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Valproic acid regulates erythro-megakaryocytic differentiation through the modulation of transcription factors and microRNA regulatory micro-networks

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ABSTRACT

Valproic acid (VPA) exhibits important pharmacological properties but has been reported to trigger side effects, notably on the hematological system. We previously reported that VPA affects hematopoietic homeostasis by inhibiting erythroid differentiation and promoting myeloid and megakaryocyte differentiation. Here, we analyzed the effect of VPA on regulatory factors involved in erythro-megakaryocytic differentiation pathways, including transcription factors and microRNAs (miRs). We demonstrate that VPA inhibited erythroid differentiation in erythropoietin (Epo)-stimulated TF1 leukemia cells and $CD34^+$/hematopoietic stem cells (HSCs) and in aclacinomycin-(Acla)-treated K562 cells. Mir-144/451 gene expression was decreased in all erythroid and megakaryocyte models in correlation with GATA-1 inhibition. In Epo-stimulated $CD34^+$/HSCs, VPA induced the expression of the ETS family transcription factors PU.1, ETS-1, GABP-α, Fli-1 and GATA-2, which are all known to be negative regulators of erythropoiesis, while it promoted the megakaryocytic pathway. PU.1 and ETS-1 expression were induced in correlation with miR-155 inhibition; however, the GATA-1/PU.1 interaction was promoted. Using megakaryoblastic Meg-01 cells, we demonstrated that VPA induced megakaryocyte morphological features and CD61 expression. GATA-2 and miR-27a expression were increased in correlation with a decrease in RUNX1 mRNA expression, suggesting megakaryocyte differentiation. Finally, by using valpromide and the Class I HDACi MS-275, we validated that the well-described HDACi activity of VPA is not required in the inhibitory effect on erythropoiesis. Overall, this report shows that VPA modulates the erythro-megakaryocytic differentiation program through regulatory micro-networks involving GATA and ETS transcription factors and miRNAs, notably the GATA-1/miR-144/451 axis.

1. Introduction

Valproic acid (2-propylvaleric acid, 2-propylpentanoic acid or *n*-dipropylacetic acid; VPA) is a short-chain fatty acid that has been used as an antiepileptic and a mood-stabilizing drug for many years due to its ability to modulate γ-aminobutyric acid (GABA)-dependent neurotransmission and to modify voltage-gated ion channels [1,2]. More recently, VPA has been reported to preferentially inhibit Class I histone deacetylases [3]. Published results have demonstrated the capacity of VPA to modulate the regulation of apoptosis, inflammation, proliferation and differentiation by affecting signaling pathways and regulatory factors in various cancer models [4]. Extended use of VPA in neuropsychiatry has allowed for the collection of clinical data related to hematological disorders in correlation with direct toxicity and decreased production of neutrophilic and

Abbreviations: Acla, aclacinomycin; Epo, erythropoietin; GPA, glycophorin-A; HSC, hematopoietic stem cell; miR, microRNA; VPA, valproic acid.

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erythrocytic marrow [4,5]. In epileptic patients treated with VPA over several years, aplastic anemia, pure red cell aplasia, coagulopathies and increased fetal hemoglobin synthesis have been described [6–10]. By studying the effect of VPA on hematopoietic differentiation pathways in leukemia, we showed that VPA modifies hematopoietic homeostasis by inducing myelo-monocytic features while preventing erythroid differentiation [11].

Erythropoiesis requires the interaction of erythropoietin (Epo) with its membrane receptor (EpoR), which triggers signaling cascades that lead to the regulation of hematopoietic transcription factor activation and gene expression [12]. Activity of the major erythroid transcription factor GATA-1 is regulated through post-translational modifications and interactions with co-factors [13,14]. Importantly, the GATA-1/PU.1 complex prevents GATA-1 binding to its consensus sequence (A/T)GATA(A/G) in target genes [15]. During erythropoiesis, GATA-1 and GATA-2 expression levels balance each other [16], which is required for the development of pluripotent hematopoietic progenitors. GATA-2 prevents the terminal differentiation of erythroid cells [17] and has been shown to increase megakaryopoiesis in the absence of GATA-1 [18].

In addition to transcription factors, microRNAs (miRs) also regulate erythroid gene expression. Mirs are regulatory non-coding RNAs that block translation or destabilize mRNAs by pairing with 3′-untranslated regions. As transcription factors regulate miR gene transcription, and miRs regulate transcription factor expression, this crosstalk results in a complex regulatory network [19]. Mirs are known to be involved in hematopoiesis [20,21], and the following miRs regulate erythropoiesis: miR-221/222, miR-144/451, miR-15a, miR-16-1, miR-126 and miR-210 [22]. The gene cluster encoding miR-144/451 is regulated by GATA-1, as reported in zebrafish and murine G1E cells. Mature miR-144 and miR-451 are specifically expressed in erythroid cells [21,23], and increased miR-451 expression is associated with a significant decrease in AKT and Bcl-2 expression [24,25]. Moreover, c-myc expression is repressed by miR-451 in correlation with predicted targeting of c-myc mRNA by miR-451 [26,27]. Moreover, PU.1 and ETS-1 have been described as targets of miR-155 [28,29], while RUNX1 regulates transcription of the miR-221/222 and miR-27a genes [30,31]. As a negative regulatory loop, miR-27a targets RUNX1 mRNA during megakaryocytic differentiation [30], and the miR-27a gene is regulated by a GATA-1/GATA-2 switch in erythropoiesis [32]. Interestingly, the GATA and ETS family of transcription factors (e.g., GATA-1, GATA-2, ETS-1, PU.1, Fli-1 and GABPα) are involved in an erythro-megakaryocytic differentiation program by activating one pathway and repressing the other [18,29,33–36].

The involvement of specific miRs and transcription factors in VPA-mediated perturbation of hematopoiesis was investigated in this study. The requirement of HDAC inhibitory (HDACi) activity of VPA was evaluated by using valpromide (VPM), a VPA analog lacking HDACi activity, and the specific Class I HDAC inhibitor MS-275. The results showed that VPA down-regulated GATA-1/miR-144/451 in correlation with inhibition of erythropoiesis in Epo-stimulated TF1 and hematopoietic stem cells (HSCs). In HSCs, VPA stabilized the GATA-1/PU.1 complex and induced overexpression of transcription factors that negatively regulate erythropoiesis, while promoting megakaryopoiesis via GATA-2 and ETS transcription factors. Modulation of transcription factors correlated with changes in related miRs including miR-144/451, miR-27a, and miR-155, leading to the conclusion that VPA modulates an erythro-megakaryocytic transcription factor/miR regulatory micro-network. Moreover, our results reveal that HDACi activity of VPA is not required for its inhibitory activity on erythropoiesis.

2. Materials and methods

2.1. Cell culture and treatments

The human leukemia cell lines TF1, K562, and MEG-01 (DSMZ, Braunschweig, Germany) were cultured in RPMI 1640 (BioWhittaker®, Lonza, Verviers, Belgium) supplemented with 10% heat-inactivated fetal calf serum (BioWhittaker®) and 1% antibiotic-antimycotic (BioWhittaker®). TF1 cells were grown in the presence of 5 ng/mL human recombinant GM-CSF (Tebu-Bio, PeproTech, Boechout, Belgium) or 10 U/mL human recombinant erythropoietin (Epo; Epoetin Beta, NeoRecormon, Roche Pharma AG, Grenzach-Whylen, Germany) in the culture medium to induce erythroid differentiation. All cells were cultured in an incubator at 37 °C, 95% humidity, and 5% CO_2. Medium was renewed every 3 days, and the cells were resuspended at a concentration of 2×10^5 cells/mL. K562 cells were induced to differentiate with 10 nM aclacinomycin A (Acla; Sigma–Aldrich Bornem, Belgium). Meg-01 cells were induced toward the megakaryocytic pathway with 100 nM of phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma–Aldrich).

Human umbilical cord blood was obtained from the Clinique Bohler, Luxembourg, with a written informed consent for use of hematopoietic stem cells (HSCs) in research, in agreement with the National Committee of Research Ethics in Luxembourg. $CD34^+$/HSCs were selected using magnetic cell sorting (MACS Miltenyi, Utrecht, The Netherlands) and cultured as previously described [37]. After 3 days of culture, the medium was renewed, and erythroid differentiation was induced by adding 2 U/mL human recombinant Epo to the medium for 4 days. All cells were treated with 1 mM VPA, 1 mM VPM (Sigma), 0.1 μM MS-275 (Enzo Life Sciences BVBA, Antwerpen, Belgium) or suberoylanilide hydroxamic acid SAHA (Cayman Chemical Company, Bio-connect, Huissen, The Netherlands). VPM is a VPA-related compound devoid of HDACi activity and MS-275 (MS) is a Class I HDAC-specific inhibitor of erythroid differentiation. VPA was used at 1 mM, in accordance with previous investigations [11] and other references related to VPA studies on hematopoietic cells differentiation [3,38,39]. VPM was used at the same concentration as VPA. MS-275 was used at 0.1 μM, which allows for HDACi activity without cytotoxic effects in our cell models. SAHA was used at 1 μM, in accordance with the literature [40] for TF1 cells.

2.2. Cell proliferation, viability, morphology, and erythroid differentiation

Cell viability was estimated by performing Trypan blue exclusion tests. Cells were processed through a semi-automated image-based cell analyzer to quantify living cells among the total population (Cedex XS Innovatis, Roche, Luxembourg). The percentage of cell viability was calculated by comparing the number of total cells with the number of dead cells. The fold increase in cell proliferation was determined in control cultures and treated cultures by calculating the ratios Nc/N0 = Rc and Nt/N0 = Rt, where Nc and Nt are the number of viable cells at the different time points in control cultures and in treated cultures, respectively. N0 is the number of viable cells in initial cultures at 0 h, and the fold change in cell proliferation at 0 h (N0/N0) is R0 = 1. When $Rt < R0$, treatment was considered cytotoxic; $R0 < Rt < Rc$ treatment was considered cytostatic, and Rt = Rc indicates that the treatment had no effect on cell proliferation.

For morphological observations, cytospin preparations of cells were stained with May-Grünwald/Giemsa (MGG) solution (Merck, Leuven, Belgium). Determination of erythroid differentiation was scored by the benzidine staining method. Images were captured using a Leica DM 2000 microscope.