with 1 mM VPA (the concentration at which the greatest expression of CD61 was observed) or with 6 ng/ml TSA. Formaldehyde was added to the cells to a final concentration of 1%, and the cells were incubated at 37°C for 10 min. Cells (2×10^6) were collected, and ChIP assay was performed with an H4 acetyl-histone immunoprecipitation assay kit (Upstate Biotechnology, Lake Placid, NY), following the manufacturer's instructions.

Immunoprecipitated DNAs were amplified by PCR with primers specific for the GpIIIa and GAPDH promoters. GpIIIa promoter: forward 5′-GCATTGACACTGCTGTTAA-3′, reverse 5′-GCTGAAGCGTCACTAGCAA-3′, probe 5′-AGGGGAGGTA-GATGGAGCTTCAGCCTGA-3′ (53°C, 154 bp). GAPDH promoter: forward 5′-GCTACTAGCGGTTTTACGGG-3′, reverse 5′-CGAA-CAGGAGGAGCAGAGAG-3′, probe 5′-GCCTGCCGCCGCGCC-CCCGG-3′ (59°C, 167 bp). As a control for DNA content, PCR reactions were also performed on chromatin samples prior to immunoprecipitation (input).

Results

Histone acetylation of GpIIIa promoter in UT-7 cells induced by VPA

According to the results of flow cytometry (performed after 3 days of VPA treatment), VPA induced the expression of the early MK-markers GpIIIa (CD61) and GpIIb/IIIa (CD41) (Fig. 1A) in a dose-dependent manner, with the greatest expression observed for the 1.0 mM concentration (Fig. 1B).

The immunoblotting analysis of nuclear extracts from VPA-treated UT-7 cells showed hyperacetylation of H3 and H4. For both histones, acetylation was present at 6 h of treatment, peaked at 24 h, and was retained at 3 days (Fig. 2A). To determine whether or not histone acetylation was associated with CD61 expression, we performed ChIP assay with an antibody specific for acetylated H4. Associated genomic DNA was then analyzed by PCR using primers specific for the promoter region of the GpIIIa gene. The promoter region of GpIIIa was more acetylated in the cells treated with VPA compared to the untreated cells (Fig. 2B).

Regarding the effect of other HDACis on the expression of MK differentiation markers, 1.0 mM NaB increased CD41 and

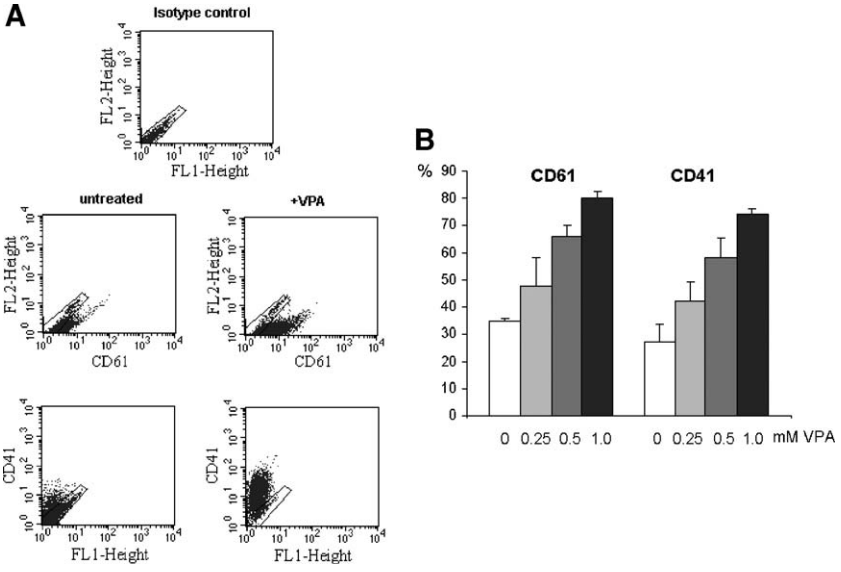

Fig. 1 – Dose-dependent VPA induction of the CD61 and CD41 expression in UT-7 cells. (A) Flow cytometric analysis of CD41 and CD61 expression in UT-7 cells, untreated or treated with VPA (1.0 mM); representative experiment of three independent ones is presented. (B) CD61 and CD41 expression in UT-7 cells after 3 days of VPA treatment (0.25, 0.5, and 1.0 mM) according to the results of flow cytometric analysis; histograms were the mean ± SEM of three independent experiments.

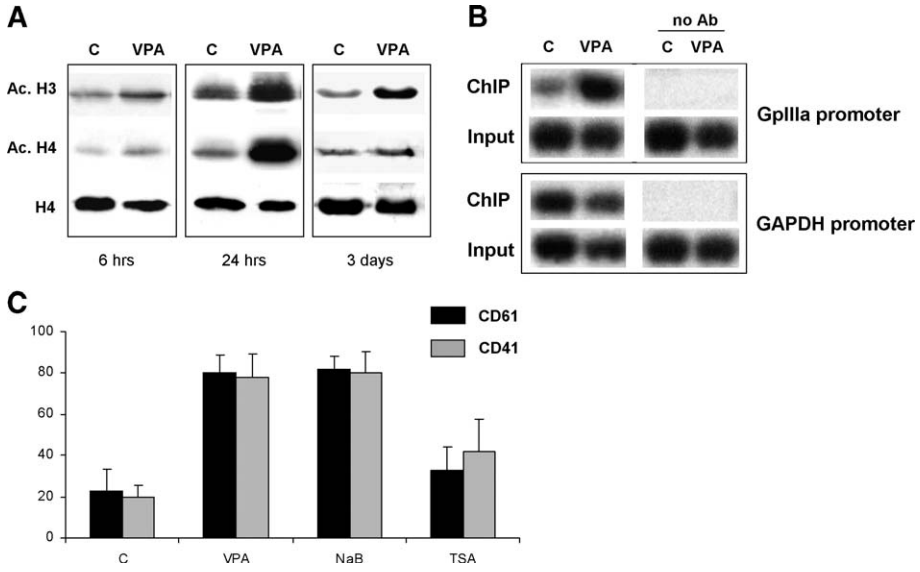

Fig. 2 – Correlation of histone acetylation in the GpIIIa promoter with CD61 and CD41 expression in UT-7 cells treated with VPA. (A) Immunoblot analysis of H3 and H4 for acetylation in untreated cells (control, C) and cells treated with 1 mM VPA, by treatment time. Filters were reprobed with an antibody recognizing total H4 histone. Representative blots are shown; at least three other experiments were performed, all of which demonstrated a similar pattern. (B) Acetylation of the GpIIIa promoter in UT-7 cells treated with VPA, as shown by ChIP assay. Anti-acetylated histone H4 antibody was used to immunoprecipitate soluble chromatins from untreated cells (control, C) and cells treated for 24 h with 1 mM VPA. The immunoprecipitated DNAs (ChIP) as well as chromatin samples before immunoprecipitation (Input) were subjected to PCR using primers for GpIIIa and GAPDH promoters. All PCR products were of the expected size (see Materials and methods). As negative control (no Ab), immunoprecipitation reaction was carried out in the absence of antibody. (C) CD41 and CD61 expression in untreated UT-7 cells (control, C) and UT-7 cells treated with VPA (1 mM), NaB (1 mM), or TSA (6 ng/ml) according to the results of flow cytometric analysis; histograms were the mean ± SEM of three independent experiments.

CD61 expression to the same extent as 1.0 mM VPA (four-fold with respect to the control), whereas 6 ng/ml TSA induced a less marked increase (Fig. 2C). Valpromide VPD (a VPA analogue with no HDACi activity) neither induced acetylation nor showed effect on CD41 or CD61 expression (data not shown).

Furthermore, we tested, in UT-7 cells, the ability of VPA to induce the expression of gelsolin, an actin-binding protein known to be a target of HDACis in human cancer cells. VPA as well as NaB and TSA induced gelsolin expression although at different levels. Notably, 1 mM VPD did not (Fig. 3).

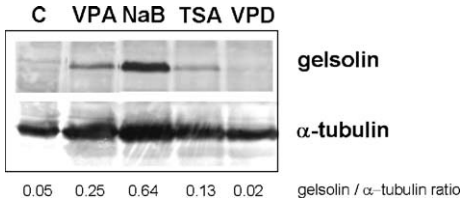

Fig. 3 – Immunoblot analysis of the gelsolin expression in UT-7 cells treated with HDACis. Whole cell lysates from untreated cells (control, C) and cells treated for 3 days with VPA (1.0 mM), NaB (1.0 mM), TSA (6 ng/ml), or VPD (1.0 mM) were analyzed by immunoblot using an anti-gelsolin antibody; filters were reprobed with α-tubulin antibody. Representative experiment of three independent ones is presented.

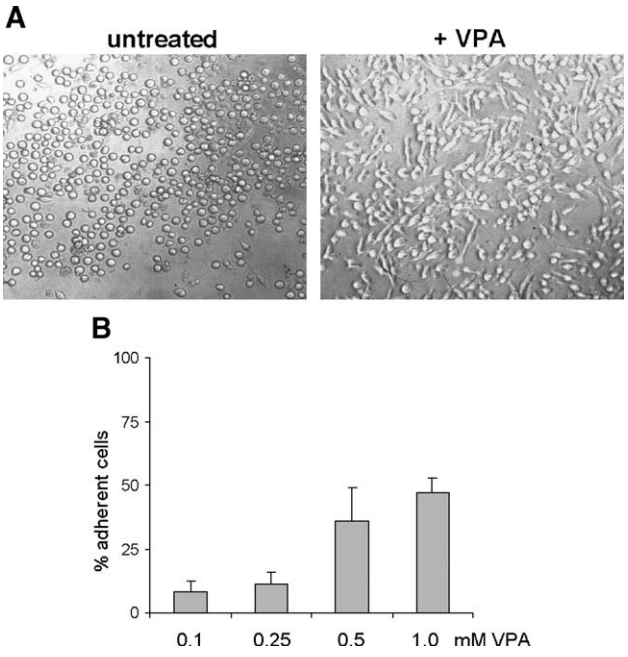

Fig. 4 – Adhesion of UT-7 cells following VPA treatment for 3 days. (A) Phase contrast micrographs of cells, untreated or treated with VPA (0.5 mM); (B) cell adhesion (expressed as the percentage of total treated cells), by VPA concentration. Histograms were the mean ± SEM of three independent experiments.

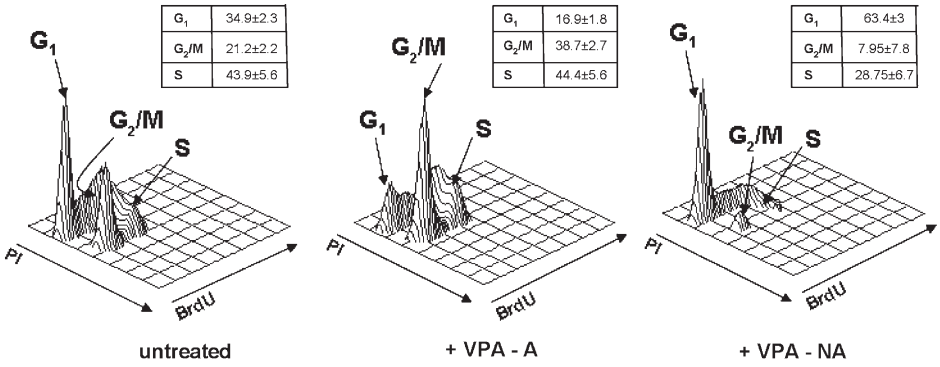

Fig. 5 – FACS analysis to measure the percentage of untreated or VPA-treated UT-7 cells in G_1, S, and G_2/M phase. Cells treated for 3 days with VPA (1.0 mM) were distinct in adherent (+VPA-A) and non-adherent (+VPA-NA) cells. Representative experiment of three independent ones is presented.

VPA effects on cell adhesion and cell cycle

On the third day of VPA treatment, the cells began to adhere to the plate surface and develop an elongated shape (Fig. 4A). Cell adhesion increased in a dose-dependent manner, reaching 47% of the total cells with 1.0 mM VPA (Fig. 4B). Same results were obtained with 1 mM NaB, whereas no cell adhesion was observed in TSA- and VPD-treated cells (not shown).

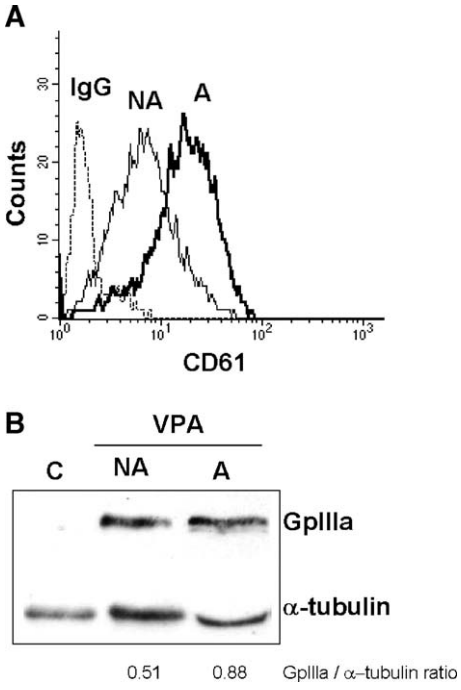

Fig. 6 – CD61 expression in adherent and non-adherent UT-7 cells treated with VPA (1.0 mM for 3 days). (A) Flow cytometric analysis of CD61 expression in adherent (A) and non-adherent (NA) cells; representative experiment of three independent ones is presented. (B) Immunoblot analysis of the GpIIIa expression in UT-7 cells treated with VPA. Whole cell lysates (100 μg) from untreated cells (control, C) and adherent (A) and non-adherent (NA) cells treated with VPA were analyzed by immunoblot using an anti-GpIIIa antibody; filters were reprobed with α-tubulin antibody. Representative experiment of three independent ones is presented.

Proliferation was inhibited at 0.5 and 1.0 mM VPA, with a decrease of about 20–30% in the total number of cells, compared to the untreated cells without significant cell toxicity as evaluated by propidium iodide exclusion; no cell toxicity and inhibition of cell growth were observed after TSA and VPD treatment (data not shown).

In the adherent cells, the cell cycle profile showed cell accumulation in the G_2/M phase (Fig. 5), although the cell cycle was not blocked in this phase, in that all of the cells progressed to the G_1/S phase after treatment with 1 mM hydroxyurea (not shown).

The adherent and non-adherent cells differed in terms of the distribution of MK markers: CD61 and CD41 were expressed in 87.8 ± 4.8% and 85.6 ± 7.5%, respectively, of the adherent cells and in 63.5 ± 11.1% and 59.5 ± 15.5% (mean ± SEM from three separate experiments) of the non-adherent cells. A representative CD61 experiment is shown in Fig. 6A. Both immunoblot (Fig. 6B) and PCR analysis (Fig. 7) showed that GpIIIa expression was higher in adherent cells than in non-adherent cells.

That VPA induced MK gene expression in UT-7 cells was also suggested by the observed down modulation of α globin gene expression, which was similar to the pattern of down modulation obtained in vitro with phorbol ester, an inductor of MK differentiation (Fig. 7). No modulation of α globin gene

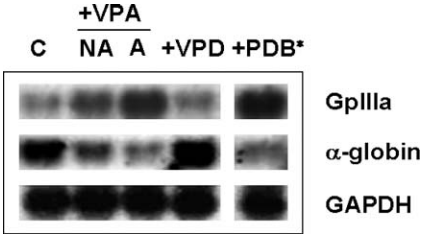

Fig. 7 – RT-PCR analysis of the expression of GpIIIa and α-globin transcripts in UT-7 cells untreated (control, C), treated with VPA (1.0 mM; adherent, A; non-adherent, NA), VPD (1.0 mM), and phorbol 12,13-dibutyrate (PDB). GAPDH gene expression was used as a quantitative control. (*the volume of the PDB sample for GpIIIa was 1/10 that of the PDB sample for GAPDH). A representative experiment is shown. The expression pattern of each gene was confirmed in two other experiments.

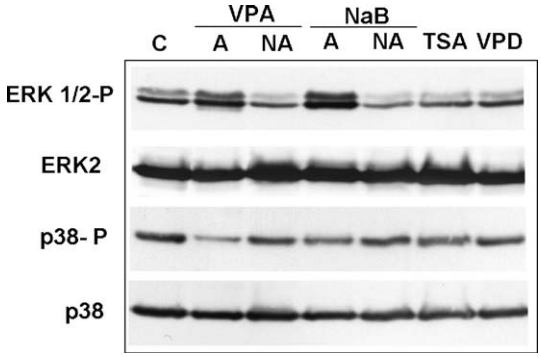

Fig. 8 – Immunoblot analysis of phosphorylated ERK1/2 (ERK1/2-P) and phosphorylated p38 (p38-P) in UT-7 cells, untreated (control, C) or treated for 3 days with VPA (1.0 mM), NaB (1.0 mM), TSA (6 ng/ml), or VPD (1.0 mM). Adherent cells, A; non-adherent cells, NA. The treated cells were harvested, and 80 μg cell lysate was subjected to immunoblot analysis, using specific antibody against phosphorylated ERK1/2; blots were stripped and reprobed with antibody against phosphorylated p38. The total ERK2 and p38 are also shown. Representative experiment of three independent ones is presented.

expression was observed after treatment with 1.0 mM of VPD (Fig. 7).

Enhancement of GpIIIa expression through the activation of the ERK pathway

To investigate whether ERK pathway is also involved in the mechanism by which VPA stimulates early differentiative MK markers, we analyzed the level of phosphorylated-activated forms of ERK1/2. Immunoblot analysis showed that, in the adherent cells, the levels of the phosphorylated forms of ERK1/2 were enhanced after prolonged treatment (3 days) with 1.0 mM VPA. Similar results were obtained using NaB, whereas there was no enhancement with TSA or VPD (Fig. 8). In adherent VPA-treated cells, phosphorylated p38, which is specific to the erythroid differentiation pathway, showed a 70% reduction; while in adherent NaB-treated cells, a 30% decrease was observed (Fig. 8).

In UT-7 cells pretreated with 10 μM U0126 (an inhibitor of the MEK/ERK pathway), the expression of both CD41 (Fig. 9A) and CD61 (not shown) was strongly impaired in cells treated with 1.0 mM VPA. Cell adhesion was also impaired (Fig. 9B). To determine whether ERK signaling affects histone acetylation, we performed immunoblotting analysis on nuclear extracts from VPA treated UT-7 cells, in the presence of 10 μM U0126. H4 acetylation was present at 6 h and 24 h and retained at 3 days (Fig. 9C).

Discussion

MK differentiation in UT-7 cells is known to be induced by phorbol esters [20] and the constitutive expression of the TPO receptor Mpl [23]. The results of the present study show that also VPA induces early MK differentiation in UT-7 cells.

Depending on differentiation stage, VPA increases proliferation and cell renewal in normal hematopoietic stem cells [24,25] or induces differentiation in leukemia blasts as well as in cell line [4,9]. Clinical observations in epileptic patients reported that prolonged VPA treatment can lead to thrombocytopenia with megakaryocyte dysplasia or bone marrow suppression with a decrease number of megakaryocytes [26,27]. A recent in vitro study on hematopoietic

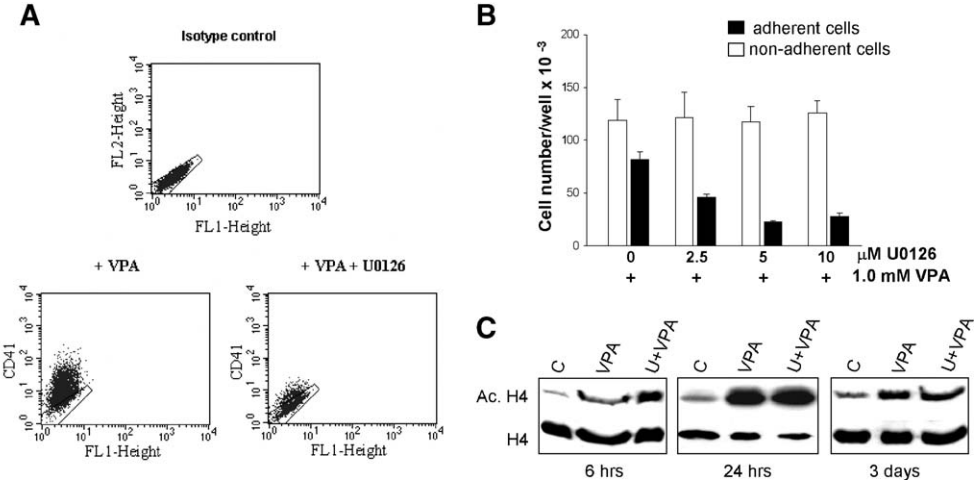

Fig. 9 – Effect of inhibition of the MEK/ERK pathway on CD41 expression, cell adhesion, and acetylation in VPA-treated UT-7 cells. (A) Dot-plot analysis of CD41 expression in cells preincubated or not with U0126 (10 μM) and treated with VPA (1 mM). The experiment was performed three times, with similar results. (B) Number of adherent and non-adherent cells preincubated with different concentrations of U0126 and treated with VPA. Each value represents the mean ± SEM of triplicates. The experiment was performed three times, with similar results. (C) Immunoblot analysis of H4 acetylation in untreated cells (control, C) and cells preincubated (1 h) or not with U0126 (10 μM) and treated with VPA (1 mM), by treatment time. Representative experiment of three independent ones is presented.

$CD34^+$ progenitor cells, amplified for 3 weeks in presence of VPA and cytokines, showed increased percentage of differentiated cells, in particular of the megakaryocytic lineage [25]. Our study is in line with the in vitro results of De Felice and coworkers [25]. UT-7 differentiation induced by VPA was associated with a slight decrease in cell growth, probably due to the slow progress in reaching the G_1/S phase. On the other hand, the increased proportion of cells in G_2/M phase represents an aspect of the induction of MK differentiation program.

VPA-induced differentiation has been attributed to the inhibition of HDACs [4,5,28]. In our study, the up-regulation of MK glycoprotein expression was found to be associated with the hyperacetylation of the nucleosomal histones H3 and H4; in particular, histone acetylation was found to have been associated with CD61 expression, in that the GpIIIa promoter showed H4 hyperacetylation. The other HDACis up-regulated CD41 and CD61 expression, yet to different extents. NaB up-regulated CD41 and CD61 expression to the same extent as VPA, whereas TSA induced a less marked increase, probably because of differences in chemical structure [29]. VPD, an analogue of VPA that does not inhibit HDACs, failed to induce MK differentiation. All of the HDACis induced the expression of gelsolin, an actin binding protein which is found in normal MKs and platelets [30] and whose expression has been related to the reversion of malignant transformation in several cancer cell lines [31–33]. The induction of gelsolin expression in UT-7 cells is a further indication that acetylation is involved in committing this leukemic cell line to an MK differentiation program.

Previous studies have provided evidence that ERK is the pathway involved in MK differentiation [15–18]. In the adherent VPA-treated UT-7 cells, we observed a sustained increase in the phosphorylated levels of ERK1/2, and when we blocked ERK activation with U0126, we did not detect up-regulation of CD61 or CD41 expression or cell adhesion. Furthermore, the specific differentiation program induced by HDACis varies with cell type: for example, in K562 cells VPA and NaB induce erythroid differentiation [9,34]. The simultaneous decrease in the phosphorylation levels of p38 after VPA treatment suggests that the activation of ERK1/2 and p38 are mutually exclusive in UT-7 cells. These data are consistent with those from other studies in which the erythroid differentiation of K562 was associated with the inhibition of the ERK pathway and the activation of p38 [34,35]. Our results demonstrate that ERK pathway activation and HDAC inhibition are associated in VPA induced MK differentiation, although the increase in histone acetylation is independent on ERK signaling. ERK inhibition by U0126 impaired expression of MK glycoproteins demonstrating the involvement of the ERK pathway in the VPA-induced MK differentiation. When using TSA, the ERK pathway was not activated, which may explain why the CD41 expression was slight.

The capacity to induce gene expression may vary among HDACis [8], perhaps reflecting different modes of action [4,36]. VPA has been shown to induce massive apoptosis in APL mice through the concomitant activation of Fas and TRAIL, members of the TNF superfamily [37], whereas TSA, which upregulates Fas expression but not TRAIL, only has a poor effect. That we did not observe ERK phosphorylation with TSA is consistent with these observations.

In conclusion, the capacity of VPA to commit UT-7 cells to MK differentiation is mediated by the long-lived activation of ERK1/2, and it is associated to its inhibitory action on HDAC.

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