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Valproic acid triggers differentiation and apoptosis in AML1/ETO-positive leukemic cells specifically

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ABSTRACT

Valproic acid (VPA) has extensive effects on leukemic blasts through its inhibition of histone deacetylases. The main goal of this study was to identify the subgroup of patients who may benefit most from VPA treatment. We examined the significance of *t*(8;21) chromosomal aberration for VPA treatment response among acute myeloid leukemia (AML) patients by direct comparison of AML1/ETO-negative vs. positive leukemic cell-lines as well as bone marrow blasts from AML patients. In *t*(8;21) AML, leukemogenesis is supposed to be induced via aberrant recruitment of histone deacetylases. AML cell lines of different genotypes (Kasumi-1, Kasumi-6, MV4;11, K562) and diagnostic bone marrow samples from patients were treated with VPA. VPA induced apoptosis in AML1/ETO-positive and MLL–AF4-positive cells in a dose-dependent manner. Differentiation, as indicated by changes in immunophenotype, was observed only in AML1/ETO-positive cells. VPA increased the expression of AML1 target genes – PU.1, C/EBPa, BPI and IGFBP7 only in AML1/ETO-positive cells. This AML1/ETO-specific effect was confirmed also using patient blasts isolated at the time of diagnosis. AML1/ETO-positive leukemia shows specific mechanism of VPA residing from differentiation followed by apoptosis that is accompanied by an increase in the expression of repressed AML1 target genes. Our data suggest that AML1/ETO-positive patients might derive the greatest benefit from VPA treatment.

1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous group of myeloid malignancies with distinct features and prognosis. The most common cytogenetic abnormality in AML patients is translocation *t*(8;21), which causes AML1/ETO fusion. This fusion gene is detected in 13% of pediatric AML patients and is characteristically present in subgroup M2 according to the French American British (FAB) classification [1,2]. The wild-type version of the AML1 (RUNX1) transcription factor plays a critical role in the differentiation of myeloid progenitor cells [3], and deletion of AML1 is lethal *in utero* in mice [4]. In AML1/ETO-positive leukemia, the DNA binding domain of AML1 is fused to ETO (MTG8), forming the chimeric protein AML1/ETO [5], which recruits a repressor complex consisting of N-CoR, mSin3 and class I histone deacetylases (HDAC) [6–8]. This aberrant recruitment interferes with the expression of AML1 target genes [9] and causes a block in myeloid differentiation [10]. Direct promoter binding and gene regulation by AML1 has been shown for only a handful of genes, including p14ARF, IL-3, GCSF1R [11–13] and the upstream regulatory element of PU.1 [14]. Several candidate AML1 target genes (including BPI and IGFBP7) for which AML1/ETO binds the promoter region have also been described [15].

Valproic acid (VPA) was found to have extensive effects on AML blasts via inhibition of the enzymatic activity of class I HDAC [16,17], which is accompanied by increased levels of acetylated histones H3 and H4 *in vitro* and *in vivo* [18,19]. VPA treatment also induces proteasomal degradation of HDAC2 [17]. After it was shown to be a potent apoptotic agent in AML-derived cell lines [20,21], VPA was tested in phases I and II clinical trials, mostly in myelodysplastic syndrome (MDS) and AML in elderly patients. Though the initial results were promising, with 30% of MDS patients responding to VPA as a monotherapy or in combination with ATRA [22], the study with AML patients showed a response in only 5% of patients (16% is considered to be effective according to the criteria of the International Working Group for MDS) [23]. VPA was used in combination with ATRA in a concomitant or sequential schedule in several trials with refractory or high risk AML patients, generating several observations: in one study, 2 out of 8 patients achieved hematological improvement [24]; in two others, 3 (including 1 complete response (CR)) of 11 patients [25] and 3 of 19 patients responded [26]. These results indicate a selective effect of VPA and point to the importance of identifying

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subgroups of AML patients who would derive the most benefit from treatment with VPA or other HDAC inhibitors.

We therefore studied the effect of VPA in various AML-derived cell lines and AML patient samples taken at the time of diagnosis. To identify the genotypic AML subgroup(s) that are sensitive to VPA treatment, we examined apoptosis and differentiation in various cell lines and AML1/ETO-positive and AML1/ETO-negative AML patients. We found a close relationship between differentiation and apoptosis in AML1/ETO-positive cells, in which differentiation is immediately followed by cellular death and accompanied by a significant increase in the expression of AML1 target genes.

2. Design and methods

2.1. Cell lines and patients' samples

The human myeloid leukemia cell lines Kasumi-1, MV4;11, K562 (DSMZ – German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and Kasumi-6 (ATCC – American Type Culture Collection, Manassas, VA, USA) were maintained in RPMI1640 (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Carlsbad, TX, USA) and 1% penicillin–streptomycin under standard conditions (37 °C and 5% carbon dioxide). Cells were treated with VPA (Sigma) at the previously used concentrations of 0.5 mM and 1.0 mM [27,28] and TPA (12-0-tetra-tetradecanoylphorbol-13-acetate; Sigma) at a concentration of 1.62 nM for 24 or 48 h. To inhibit apoptosis in Kasumi-1 cells, 20 μM or 100 μM benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD-fmk; Sigma) was added to the culture 1 h before the addition of VPA.

Four primary bone marrow samples taken from AML patients at the time of diagnosis (M2 stage according to the FAB classification; two AML1/ETO-negatives, patient number 1: 46XX, patient number 2: 46XY, two AML1/ETO-positives, patient number 3: 46XX; *t*(8;21), patient number 4: 46XY; *t*(8;21)) were collected and cryopreserved under protocols approved by the ethics committee of the institute. Informed consent was obtained from the children's parents or guardians, in accordance with the Declaration of Helsinki. The bone marrow samples were thawed and maintained in Iscoves modified Dulbecco's medium (IMDM) supplemented with 30 ng per μl of the cytokines trombopoietin (TPO), interleukin-6 (IL-6), stem cell factor (SCF) and fms-related tyrosine kinase three ligand (FLT3-L) and 30% FBS (Sigma) with or without 1 mM valproic acid (Sigma) at 37 °C in an atmosphere containing 5% carbon dioxide.

2.2. Cell cycle analysis

A CycleTEST™ PLUS DNA Reagent Kit (Becton Dickinson Immunocytometry Systems, CA, USA) was used for analysis of nuclear DNA prepared from cell suspensions according to the manufacturer's instructions. Propidium iodide bound to DNA was visualized by measurement of orange fluorescence using an LSR II instrument (BD, USA). The cell cycle distribution was analyzed using FlowJo 8.1.1 (Treestar, Ashland, OR, USA) and Modfit (Verity House, Topsham, ME, USA) software.

2.3. Detection of differentiation by flow cytometry

An LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) was used to assess the expression of surface differentiation markers by using antibodies against CD11a, CD11b, CD15, CD38, CD33, CD34, CD117, CD14, CD13, HLA-DR, CD 45 and glycophorin A (GPA) (Immunotech, Marseille, France; BD). Harvested cells (cell lines and bone marrow samples) were resuspended in 50 μl PBS, stained with the indicated combinations of antibodies, incubated for 30 min and washed. To identify nonviable cells, 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI; Sigma) was added just prior to measurement. Samples were analyzed in six-color combination tubes in the LSRII flow cytometer (BD Biosciences). Analysis was performed using FlowJo 8.1.1 software (Treestar).

2.4. Flow cytometry analysis using Vybrant DyeCycle Violet, annexin V and surface markers

Vybrant DyeCycle Violet (DCV; Invitrogen, Carlsbad, CA, USA), APC annexin V (BD Bioscience), CD33-FITC, CD117-PC7 and CD11B-PE were used to visualize changes in immunophenotype across the cell cycle. Briefly, cells cultured at a concentration of 1×10^5/ml in 24-well plates were labeled with 1 μl DCV for 30 min at 37 °C. Subsequently, cells were resuspended in annexin V binding buffer (BD Bioscience), stained with annexin V and antibodies to CD33, CD117 and CD11B and maintained at 4 °C until flow cytometry analysis LSRII cytometer (BD Biosciences).

2.5. Quantification of gene expression by real-time quantitative reverse transcriptase PCR

Total RNA was isolated from treated and untreated cells using a Qiagen RNeasy Mini Kit with the addition of DNase Reaction Mix (Qiagen, Hilden, Germany). RNA concentration and quality were determined by analyzing isolated RNA in an Agilent 2100 Bioanalyzer (Agilent Technologies, Boblingen, Germany) using an RNA 6000 Nano LabChip Kit (Agilent Technologies). cDNA was synthesized from total RNA using an iScript cDNA Synthesis Kit containing oligo(dT) and purified MMLV RNase H+ reverse transcriptase (Bio-Rad Laboratories, UK). Real-time quantitative PCR (RQ-PCR) was performed using the SYBRGreen system to quantify the mRNA levels of the genes of interest and of the reference genes β2-microglobulin and GAPDH. RQ-PCR was performed in a LightCycler 480 (Roche Diagnostics, Mannheim, Germany) using a PowerSYBRGreen PCR Reagent Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. To normalize genes expression, value of investigated gene expression was divided by expression of housekeeping gene.

2.6. Nuclear and nucleolar morphology

The nuclear chromatin structure was visualized in methanol-fixed cytospins by staining DNA with acidified methylene blue after hydrolysis with HCl according to the method of Smetana et al. [29]. Nucleoli were visualized in cytospins with acidified methylene blue according to the same method without methanol fixation [30].

2.7. Statistical analysis

The Mann–Whitney and Student's *t* tests were performed for statistical analyses using StatView software (SAS Institute, Cary, NC, USA).

3. Results

3.1. Valproic acid induces apoptosis in acute myeloid leukemia cell lines without regard to their genotypic background

Previous studies have reported that VPA is capable of inducing apoptosis in several cell lines derived from myeloid malignancies [21]. In our experiment, changes in the cell cycle upon treatment with 0.5 mM and 1.0 mM VPA were studied in four cell lines derived from myeloid malignancies: Kasumi-1 (FAB M2; AML1/ETO-positive), Kasumi-6 (FAB M2; AML1/ETO-negative), MV4;11 (FAB M5; MLL/AF4-positive) and K562 (chronic myeloid leukemia in blast crisis with BCR/ABL fusion). Different effects of VPA in different cell lines were observed. The most profound effect on apoptosis observed was in Kasumi-1 AML1/ETO-positive cells, in which 1 mM VPA induced apoptosis in 56% ± 3.2% of cells after 48 h of treatment. In MV4;11 cells, 1 mM VPA induced apoptosis in 43% ± 1% of cells. In K562 cells, only a minor induction of apoptosis (6% ± 1.1%) was observed, and in Kasumi-6 cells, 20.8% ± 0.4% of cells underwent apoptosis compared to control untreated cells, in which the amount of cells in subG1 reached 16.6% ± 0.3% due to high debris formation in the control cells during cultivation. Other changes in the cell cycle were observed after VPA treatment. Decreased proliferation was detected in three of the four cell lines under examination. VPA (1 mM) decreased the percentage of proliferating Kasumi-1 cells: the amount of cells in G2/M/S phase decreased from 27% ± 2.3% to 12% ± 1.8% after 48 h of treatment. In MV4;11 and Kasumi-6 cells, VPA treatment decreased the proportion of proliferating cells from 22.3% ± 2.1% to 10% ± 0.9% and from 23.8% ± 0.5% to 17.7% ± 0.6%, respectively. Kasumi-1 cells accumulated in G1/G0 upon treatment with 0.5 mM VPA (percentage of cells in G1/G0: 63% ± 2.6% in VPA-treated compared to 58.5% ± 3.1% in control) (Fig. 1). These data clearly demonstrate that VPA is a potent inducer of apoptosis in myeloid cells *in vitro* and has its most profound effects in AML1/ETO-positive cells.