Histone Deacetylase Inhibitor Valproic Acid Enhances the Cytokine-Induced Expansion of Human Hematopoietic Stem Cells

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Abstract

Ex vivo amplification of human hematopoietic stem cells (HSC) without loss of their self-renewing potential represents an important target for transplantation, gene and cellular therapies. Valproic acid is a safe and widely used neurologic agent that acts as a potent inhibitor of histone deacetylase activities. Here, we show that valproic acid addition to liquid cultures of human CD34+ cells isolated from cord blood, mobilized peripheral blood, and bone marrow strongly enhances the ex vivo expansion potential of different cytokine cocktails as shown by morphologic, cytochemical, immunophenotypical, clonogenic, and gene expression analyses. Notably, valproic acid highly preserves the CD34 positivity after 1 week (range, 40-89%) or 3 weeks (range, 21-52%) amplification cultures with two (Flt3L + thrombopoietin) or four cytokines (Flt3L + thrombopoietin + stem cell factor + interleukin 3). Moreover, valproic acid treatment increases histone H4 acetylation levels at specific regulatory sites on HOXB4, a transcription factor gene with a key role in the regulation of HSC self-renewal and AC133, a recognized marker gene for stem cell populations. Overall, our results relate the changes induced by valproic acid on chromatin accessibility with the enhancement of the cytokine effect on the maintenance and expansion of a primitive hematopoietic stem cell population. These findings underscore the potential of novel epigenetic approaches to modify HSC fate in vitro. (Cancer Res 2005; 65(4): 1505-13)

Introduction

Hematopoietic stem cell (HSC) ex vivo expansion is an attractive strategy to increase the number of self-renewing HSCs finalized to transplantation, gene, and stem cell therapies.

Following the widespread use of the umbilical cord blood (CB) as HSC source alternative to bone marrow (BM) in allogeneic transplantation, the cell dose infused has been markedly evidenced as pivotal item on the engraftment rate and clinical outcome (1–4). The administration of cytokines in the mobilization and ex vivo culture of CB cells, including acute myeloid leukemias, has been documented in vivo and in vitro (28–34). However, nothing is known yet about the effect of valproic acid on proliferation, differentiation, and survival of normal human HSCs.

Materials and Methods

Cell Sources and Selection of CD34+ Cells

Human BM and mobilized peripheral blood (MPB) samples were obtained, after informed consent from allogeneic transplant donors. The cord blood units not eligible for the Italian CB Bank, were processed as fresh samples or cryopreserved. Cryopreserved CB units were thawed...
according to Buhbine et al. (35). Low-density mononuclear cells were separated with Ficoll-Hypaque density gradient centrifugation and incubated overnight to remove the monocytes by adherence on plastic flasks. CD34+ cells were positively selected by immunomagnetic separation using the mini or midi MACS CD34 progenitor cell isolation kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions.

**Ex vivo Expansion of CD34+ Cells**

CD34+ cells (2 × 10^6 cell/mL) were cultured in Iscove's modified Dulbecco's medium (Life Technologies, Gaithersburg, MD) supplemented with 10% human pooled AB serum. Recombinant human (rhu) Flt3L (50 ng/mL; R&D Systems, Abingdon, United Kingdom), rhu-thrombopoietin (50 ng/mL), rhu-SF (100 ng/mL), and rhu-IL-3 (20 ng/mL; PeproTech, London, United Kingdom) were used in the following combination: Flt3L + thrombopoietin (FT) or Flt3L + thrombopoietin + SCF + IL-3 (FTS3). Valproic acid (1 mmol/L, Sigma-Aldrich, Milan, Italy) was added 30 minutes before the addition of cytokines. The cultures were incubated at 37°C and 5% CO2 for 7 days. In long-term expansion, the cultures were fed every week with an equal volume of fresh medium supplemented with valproic acid and cytokines. On days 7, 14, and 21 the nucleated cell number, viability, clonogenic potential, immunophenotype, and gene expression of amplified cells were determined. Cell expansion was expressed as fold increase, calculated by dividing the output absolute number of cells and progenitors after amplification cultures by the respective input cell number on day 0.

**Clonogenic Assay**

Cells were suspended in 1 mL of methylcellulose medium supplemented with SCF, GM-CSF, IL-3, and EPO (Methocult GFH4434, Stem Cell Technologies, Vancouver, British Columbia, Canada), and plated in duplicate in 35-mm culture dishes. Each plate was scored for granulocyte macrophage colony-forming unit, burst forming unit-erythro, and granulocyte, erythro, macrophage, megacaryocyte colony-forming unit growth after 10 and 14 days incubation.

**Replating Tests.** The ability of primary granulocyte macrophage colony-forming unit colony-forming unit, burst forming unit-erythroid, and granulocyte, erythro, macrophage, megacaryocyte colony-forming unit to give rise to secondary colonies was evaluated by picking up individual colonies from the Petri dishes. Each colony was dispersed to single cell suspension in 200 μL of methylcellulose medium (Methocult) and seeded into separate wells. The percentage of positive wells and the number of secondary colonies derived from each primary colony was established after 10 and 14 days incubation.

**Flow Cytometry**

Cell immunophenotype was assessed by two-color fluorescence analysis using FITC-conjugated mouse anti-human CD34, CD38, CD90, CD45, CD15 (Becton Dickenson, Mountain View, CA), and phycoerythrin-conjugated mouse anti-human CD34, CD38, CD13 (BD, CD13/Alex (AC-133)-phycoerythrin (Miltenyi Biotec), CD117-phycoerythrin (Beckman Coulter, Fullerton, CA). Isotype controls were mouse immunoglobulin G conjugated to FITC or phycoerythrin. A minimum of 50,000 events were collected for each sample by a FACScan flow cytometer (Becton Dickenson) using CellFit software (Becton Dickenson) for data acquisition and analysis. For cell cycle analysis and apoptosis, cells were resuspended in 50% FCS, fixed in 70% ethanol for 24 hours, incubated with 50 μg/mL propidium iodide (Sigma-Aldrich) and 50 units/mL DNase-free RNase A (Sigma-Aldrich) and analyzed after 3 hours (10,000 events) using a Epics XL Cytometer (Beckman Coulter).

**Cell Differentiation Assays by Morphologic and Cytochemical Analysis.**

Myelograms were evaluated on Wright-Giemsa stained cytospins by light microscopy by counting at least 200 cells per experimental condition. Cells were classified as immature, granulocytes and monocytes based on chromatin and cytoplasm characteristics. Cytospins were evaluated for positive staining of (i) MPO, an enzyme restricted to the primary granules of granulocytes and monocytes; (ii) a naphthyl acetate esterase positive in cells of the monocytic lineage and virtually absent in granulocytes; (iii) a naphthyl acetate esterase inhibition by sodium fluoride, occurring in monocytes, as per manufacturer's instructions (Sigma-Aldrich).

Immunofluorescence and Immunoblot analyses were done using antibodies recognizing histone H3 or the acetylated forms of histone H3 and H4 (Upstate) or HOXB4 (Santa Cruz Biotechnology, Santa Cruz, CA) as described (33).

**Quantitative reverse transcription-PCR**

RNA was extracted at days 0, 7, 14, and 21 as described (33). Quantitative reverse transcription-PCR was done in ABI PRISM 7000 (Applied Biosystems, Foster City, CA) using Taqman oligonucleotides (Assay on Demand, Applied Biosystems) for gGlyceraldehyde phosphate dehydrogenase (GAPDH), CD34 antigen (CD34), c-kit receptor (CD117), AC133 (CD133), kinase domain receptor (KDR), GATA binding protein 1 (GATA1), colony-stimulating factor 2 receptor (GM-CSFr), and colony-stimulating factor 3 receptor (G-CSFr), glycoprotein B (GP1b). Delta-delta Ct values were normalized with those obtained from the amplification of GAPDH. For the SYBR green dye detection method, primers were designed using the Primer Express software (Applied Biosystems): GAPDH (forward) 5'-TACGAACATCTCCCTGTCGAC-3', GAPDH (reverse) 5'-TGCGATGCGACTGTGTCATG-3', monocyte-specific esterase 1 (MSE, forward) 5'-GAACCACAGATGCTGGAGC-3', and MSE (reverse) 5'-TCCCCGTGTCCTCCTC-3'. Reactions were done in triplicates.

**Chromatin Immunoprecipitation.** Cells (2 × 10^6) were treated with valproic acid and/or FT3 alone or in combination for 2 hours. Cross-linking of histones to DNA was obtained by the addition to the medium of formaldehyde at 1% final concentration at 37°C to cross-link the histones to DNA. Chromatin was sonicated and immunoprecipitated overnight with an anti-acetylated histone H4 antibody (Upstate, Lake Placid, NY) as recommended by the manufacturer. DNA was analyzed by PCR using primers for HOXB4 P1 252-bp promoter region encompassing both HoxE1 and E-box regions (36, 37). HOXB4-P1 (forward, -208 to -184 bp) 5'-GAAAATACCTCCCTTGGTCGAGTG-3'; HOXB4-P1 (reverse, -21 to +44 bp) 5'-GGTGTCCCCATATGATGGTGT-3'; HOXB4-P5 upstream 236-bp promoter region used as control: HOXB4-P5 (forward, -748 to -726 bp) 5'-GGCAAGCTCCCCGAAAT-TAGTG-3'; HOXB4-P5 (reverse, -512 to -488 bp) 5'-GTCCTCTATTGG-GAGTGTGGTACT-3', AC133-1A, 191-bp promoter 1/exon 1A region used by CD34+ progenitors (38): AC133-EX1A (forward) 5'-CTACAGGAATG-

**Table 1. Immunophenotypic features and proliferative potential of HSC from different cell sources**

<table>
<thead>
<tr>
<th>Source</th>
<th>CB</th>
<th>MPB</th>
<th>BM</th>
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<tbody>
<tr>
<td>CD34+ (%)</td>
<td>73.4 ± 9.0</td>
<td>90.4 ± 3.7</td>
<td>95.9 ± 1.4</td>
</tr>
<tr>
<td>CD34+90+ (%)</td>
<td>28.7 ± 4.4</td>
<td>44.4 ± 6.2</td>
<td>13.3 ± 2.5</td>
</tr>
<tr>
<td>CD34+28~ (%)</td>
<td>17.4 ± 2.9</td>
<td>16.1 ± 8.1</td>
<td>5.7 ± 1.5</td>
</tr>
<tr>
<td>GM-CFU (n. colonies per 500 CD34+)</td>
<td>224.6 ± 98.7</td>
<td>63.9 ± 13.4</td>
<td>12.8 ± 0.7</td>
</tr>
<tr>
<td>BFU-E (n. colonies per 500 CD34+)</td>
<td>37.7 ± 9.7</td>
<td>86.7 ± 22.6</td>
<td>13.7 ± 5.2</td>
</tr>
<tr>
<td>CFU-GEMM (n. colonies per 500 CD34+)</td>
<td>31.5 ± 6.2</td>
<td>12.3 ± 3.5</td>
<td>3.0 ± 2.7</td>
</tr>
<tr>
<td>Total CFU (n. colonies per 500 CD34+)</td>
<td>292.5 ± 112.4</td>
<td>163.0 ± 38.8</td>
<td>29.4 ± 3.2</td>
</tr>
</tbody>
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NOTE: Mean ± SE.

Abbreviation: BFU-E, burst forming unit-erythroid; GM-CFU, granulocyte macrophage colony-forming unit; CFU-GEMM, granulocyte, erythroid, macrophage, megacaryocyte colony-forming unit.

*P = 0.030.

1P = 0.041.

2P = 0.034.
GATGCTGTCCAG-3; AC133-EX1A (reverse) 5'-GGATTCCCTAACATATA-CACCGC-3; AC133-EXE, alternative 251-bp region on exon E used as control: AC133-EXE (forward) 5'-GCTCTGATGCTCTGAGAC-3'; AC133-EXE (reverse) 5'-CTCTATGGCTCTCGAATCGTCTG-3'; GAPDH to detect nonrelevant cellular DNA sequences: GAPDH (forward) 5'-ACAGTCCATGCCCACCTGCCC-3'; GAPDH (reverse) 5'-GCCCGCTTAC-CACCTCTCTCTG-3'.

Statistics
Descriptive statistics are reported as mean ± SE unless otherwise stated. Student's t test was used to test the probability of significant differences between samples. Differences were considered significant if P ≤ 0.05.

Results
Effect of Valproic Acid on Short-term Amplification Induced by Four Cytokines.
The effect of valproic acid on cytokine-induced HSCs amplification was assessed in 7-day cultures stimulated by the combination of four growth factors: Flt3L, thrombopoietin, SCF, and IL-3 (FTS3). The baseline characteristics of the CD34+ cells selected from CB (n = 3), MPB (n = 4), and BM (n = 2) are shown in Table 1. CB and MPB were more enriched in the early stem cell fraction than BM as documented by the higher percentage of CD34-CD90+ and CD34+CD38– cells. The proliferative potential of committed progenitors was higher in CB than in the other cell sources with a frequency of one clonogenic cell out of 1.7 CB CD34+ cells, one of three MB CD34+ cells, and 1 of 17 BM CD34+ cells.

The acetylation status of histone H3 or H4 was evaluated in CD34+ cells by immunofluorescence and immunoblotting analysis using specific antibodies. Low levels of the acetylated form of histone H3 and H4 were detectable in untreated CD34+ cells from all the cell sources. Sixteen hours treatment with either FT or FTS3 induced a moderate increase of the acetylated forms of both these histones that was further enhanced in the presence of valproic acid (Fig. 1A and 1B).

Because HDAC inhibitors have been reported to induce either differentiation or apoptosis in cancer cells and in leukemic progenitors (28–33), the effect of FTS3 + valproic acid (FTS3 + VPA) on differentiation and apoptosis of HSC was analyzed. In sharp contrast with the results obtained in tumor cells, the morphology of normal HSC treated for 7 days with FTS3 + VPA seemed significantly more immature than after treatment with FTS3 alone. Indeed, morphologic features of myeloid differentiation (chromatin condensation with nuclear segmentation, decreased nucleus/cytoplasm ratio, decreased cytosolic basophilia, and appearance of specific granules) were detectable in cytokine-treated HSC but not in FTS3 + VPA–treated cells (Fig. 1C).

Fluorescence-activated cell sorting analysis of propidium iodide–stained cells revealed a consistent increase of cells in G0-G1 phase of the cell cycle after treatment with FTS3 + VPA, whereas in FTS3-treated cultures a higher percentage of cells was in S-G2M phase. Valproic acid in combination with FTS3 also reduced of 1.5- to 6-fold the frequency of apoptotic cells induced by 7 days of FTS3 treatment (median, 7.1; range, 0-22.8%) in all the HSC sources (data not shown).

Accordingly, immunophenotypic analysis of 7-day FTS3 amplification cultures showed that the percentage of total CD34+ cells was significantly decreased whereas in cultures treated with FTS3 + VPA the CD34+ cells were highly preserved either for CB (76.6% versus 11.2%, P < 0.001), MPB (88.9% versus 42.2%, P = 0.003), or BM (40% versus 19.6%, P = 0.007; Fig. 2A). More impressive was the effect of valproic acid on the early stem cell subsets CD34+CD90+.
and CD34+CD38−. The percentage of CD34+CD90+ detected in cultures with FTS3 + VPA or FTS3 alone were respectively 37.2% versus 0.26% (P < 0.001) for CB, 65.3% versus 1.9% (P < 0.001) for MPB and 25.6% versus 1.3% (P = 0.015) for BM. Similar results were obtained for the CD34+CD38− cell fraction with higher values in FTS3 + VPA than in FTS3 cultures (Fig. 2A and B). Interestingly, in all the three HSC sources the percentages of the early cell fraction CD34+CD90+ after 7 days of amplification with FTS3 + VPA were higher than at the start of cultures.

Cell expansion was evaluated in terms of fold increase by dividing the total number of nucleated cells recovered at day 7 by the initial cell number. As shown in Fig. 3, a lower cell proliferation was detected in FTS3 + VPA cultures than in cultures treated with cytokines alone (with an increase of nucleated cells of 25.1- versus 35% of FTS3-treated cells, and in 4% to 8% of cells treated with FTS3 + VPA cultures.

Several parameters were evaluated to assess the efficiency of cell expansion. The percentage of CD34+CD90+ after 7 days of amplification with FTS3 + VPA was 50% of cells amplified with FTS3 + VPA and after 7 and 21 days amplification with FTS3 ± VPA. c. Effect of VPA on cytokine-induced amplification evaluated after 7 days amplification with only two early acting cytokines (FT) in three MPB and one CB samples. The results have been expressed as mean of the four cases because no significant difference was detected among the different cell sources. Although the CD34+ cells were more effectively preserved in cultures stimulated by FT compared with FTS3, the addition of valproic acid further increased the percentage of either CD34+ cells (79% versus 61.9%) or the early cell fraction CD34+CD90+ (65.4% versus 8.6%, P < 0.001; Fig. 2C). The plating efficiency of CD34+ cells amplified with FT + VPA was 50% of cells amplified with FT alone. Notably, immunoblot analysis, showed that valproic acid was effective in increasing the histone acetylation levels induced by 16 hours FT treatment (Fig. 1A).

**Effect of Valproic Acid on Short-term Amplification Induced by the Early Acting Cytokines FL and Thrombopoietin.** The effect of valproic acid was also evaluated in expansion cultures stimulated only by early acting cytokines FL + thrombopoietin (FT) in three MPB, and one CB samples. The results have been expressed as mean of the four cases because no significant difference was detected among the different cell sources. Although the CD34+ cells were more effectively preserved in cultures stimulated by FT compared with FTS3, the addition of valproic acid further increased the percentage of either CD34+ cells (79% versus 61.9%) or the early cell fraction CD34+CD90+ (65.4% versus 8.6%, P < 0.001; Fig. 2C). The plating efficiency of CD34+ cells amplified with FT + VPA was 50% of cells amplified with FT alone. Notably, immunoblot analysis, showed that valproic acid was effective in increasing the histone acetylation levels induced by 16 hours FT treatment (Fig. 1A).

**Effect of Valproic Acid on Hematopoietic Stem Cell Expansion over 3-Week Cultures.** The long-term effect of valproic acid on HSCs amplification was investigated in 3-week cultures with FTS3 in six cases (MPB = 4 and CB = 2). At day 21, morphologic analysis showed an immature phenotype in <50% of MPB or CB cells amplified with FTS3 alone, and in more than 75% of cells expanded with FTS3 + VPA (Fig. 4A). Accordingly, more pronounced features of myelomonocytic differentiation were detected in FTS3-treated cells than in cultures supplemented with FTS3 + VPA. Cytochemical staining that allow a functional characterization of myeloid cells was also done. Myeloperoxidase (MPO) activity was detected in about 35% of FTS3-treated cells, and in 4% to 8% of cells treated with FTS3 + VPA. Likewise, staining for sodium fluoride–inhibited naphthyl acetate esterase activity revealed a higher percentage of monocytes in FTS3-treated cells than in cells treated with FTS3 + VPA (Fig. 4B).

Immunophenotypic analysis was done with a wide panel of monoclonal antibodies to evaluate the early stem cell pool, the intermediate progenitor cell compartment, and the differentiated...
cells generated along the expansion. A significantly slower decrease over time of the number of CD34+ cells was observed in cultures supplemented weekly with FTS3 + VPA (1st week, 84.6%; 2nd week, 46.8%; 3rd week, 32.5%) compared with cultures stimulated with FTS3 alone (1st week, 32.3%; P = 0.005; 2nd week, 3.1%; P < 0.001; 3rd week, 0.6% P = 0.002; Fig. 5). On the 3rd week of FTS3 + VPA cultures, the percentage of CD34+ cells (32.5 ± 7.5%) was comparable to the number of positive cells (32.2 ± 11.0%) detected at day 7 in amplification cultures with FTS3 alone.

Notably, at the 3rd week of culture, a higher number of cells amplified in presence of valproic acid maintained the characteristics of primitive stem cells as documented by the significant increase of CD34+CD90+ cells (17.7% versus 0.14%, P = 0.002) and CD34+CD38− cells (3.4% versus 0.04%, P = 0.018). Finally, a consistent fraction (10.2%) of the CD34+ cells generated in presence of valproic acid coexpressed the AC133, which is an early hematopoietic cell marker rapidly down regulated during differentiation (Figs. 2B and 5). The c-kit receptor (c-kitR/CD117) was detected at a low level in valproic acid cultures compared with the lack of expression of this marker among cells amplified with FTS3 alone (CD34+CD117+ 2.0% versus 0%).

Despite the significantly lower number of total nucleated cells recovered after amplification with FTS3 + VPA, the CD34+ cells generated after 3 week expansion increased over the input cell number 35.1-fold compared with 4.5-fold in presence of FTS3. Notably, the early subpopulations CD34+CD90+, CD34+CD38−, and CD34+CD133+ were amplified respectively 16.1-, 7.2-, and 16.2-fold by the 3rd week, whereas no amplification of these cell fractions was detected among cells expanded with FTS3.

The progenitor cells (CD34+CD33+) and early myeloid cells (CD33+ and CD13+) were more represented in valproic acid amplification cultures although the differences were not statistically significant. Indeed, a significantly lower percentage of differentiated cells (CD15+) was produced along the 3 weeks of amplification in presence of valproic acid than after expansion with only FTS3 (1st week, 10.5% versus 34%, P = 0.05; 2nd week, 12.1% versus 48.4%, P = 0.001; 3rd week, 15.4% versus 57.9%, P < 0.001). By contrast, differentiated cells of erythroid (CD235a+) and megakaryocytic lineages (CD61+) were more frequent in the presence of valproic acid than in cultures treated with FTS3 alone (Fig. 5).

In amplification cultures with FTS3 the plating efficiency of CD34+ cells, after a drop on day 7, increased progressively and was comparable to baseline level by the 3rd week. In agreement with the higher immatureity of the cells, a progressive decrease of the plating efficiency was detectable in valproic acid cultures (Fig. 5).

**Effect of Valproic Acid on Genes Expressed at Different Stages of Hematopoiesis.** The effect of FTS3 and FTS3 + VPA on the expression of genes implicated in the early developmental stages of HSC (CD34, KDR, c-kit R, and AC133) or during lineage commitment and differentiation (GATA1, MPO, GM-CSFr, G-CSFr, MSE, and Gp1ab) was investigated in four cases (MPB = 2 and CB = 2) by quantitative reverse transcription-PCR. At days 7, 14, and 21, either FTS3 or FTS3 + VPA modulated the expression of most genes when compared with freshly isolated MPB or CB CD34+ cells (day 0), although with differences in potency and kinetics between the two cell sources (Fig. 6A and B). In agreement with the results of immunophenotypic analysis, CD34 expression decreased during the long-term cultures in both FTS3 and FTS3 + VPA–treated cells. However, CD34 mRNA was about 3- to 7-fold more abundant in valproic acid-treated cells than in cultures treated with cytokines alone either in MPB or CB expansion cultures (Fig. 6 and data not shown). Remarkably, the expression of KDR, AC133 and c-kitR were induced by FTS3 + VPA with levels higher than those measured in freshly isolated CD34+ cells on days 14 and 21. GATA1, which is present either in HSCs or in erythroid and megakaryocytic progenitors was also increased by FTS3 + VPA at 14 to 21 days compared with FTS3 cultures or freshly isolated MPB or CB CD34+ cells (data not shown), whereas GM-CSFr, a common myeloid progenitor-affiliated gene expressed during the early and late stages of granulocytic and monocytic differentiation was expressed at comparable levels in valproic acid and control cultures (Fig. 6A and B). Expression of genes characterizing late stages of hematopoietic differentiation, such as G-CSFr, glycoprotein 1ab (Gp1ab), MPO, and MSE was significantly higher in FTS3-treated cells than in cells treated with FTS3 + VPA along 3 weeks amplification (Fig. 6B).

We next assessed the effect of valproic acid on the expression of genes involved in HSC self-renewal such as Wnt3A, β catenin, Notch-1, and HOXB4 (18, 19, 39). The expression of β catenin and Notch-1 remained unchanged and Wnt3A was undetectable (Fig. 6C and data not shown). In contrast, the homeogene HOXB4 was significantly induced in FTS3 + VPA cultures at both
RNA and protein levels (Fig. 6C and D), with a strong increase at day 21 compared with a minimal and transient increase in FTS3-treated cells.

Valproic Acid Increases Histone Acetylation Levels on Chromatin at AC133 and HOXB4 Regulatory Sites. To address whether chromatin remodeling events are involved in the effect of valproic acid on the ex vivo amplification of CD34+ cells by cytokines, we investigated by ChIP analysis on chromatin prepared from CD34+ cells isolated from MPB (two cases), the changes in the acetylation status of histone H4 at regions surrounding the exon1A of AC133 whose expression seems restricted to CD34+ cells and the HOXB4 promoter, which contain the promoter response element 1 (HxRE-1) and the E-box regulatory regions (36–38). Upon 2 hours treatment, FTS3 changed the acetylation levels on histone H4 at these regulatory sites on both AC133 and HOXB4 that became hyperacetylated after the addition of valproic acid either in absence or presence of FTS3 (Fig. 6E). The specificity of the chromatin changes at these sites was indicated by the absence of modulation in the acetylation status of histone H4 in the same samples when distal sequences were amplified by PCR using specific primers. Thus, the functional effects of valproic acid on AC133 and HOXB4 gene expression positively correlated with the acetylation status of histone H4 at regulatory sites present on their promoters.

To relate the changes on chromatin accessibility by valproic acid with the enhancement of the cytokine effect on the maintenance of a primitive HSC population, we analyzed the effect of a single exposure to valproic acid on 3 weeks amplification of CD34+ cells from 3 MPB and 1 CB. The immunophenotype of cells exposed to a single dose of valproic acid and cultured 3 weeks in its absence was similar to that of control cells never exposed to valproic acid and from 3 MPB and 1 CB. The immunophenotype of cells exposed to a single dose of valproic acid and cultured 3 weeks in its absence was similar to that of control cells never exposed to valproic acid and from 3 MPB and 1 CB. The immunophenotype of cells exposed to a single dose of valproic acid and cultured 3 weeks in its absence was similar to that of control cells never exposed to valproic acid.

Figure 4. Valproic acid (VPA) addition maintains a high percentage of morphologically and functionally immature CD34+ HSC in amplification cultures with multiple cytokines. Morphologic and functional analyses were done in CD34+ cells from MPB (n = 2) and CB (n = 2) before (day 0) and after 7, 14, and 21 days of treatment with FTS3 or FTS3 + VPA. A. % immature cultures (immature, □), myelomonocytic cells of the granulocytic (Gr, □) and mononuclear (Mo, □) lineage. B, functional characterization of myelomonocytic cells was done by cytochemical staining of MPB and CB cells. Cells were evaluated for positive staining of MPO, α naphthyl acetate esterase (ANAE) with or without sodium fluoride (NaF) as described in Materials and Methods. At day 0, all the cells were negative for these cytochemical staining. Means ± SD of two separate experiments.

Discussion

A requisite for successful in vitro expansion of human HSC for application in stem cell transplantation settings and gene therapy would be to efficiently induce stem cell proliferation without a concomitant loss of the self-renewing potential. Ex vivo amplification protocols have shown that several cytokine combinations may induce a rapid cell cycling of HSCs resulting in a significant expansion of a partially differentiated HSC compartment that can be used to shorten the period of post-transplant aplasia (40). Nevertheless, the expansion or maintenance of the early HSCs with long-term repopulating ability is still controversial (40).

In this study, we have investigated the effect of valproic acid, an inhibitor of HDAC, in amplification cultures stimulated by combinations of either four (FTS3) or two early acting cytokines (FT). Our results provide evidence that valproic acid significantly enhances the effect of cytokines on the amplification of HSC and selectively increases the early HSC compartment in 1- to 3-week expansion cultures. This effect of valproic acid has been documented in all the HSC sources and is correlated with the hyperacetylation of the nucleosomal histones H3 and H4.

In all the HSC sources, the addition of valproic acid to cultures stimulated by either FTS3 or FT decreased the overall number of ex vivo generated cells. However, the percentage of the cells retaining the expression of CD34 antigen after 7-day cultures was significantly higher (1.5- to 3-fold) than with FT and FTS3 alone.

Previous studies on CB amplification reported percentages of CD34+ cells consistently <20% with a 10-fold expansion of CB CD34+ (41, 42). In agreement with these results, our data showed a 9.4-fold increase of CB CD34+ cells after 7 days of expansion with FTS3. Notably, the addiction of valproic acid significantly improved the amplification of CD34+ cells with an increase of about 25-fold in samples from either fresh or thawed CB. Furthermore, the expression of CD34+ cells was kept significantly higher in presence of valproic acid till the 3rd week of liquid culture, whereas previous amplification studies showed a marked decrease with time of the percentage of CD34+ cells, independently from the cytokine combination used (43).

Surprisingly, further characterization of the CD34+ cells showed that the CD34+ subpopulations lacking the CD38 antigen or coexpressing the antigens CD90 and AC133 were significantly more preserved in amplification cultures with FTS3 and valproic acid than with growth factors alone either after 1- or 3-week cultures in all the HSC sources. Several studies have documented that these
markers identify cell subsets in early stages of hematopoietic development. The AC133 has been shown to be expressed on stem/progenitor cell fractions including CD34+CD38- cells that have a long-term repopulating potential as assessed in fetal sheep and NOD/SCID transplantation models (44, 45). Interestingly, we found that in MPB samples, valproic acid addition greatly hyperacetylate the chromatin surrounding the exon 1A, a region generating the AC133 mRNA isoform specifically expressed in CD34+ HSC (38). In our study, the higher immaturity of CD34+ cells generated in cultures supplemented with FTS3 + VPA was also confirmed by the 2- to 4-fold increased expression of genes associated to an early hematopoietic stem cell compartment (KDR, CD117, and HOXB4), compared with the levels detected in cultures with FTS3 alone. Recently, several studies have shown differences in long-term engrafment potential of resting and cycling hematopoietic stem cells providing evidences that phenotypic changes associated with cell cycle phases are reversible (46, 47). Growth factors exposure induces quiescent, early HSC to proceed through cell cycle giving rise either to cells retaining HSC properties, or to cells committed toward differentiation. In amplification cultures supplemented with valproic acid, a lower number of cells were in the active phase of cell cycle (S-G2-M) and a significantly higher percentage of cells still was in G1 phase on the 3rd week of expansion compared with the higher proliferative rate induced by growth factors alone (data not shown). The lower rate of proliferation induced by valproic acid correlates with the immaturity of the cells generated in cultures. Indeed, the lower plating efficiency of committed hematopoietic progenitors in primary cultures and the higher number of secondary colonies originated from individual clones suggests that valproic acid treatment induces one or more self-renewal divisions

Figure 5. Immunophenotypic profile of cells amplified over 3 weeks with FTS3 alone or in association with valproic acid (VPA). The characterization of the amplified cells from MPB (n = 4) and CB (n = 2) was done weekly on cells generated in vitro along 3 weeks expansion cultures supplemented with FTS3 alone (-) and after coexposure to FTS3 and VPA either added to cultures every week (-----) or only once at the start of cultures (---). Early, intermediate, and late hematopoietic cells were characterized by a wide panel of MoAbs. Points, mean; bars, ±SE. The statistical differences between the mean values of cell subpopulations detected in FTS3 and FTS3 + VPA amplification cultures were evaluated by Student’s t test. *, P < 0.05; **, P < 0.001. Fold amplification (F) of the different cell subsets over the input cell number.

Figure 6. Valproic acid (VPA) modifies the gene expression profiles induced by cytokines in CD34+ HSC cells. Time-dependent effect of FTS3 (-) and FTS3 + VPA (-----) on the expression of (A) genes of HSC early developmental stages (CD34, KDR, AC133, CD117, and GATA-1), (B) genes of different stages during late hematopoietic differentiation (MPO, GM-CSF, G-CSF, MSE, and Gp1ab), and (C) genes potentially involved in HSC self-renewal (HOXB4, catenin, and Notch-1). Total RNA was extracted from CD34+ MPB cells before (day 0) or after 7, 14, and 21 days treatment with FTS3 or FTS3 + VPA. Relative gene expression in treated cells compared with freshly isolated CD34+ cells (day 0) was determined by quantitative reverse transcriptase-PCR. Points, means of two separate experiments; bars, ±SD. D, immunoblot analysis of HOXB4 expression in CD34+ MPB cells treated or not with FTS3 in the presence or absence of VPA done as described in Materials and Methods. Histone H3 was probed as a protein loading control. Representative case of two that gave similar results. E, effect of cytokines and/or VPA on histone H4 hyperacetylation at regulatory chromatin sites of HOXB4 and AC133 genes. CD34+ MPB cells were treated or not (control) with VPA, FTS3, and FTS3 + VPA for 2 hours. ChIP assay was done with an anti-acetyl-histone H4 antibody (H4Ac) or no antibody (no Ab) and analyzed by PCR using primers corresponding to the regulatory sites on the HOXB4 (P1) and AC133 (Ex1A) genes that are active in hematopoietic cells or to distal regions on HOXB4 (P5) and AC133 (ExE) as described in Materials and Methods. The amplification of GAPDH was used to detect nonrelevant cellular DNA sequences present in the samples. A sample representing 0.02% of total input chromatin (input) was included in the PCR analysis. Representative case of two that gave similar results.
of the colony-forming cells before the enter a pathway of terminal differentiation. Although these results suggest that valproic acid affects the amplification of HSC by promoting self-renewal, the differentiation capacity toward all the hematopoietic lineages is maintained as indicated by the detection of mature cells expressing specific markers for all the hematopoietic lineages (CD13, CD15, CD235a, and CD61).

While this article was in preparation, another group (48) reported that human bone marrow CD34+ cells amplified in a two-step culture system with cytokine cocktails specific to promote HSC cycling (1st step) or differentiation (2nd step) and sequentially exposed to 5-aza-2’-deoxycytidine alone or in combination with Trichostatin A, increased hematopoietic progenitor cells with a primitive phenotype (CD34+CD90+) that were able to engraft NOD/SCID mice (48). 5-Aza-2’-deoxycytidine is a chromatin remodeling agent that function as an inhibitor of DNA methyltransferase activities. Trichostatin A is an HDAC inhibitor structurally and functionally different from valproic acid (29, 32, 49).

The effectiveness of different agents altering chromatin structure in enhancing the amplification of primitive HSC subsets is in agreement with the hypothesis that reversible phenotypic shifts in hematopoietic cells depend on chromatin remodeling and the consequent expression of genes in response to environment-derived stimuli (50). Chromatin itself may serve as a genomic integrator of various extracellular and intracellular pathways. Among the intracellular factors that induce self-renewal of HSC, ectopic expression of the transcription factor HOXB4 increases the number of transplantable HSCs retaining the ability to repopulate in vivo myeloid and lymphoid lineages (24, 25). Here we show that in MPB CD34+ cells the acetylation status of histones on specific DNA binding sites within the HOXB4 promoter is increased by 2 hours of exposure to valproic acid. Notably, the addition of FTS3 further enhances the valproic acid effect on the chromatin acetylation status at this site. Increased expression levels of HOXB4 gene and protein were also detectable after 1 or 3 weeks of culture in FTS3 + VPA–treated cells, compared with FTS3 alone. Thus, it seems that valproic acid by inducing chromatin modifications at critical DNA-binding sites leads to transcriptional derepression of specific target genes and enhances the effect of early acting cytokines. Accordingly, a single exposure to valproic acid at the start of the amplification culture maintained a significantly higher fraction of CD34+CD90+ cells over 3 weeks compared with cultures never exposed to valproic acid.

A significant role of histone deacetylases in hematopoiesis is further highlighted by findings showing that an abnormal regulation of HDAC activities is a leukemogenic event underlying the block of differentiation in acute myeloid leukemias. Regardless of their primary genetic lesion, acute myeloid leukemias can be also induced to differentiate by treatment with HDAC inhibitors valproic acid and Trichostatin A in vitro and in vivo (28–33).

These evidences suggest a scenario where valproic acid can act as epigenetic regulator of both stem cell maintenance and differentiation of committed progenitors into different lineages (28–33). The chromatin accessibility at specific DNA-binding sites might represent the key event for the activity of cytokines or transcription factors either involved in the maintenance of early HSC population or in lineage commitment. Some of these factors might be present in the same cellular context and dictate cell fate choice by targeting enzymes with chromatin remodeling activity such as HDACs, histone acetyltransferases, or methylases at specific gene loci. Valproic acid can therefore initiate a series of events leading to the maintenance of the undifferentiated state in early HSC, or generating a chromatin code coupled to specific differentiation decision in HPC and acute myeloid leukemia blasts.

In conclusion, this study present in vitro evidence for a new and relevant effect of valproic acid on human hematopoietic stem cells and highlight the potentiality of novel epigenetic approaches for the ex vivo amplification of HSC aimed to transplantation, gene and stem cell therapies. However, whether valproic acid enhances the plasticity, self-renewal, and/or engraftment potential of long-term repopulating stem cells in vivo are relevant questions that need further investigation in animal models.

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