Valproic Acid Stimulates Proliferation and Self-renewal of Hematopoietic Stem Cells

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Abstract
Histone deacetylase inhibitors have attracted considerable attention because of their ability to overcome the differentiation block in leukemic blasts, an effect achieved either alone or in combination with differentiating agents, such as all-trans retinoic acid. We have previously reported favorable effects of the potent histone deacetylase inhibitor valproic acid in combination with all-trans retinoic acid in patients with advanced acute myeloid leukemia leading to blast cell reduction and improvement of hemoglobin. These effects were accompanied by hypergranulocytosis most likely due to an enhancement of nonleukemic myelopoiesis and the suppression of malignant hematopoiesis rather than enforced differentiation of the leukemic cells. These data prompted us to investigate the effect of valproic acid on normal hematopoietic stem cells (HSC). Here we show that valproic acid increases both proliferation and self-renewal of HSC. It accelerates cell cycle progression of HSC accompanied by a down-regulation of p21cip-1/waf-1. Furthermore, valproic acid inhibits GSK3β by phosphorylation on Ser9 accompanied by an activation of the Wnt signaling pathway as well as by an up-regulation of FoxB1, a target gene of Wnt signaling. Both are known to directly stimulate the proliferation or apoptosis in leukemic blasts, stimulates the proliferation of normal HSC, an effect with a potential effect on its future role in the treatment of acute myeloid leukemia. (Cancer Res 2005; 65(7): 2537-41)

Introduction
Acute myeloid leukemia (AML) is characterized by a differentiation block to the accumulation of immature blasts in the bone marrow. The vast majority of AML-patients are over 60 years old and experience a median survival below 1 year even if treated with intensive chemotherapy (1). Therefore, there is the necessity to develop alternative treatment strategies, such as a differentiation-inducing therapy employing histone deacetylase inhibitors (HDI). HDI have been shown to promote differentiation either alone or in combination with differentiating agents such as all-trans retinoic acid (t-RA; refs. 2, 3). Valproic acid (4), a potent HDI which has been safely used for over two decades in the therapy of epilepsy and bipolar disorders, is now under clinical evaluation (5). In a current clinical study we are evaluating a valproic acid/t-RA combination therapy on patients suffering from advanced myeloid malignancies (6). Patients responding to this therapy frequently developed constant or increased bone marrow cellularity despite a remarkable blast cell reduction and peripheral hypergranulocytosis (>100,000 cells/L; ref. 6). In one patient, it was possible to distinguish malignant from normal hematopoiesis by the presence of the isochromosome (17)(q10) in the leukemic blasts. The analysis revealed that whereas the CD34+ progenitor cells contained residual i(17)(q10), all granulocytes had a normal karyotype, suggesting dominance of normal hematopoiesis over the malignant clone.

Based on the hypothesis that this clinical picture might be related to a still unknown effect of valproic acid, we here investigated its activity on hematopoietic stem cells (HSC) with respect to differentiation, proliferation as well as to self-renewal.

Materials and Methods
Enrichment of human and murine hematopoietic stem cells. Bone marrow was obtained from healthy donors and umbilical cord blood was collected with informed consent of the donors or mothers, respectively. Isolation of mononuclear cells, CD34+ cell selection, and isolation of Sca1+/lin-HSC from C57BL/6J (Ly5.1) female mice or congenic C57BL/6.SJL-Ly5.1 mice (Charles River, Sulzfeld, Germany) were done as previously described (7, 8).

Culture and colony assay of CD34+ hematopoietic stem cells. CD34+ cells were cultured and colony formation of CD34+ HSC was assessed as previously described (7). Cells were harvested after 7 days, counted, and assayed for CD34 and CD14 expression as previously described (7).

Competitive repopulation assay. After 2 days of culture, all cells that grew from 1,000 Ly5.2 Sca1+/lin− cells were injected into lethally irradiated (10 Gy) female Ly5.1 recipients 8 to 12 weeks of age. Transplanted mice were euthanized 12 weeks later. Spleens were either fixed in Bouin’s fixative for 5 minutes, then transferred to 10% neutral buffered formalin (Sigma, Steinheim, Germany; ref. 9) or cells were prepared for surface marker analysis as described (8).

Competitive repopulation assay. After 2 days of culture, all cells that grew in culture from 1,000 Ly5.2 Sca1+/lin− cells under each culture condition were injected into lethally irradiated Ly5.1 female recipients 8 to 12 weeks together with 1 × 106 normal Ly5.1 bone marrow cells (9). Transplanted mice were euthanized 12 weeks later and mononuclear bone marrow cells were isolated and stained with conjugated monoclonal antibodies specific for Ly5.2 and Ly5.1 or mouse IgG2a (all from PharMingen, San Diego, CA) for 30 minutes at 4°C for fluorescence-activated cell sorting analysis.

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Cell cycle analysis. Cell cycle analysis was done as recently described (8).

Cell culture and chemicals. KG-1 cells were maintained in RPMI 1640 (Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS (Invitrogen). Valproic acid was provided by Sigma.

Western blotting. Western blotting was done according to widely used protocols using the following antibodies: anti-p21 cip-1/waf-1, anti-GSK3β, anti–Ser9-phospho GSK3β, anti-Akt, anti–phospho Akt, anti–β-catenin, anti–phospho β-catenin (all from Santa Cruz Biotech, Santa Cruz, CA).

Figure 1. Proliferation and differentiation of HSC on exposure to valproic acid. A, expression of CD34 and CD14 in bone marrow CD34+ on increasing concentrations of valproic acid (VPA; 0, 30, 75, and 150 μg/mL). B, percentage of CD34+ cells in colonies of bone marrow CD34+ plated in semisolid medium and cultured in the presence of FCS and G-CSF for 10 days on increasing concentrations of valproic acid (0, 30, 75, and 150 μg/mL). C, total number of the umbilical cord blood CD34+ cells plated in semisolid medium and cultured in the presence of FCS and G-CSF for 10 days on increasing concentrations of valproic acid (0, 30, 75, and 150 μg/mL).

Figure 2. Proliferation and self-renewal potential of Sca+/lin− HSC exposed to valproic acid or t-RA for 2 days in vitro. A, replating efficiency of murine Sca+/lin− HSC on exposure to valproic acid (150 μg/mL). Reported are numbers of platings (I, II) and CFU. B, differentiation of Sca+/lin− HSC on exposure to valproic acid (150 μg/mL) cultured in semisolid medium for 10 days; c-Kit as well as Sca1 were used as stem cell markers and Gr1 and Mac1 as myeloid differentiation markers. Columns, average of three independent experiments; bars, SD. C, CFU-S assay on Sca+/lin− HSC exposed to valproic acid or t-RA for 2 days in vitro. nt-controls, not transplanted recipients; control, untreated Sca+/lin− HSC; t-RA, t-RA–treated Sca+/lin− HSC; valproic acid, valproic acid–treated Sca+/lin− HSC. Given is one of two experiments which yielded similar results. D, analysis of surface marker expression in the CFU-S Sca+/lin− HSC exposed to valproic acid or t-RA for 2 days in vitro. Sca1 and c-Kit: stem cell markers; Mac1 and Gr1: myeloid differentiation markers. Columns, mean (6 mice/group); bars, SD. Given is one experiment of two which yielded similar results.
Valproic Acid Stimulates Proliferation of HSC

Results

Valproic acid increases the proliferation of human CD34+ hematopoietic stem cells. To determine the effects of valproic acid on HSC, their proliferation and differentiation potential was analyzed in human CD34+ HSC in the presence of increasing doses of valproic acid (30-150 μg/mL). Valproic acid prevented differentiation of bone marrow CD34+ cells in a dose-dependent manner as revealed by the increase of CD34+ cells and the concomitant decrease of monocytic CD14+ cells in a 7-day liquid culture (Fig. 1A).

Even on higher differentiation pressure in a semisolid medium supplemented with FCS and granulocyte colony-stimulating factor (G-CSF), 150 μg/mL of valproic acid increased the percentage of CD34+ cells from 0.5% to more than 21% (Fig. 1B).

In umbilical cord blood CD34+ cells harvested after 10 days of culture in semisolid medium, valproic acid augmented the number of CD34+ cells in a dose-dependent manner to more than 1 log with respect to untreated cells (Fig. 1C).

These data clearly indicate that valproic acid does not induce differentiation, but proliferation in HSC.

Treatment with valproic acid increases the reploting efficiency as well as the self-renewal potential of murine hematopoietic stem cells. To compare the effect of valproic acid on HSC with the known expanding effect of t-RA on murine HSC (10), we extended our investigations to murine Sca1+/lin- HSC. Thus, we compared the effect of valproic acid (150 μg/mL) on the reploting efficiency of Sca1+/lin- HSC in semisolid medium with that of t-RA (1 μmol/L). Valproic acid not only enhanced the number of colony-forming units (CFU; Fig. 2A) but, in contrast to the controls and t-RA, valproic acid also increased another plating with a constant number of CFU (Fig. 2A). Upon exposure to valproic acid, it was possible to replate Sca1+/lin- HSC up to four times (data not shown). The valproic acid–treated cells did not differentiate as shown by the high levels of the stem cell markers Sca1 and c-Kit (Fig. 2B).

To investigate whether the effect of valproic acid on HSC is related to an increase of their potential for self-renewal, we inoculated HSC treated for 2 days with t-RA or with valproic acid into lethally irradiated recipient mice. At day 12 we analyzed the spleen colony-forming units (CFU-S). Valproic acid–treated HSC gave origin to a higher number of CFU-S as compared with control and t-RA–treated HSC as revealed by the differences in the spleen size (Fig. 2C). Exposure to valproic acid led to a higher percentage of c-Kit− and Sca1-expressing cells in the CFU-S as compared with controls (Fig. 2D). No difference was seen regarding CD3ε, B220, or TER119 expression between the different conditions (data not shown), indicating that the treatment did not influence the multipotency of the cells (11).

To confirm the effect of valproic acid on the long-term HSC, a competitive repopulation assay on six mice per group was done and analyzed at 12 weeks after the transplantation. As compared with the untreated controls (8%), exposure to valproic acid increased the long-term potential of HSC (18%) even to a lower extent than t-RA (23%; Fig. 2E).

In summary, these data suggest that the exposure to valproic acid not only induces proliferation of murine HSC but also increases their self-renewal potential.

Valproic acid accelerates cell cycle progression of hematopoietic stem cells and down-regulates p21cip-1/waf-1. Next we investigated the effect of valproic acid on the cell cycle progression of the HSC. The cell cycle analysis was assessed using Sca1+/lin− HSC cultured for 7 days in semisolid medium in the presence or absence of 150 μg/mL valproic acid. In contrast to t-RA, valproic acid increased the percentage of the cells in S phase (23% and 38%, respectively) as compared with untreated control cells (20%) with a concomitant reduction of cells in G1 phase (Fig. 3A).

Next we examined the effect of valproic acid on the expression of the CDK inhibitor p21cip-1/waf-1 in Sca1+/lin− HSC cultured in the semisolid medium for 7 days as well as in bone marrow CD34+ cells cultured for 2 days by Western blotting. In contrast to t-RA, valproic acid reduced the expression level of p21cip-1/waf-1, an effect still notable at day 7 in murine HSC (Fig. 2B) and at day 2 in CD34+ cells (Fig. 2C).

Taken together, these data indicate that valproic acid accelerates cell cycle progression of HSC.

Valproic acid activates GSΚ3β-dependent signaling pathways and up-regulates HoxB4 in hematopoietic stem cells. GSΚ3β becomes inhibited by phosphorylation on Ser9 on exposure to valproic acid (10), we extended our investigations to murine Sca1+/lin− HSC on exposure to valproic acid (150 μg/mL). Valproic acid prevented the expression of p21cip-1/waf-1, an effect still notable at day 7 in murine HSC (Fig. 3B) and at day 2 in CD34+ cells (Fig. 3C).

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Figure 3. Cell cycle progression of Sca1+/lin− HSC on exposure to valproic acid (150 μg/mL) and t-RA (1 μmol/L). A, propidium iodide staining and BrdUrd incorporation of Sca1+/lin− HSC on exposure to valproic acid (150 μg/mL) and t-RA (1 μmol/L). The percentages of cells in G1, S, and G2-M are given in the respective gates. Representative experiment of three yielding similar results. B, regulation of p21cip-1/waf-1 expression in Sca1+/lin− HSC on exposure to valproic acid (150 μg/mL) and t-RA (1 μmol/L). Assessment of -tubulin was used as loading control. C, regulation of p21cip-1/waf-1 expression in CD34+ HSC on exposure to valproic acid (150 μg/mL). β-tubulin staining serves as loading control.
to valproic acid (12). To disclose the mechanisms by which valproic acid induces proliferation of HSC, we studied the effects of valproic acid on GSK3β in HSC as well as in KG-1 cells cultured under conditions under which about 80% of the cell population were CD34+/CD38− (data not shown).

Valproic acid induced a strong modification of GSK3β in bone marrow CD34+ cells at 48 hours (Fig. 4A) as well as in the murine Sca1+/lin− at day 7 of exposure (Fig. 4B). In fact, GSK3β was Ser9-phosphorylated in HSC on exposure to valproic acid but not to t-RA (Fig. 4B), indicating an inhibition of GSK3β. This effect was also seen in KG-1 cells (Fig. 4C).

GSK3β is known to be Ser9-phosphorylated by activated Akt (13). In CD34+ Akt was activated after 48 hours, whereas in Sca1+/lin− no activated Akt was seen at day 7 of exposure, most likely due to the late time point of analysis as shown in KG-1 cells in which activated Akt returned to control levels after 96 hours (Fig. 4F).

Ser9-phosphorylated GSK3β stabilizes β-catenin. Given the fact that in both CD34+ and Sca1+/lin− endogenous β-catenin was hardly detectable using a variety of antibodies (Fig. 4E and data not shown), we confirmed the up-regulation of β-catenin on exposure to valproic acid in KG-1 cells, which are >80% CD34+ / CD38− (Fig. 4F).

**Discussion**

Valproic acid is one of the most promising HDI, raising the hope that a successful differentiation therapy of AML might be feasible. Here we show that valproic acid has unexpected effects on HSC requesting reconsideration of its role in the treatment of AML.

In contrast to recent reports which show that valproic acid induces differentiation in leukemic blasts (3), valproic acid blocks the activation of Akt and on the expression level of β-catenin in Sca1+/lin− HSC at day 7 of culture in semisolid medium; β-tubulin: loading control.

**Figure 4.** Regulation of GSK3β-dependent pathways. A. influence of valproic acid (150 μg/mL) on the expression as well as the Ser9 phosphorylation of GSK3β in CD34+ at 48 h; β-tubulin: loading control. B. influence of valproic acid (150 μg/mL) on the expression as well as the Ser9 phosphorylation of GSK3β in Sca1+/lin− HSC at day 7 of culture in semisolid medium; β-tubulin: loading control. C. influence of valproic acid (150 μg/mL) on the expression as well as the Ser9 phosphorylation of GSK3β in CD34+/CD38− KG-1 cells on exposure to valproic acid (150 μg/mL) at 48 h; Coomassie: loading control. D. influence of valproic acid (150 μg/mL) on the activation of Akt (direct proportion between p-Akt and Akt) in CD34+ at 48 hours; β-tubulin: loading control. E. influence of valproic acid (150 μg/mL) on the activation of Akt and on the expression level of β-catenin in Sca1+/lin− HSC at day 7 of culture in semisolid medium; β-tubulin: loading control. F. influence of valproic acid (150 μg/mL) on the activation of Akt and on the expression level of β-catenin in CD34+/CD38− KG-1 cells on exposure to valproic acid (150 μg/mL) at 48 hours; β-tubulin: loading control. G. induction of HoxB4 by valproic acid (150 μg/mL) in CD34+ HSC in comparison with untreated cells (Control) monitored by real-time PCR; number of copies defined by the comparison to a plasmid-based standard curve. GAPDH expression was done as reference. The fold change between untreated controls and valproic acid-treated cells is represented graphically. Representative experiment of three yielding similar results. H. induction of HoxB4 by valproic acid (150 μg/mL) in Sca1+/lin− HSC by Western blotting; β-tubulin: loading control.
differentiation of normal HSC as revealed by the fact that it increases the fraction of HSC rather than differentiated cells. Furthermore, valproic acid increased the replating efficiency of murine HSC. An asymmetrical induction of apoptosis in the HSC population by valproic acid was excluded by the evidence that valproic acid accelerates the cycle progression of HSC accompanied by a down-regulation of p21cip-1/waf-1. This is in contrast to the effect of valproic acid and other HDI, such as Laq824, which increase p21cip-1/waf-1 expression in cell line models of acute leukemia followed by differentiation or apoptosis (15, 16). Thus, valproic acid has differential effects on stem cells as compared with blast cells. Laq824 and CG1521, two potent hydroxamic acid–derived HDI, which induce differentiation and/or apoptosis in leukemia cell lines, share the properties of valproic acid on HSC (ref. 17 and data not shown). There is a relationship between the differentiation level and the response to HDI, meaning that very immature cells respond to HDI with a down-regulation of p21cip-1/waf-1 and cell cycle progression, whereas at a more advanced differentiation stage, cells respond to valproic acid and Laq824 with a down-regulation of p21cip-1/waf-1 and differentiation or apoptosis (15, 16).

One can hypothesize that valproic acid increases self-renewal of HSC by (a) a “transcriptional reprogramming” of these cells through its capacity to induce histone acetylation as well as DNA demethylation in a dose-dependent manner (18), or (b) by the inhibition of GSK3β. The Ser9 phosphorylation of GSK3β by GSK3β reduces its kinase activity on β-catenin (13) enabling it to transcriptionally activate Wnt target genes (19). The activation of the Wnt signaling pathway on valproic acid is confirmed also by the fact that HoxB4, a target gene of the Wnt signaling pathway in HSC (14), is up-regulated on exposure to valproic acid. Both HoxB4 and Wnt signaling are crucial for the self-renewal potential of HSC (14). In fact, similarly to t-RA (9), valproic acid increases self-renewal of HSC as shown in the CFU-S as well as in the competitive repopulation assays.

The increase in the proliferation and self-renewal potential of HSC by valproic acid may have important therapeutic consequences. The valproic acid–induced entry of quiescent hematopoietic and leukemic stem cells into the cell cycle could render them more susceptible to conventional chemotherapy, resulting either in a prolonged aplasia due to a higher efficiency of the therapy or in a shortened aplasia owing to the enforced proliferation of the normal hematopoiesis. Our recent clinical data indicate that the exposure to valproic acid increases the response to chemotherapeutic agents (6).

The here presented data suggest to redirect the role of HDI from a desired differentiation inducer in a coadjuvant factor for increasing the response to conventional therapy.

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