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Differential Effects of Epigenetic Modifiers on the Expansion and Maintenance of Human Cord Blood Stem/Progenitor Cells

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A B S T R A C T

Epigenetic therapies, including DNA methyltransferase and histone deacetylase (HDAC) inhibitors, are increasingly being considered to treat hematological malignancies, but their effects on normal hematopoietic stem cells (HSCs) remain largely unexplored. We compared the effects of several HDAC inhibitors, including valproic acid (VPA) and trichostatin A (TSA), alone or in combination with 5-aza-2′-deoxycytidine (5azaD) on the expansion of HSCs. VPA induced the highest expansion of CD34+CD90+ cells and progenitor cells compared with other HDAC inhibitors or the sequential addition of 5azaD/TSA in culture. Xenotransplantation studies demonstrated that VPA prevents HSC loss, whereas 5azaD/TSA treatment leads to a net expansion of HSCs that retain serial transplantation ability. 5azaD/TSA-mediated HSC expansion was associated with increased histone acetylation and transient DNA demethylation, which corresponded with higher gene transcript levels. However, some genes with increased transcript levels lacked changes in methylation. Importantly, a global microarray analysis revealed a set of differentially expressed genes in 5azaD/TSA- and VPA-expanded CD34+ cells that might be involved in the expansion and maintenance of transplantable HSCs, respectively. In summary, our data indicate that treatment of HSCs with different chromatin-modifying agents results in either the expansion or maintenance of HSCs, an observation of potential therapeutic importance.

INTRODUCTION

The mechanisms that govern hematopoietic stem cell (HSC) fate decisions, including self-renewal and differentiation, are likely under tight control but remain potentially alterable. Self-renewal and differentiation decisions in HSCs seem to occur independently of cytokines and are postulated to be predetermined by cell-intrinsic properties [1,2], which can also be influenced by external regulatory factors. We demonstrated that environmental cues likely influence intrinsic factors, such as epigenetic events, to influence HSC fate choices [3].

The limited number of HSCs within a cord blood (CB) unit likely contributes to the high rate of graft failure and delayed engraftment upon CB transplantation, particularly in adults [4,5]. However, this limitation could be overcome if the number of transplantable HSCs within a CB unit were expanded. Studies have led to the identification of several molecules, including notch ligand, prostaglandin E_2, pleiotrophin, and an aryl hydrocarbon receptor antagonist, as positive stimulators of HSC expansion, although none of these molecules alone exhibited potency [6-9]. Current *ex vivo* expansion strategies in clinical trials using notch ligand or marrow stromal cell co-culture primarily expand short-term progenitors and rely on a second unmanipulated CB graft for long-term blood cell production [7,10].

Epigenetic therapies, including the hypomethylating drug 5-aza-2′-deoxycytidine (5azaD) and histone deacetylase (HDAC) inhibitors, used alone or in combination, are increasingly being considered for the treatment of cancers, including hematological malignancies [11,12]. Valproic acid (VPA) alone or in combination with all-*trans* retinoic acid has demonstrated potency in inducing cell death in leukemia, myeloproliferative diseases, and solid tumors [13-15]. However, the effects of such epigenetic modifiers on normal HSCs are not clear.

We previously demonstrated that the sequential addition of 5azaD followed by the HDAC inhibitor trichostatin A (TSA) in CD34+ cell culture expands normal HSCs possessing marrow repopulation capacity [16,17]. The objective of our current study was to examine the potency of various chromatin-modifying agents (CMAs) either alone or in combination with a DNA methyltransferase inhibitors and to compare these results with previously established strategies involving the sequential addition of CMAs, including the hypomethylating drug 5azaD and the HDAC inhibitor TSA in culture [18,19]. Toward this goal, we compared the efficacy of several CMAs, including VPA, TSA, Nicotinamide (NA), suberoylanilide hydroxamic acid (SAHA), and 5azaD as single agents or in combination to study their ability to expand

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repopulating HSCs. The potential of various CMAs to alter normal HSC fate choices has been explored [20-23], but the ability of VPA to maintain hematopoietic stem/progenitor cells in culture has not been previously described. The significance of our current study is the demonstration that different CMAs have variable effects on HSC outcomes and that this difference can be used to identify genes presumably involved in distinct biological processes, including HSC expansion or maintenance. More importantly, we show for the first time that various HDAC inhibitors alone or in combination with DNA methyltransferase inhibitors can exert distinct effects on HSC outcomes in culture, which might be relevant for the design of future clinical trials using epigenetic therapy to treat hematological malignancies.

METHODS

Isolation and Culture of CD34+ Cells

Human CB was obtained according to Institutional Review Board guidelines. Low-density cells (<1.077 g/mL) were obtained by density centrifugation on Ficoll-Paque (GE Healthcare, Uppsala, Sweden), and CD34+ cells were immunomagnetically (Miltenyi Biotech, Inc., Auburn, CA) isolated from this population as described previously [3]. The purity of the isolated CD34+ cells ranged from 90% to 99%. CD34+ cell expansion culture with or without CMA was performed in medium containing 30% FBS (HyClone Laboratories, Logan, UT) supplemented with a cocktail of recombinant human cytokines as previously described [3].

Flow Cytometric Analysis

Flow cytometric analysis was conducted as previously described [18]. All antibodies were purchased from BD Bioscience (San Jose, CA). All analyses were paired with the corresponding matched isotype control, and at least 10,000 live cells were acquired for each analysis (CellQuest software, Becton Dickinson San Jose, CA).

Colony-Forming Cell and Cobblestone Area–Forming Cell Assays

Colony-forming cells (CFCs) were assayed by plating cells in semisolid methyl cellulose (1.1%)-based medium containing 30% FBS (Stem Cell Technologies, Vancouver, BC, Canada) and a cocktail of recombinant human cytokines as described previously [3]. The colonies were counted after 14 days [3]. The number of cobblestone area–forming cells (CAFCs) was quantitated by plating in a limiting dilution as described previously [3,24,25].

Nonobese Diabetic/SCID Assays

Immunodeficient nonobese diabetic/*ltsz-scid*/*scid* (NOD/SCID) mice were purchased from Jackson Laboratories (Bar Harbor, ME), and transplantation assays were performed as previously described [3].

RNA Preparation and Real-Time PCR

RNA preparation and real-time quantitative PCR (qPCR) assays were performed using SYBR Green dye (Life Technologies, Carlsbad, CA) and an ABI 7500 Fast Real-Time PCR system (Life Technologies) to quantitate gene expression using reverse-transcribed mRNA as described previously [18]. The primer sequences used in real-time RT-PCR are shown in Supplemental Table 1.

Chromatin Immunoprecipitation Assays

The chromatin immunoprecipitation assay was conducted using a commercial assay kit following the manufacturer's instructions (Millipore, Billerica, MA) as described previously [26]. Briefly, after 72 hours of culture, CD34+ cells were cross-linked with formaldehyde, and the cell pellet was lysed, sonicated to produce genomic fragments, and immunoprecipitated using an anti–acetyl-histone H4 antibody (Millipore, Billerica, MA).

Microarray Studies

Global gene expression microarray studies using a U133 Plus 2.0 array (Affymetrix, Santa Clara, CA) were performed in collaboration with the UCLA Clinical Microarray Core as described previously with minor modifications [27]. For more details, please refer to Supplemental Methods, below.

DNA Methylation Analysis

Briefly, genomic DNA was isolated from primary or CMA-expanded enriched CD34+ cells using the Blood and Cell Culture DNA kit (Qiagen, Valencia, CA). Genomic DNA was bisulfite treated using an EZ Methylation Kit (Zymo Research, Irvine, CA), and biotinylated gene-specific primers or long interspersed nucleotide element 1 (LINE-1) primers were used to PCR amplify the regions of interest as described previously [28,29]. DNA methylation analysis was performed by EpigenDx (Worcester, MA) using quantitative pyrosequencing and the PSQ-HS96 system according to standard operating procedures [28]. Biotinylated gene-specific primers were developed for the CpG sites near the promoter area as detailed in Supplemental Table 3, or LINE-1 primers were used to amplify regions of interest for analysis as described previously [29].

Measurement of Leukotriene B4 by ELISA

The concentration of leukotriene B4 in conditioned medium was measured with an acetylcholine esterase competitive enzyme immunoassay following the manufacturer's instructions (Cayman Chemical Co., Ann Arbor, MI).

Statistical Analysis

Results are expressed as means ± standard error (SE) when applicable. The statistical significance ($P < .05$) between groups was determined using a 2-tailed Student's *t*-test.

RESULTS

VPA Induces Significant Expansion of CD34+CD90+ Cells

We compared the effects of various CMAs on HSC expansion using HDAC inhibitors as single agents or in combination with 5azaD with the effects of the sequential addition of 5azaD/TSA, a prototype CMA [3], under identical culture conditions. As shown in Figure 1C, after culture, 1.17% ± .32% of the total nucleated cells exposed to cytokines alone co-expressed CD34 and CD90, whereas 2%, 5%, 6%, 13%, and 3% of cells cultured with cytokines plus 5azaD, TSA, NA, 5azaD/NA, or SAHA, respectively, co-expressed the CD34 and CD90 antigens (Figure 1C). Cultures containing VPA, 5azaD/TSA, or 5azaD/VPA had a higher percentage of CD34+CD90+ cells compared with control (42.2% ± 13.5%, 28.2% ± 3.6%, and 52.4% ± 9.5%, respectively) (Figure 1C). Total nucleated cells were highest in the control culture and lowest in the culture treated with 5aza/VPA (Figure 1A). Because total nucleated cells and the proportion of CD34+CD90+ cells varied significantly in cultures containing various CMAs, in contrast to the control (Figure 1C), the fold expansion of primitive CD34+CD90+ cells was analyzed in comparison with their initial numbers before culture (Figure 1D).

Significant differences in the expansion of CD34+CD90+ cells were observed between cultures treated with various CMAs (Figure 1D). The addition of VPA resulted in a much higher expansion (64.6 ± 3.7-fold; $P = .001$; Figure 1D). However, when VPA was added after 5azaD to the culture (5azaD/VPA), the expansion of CD34+CD90+ cells decreased significantly (6.4 ± 1.3-fold), despite having the highest percentage of CD34+CD90+ cells (Figure 1C). VPA treatment alone provided the maximal expansion of CD34+CD90+ cells (Figure 1D). Notably, NA (2.1-fold) and SAHA (1.2-fold) as single agents or in combination with 5azaD (5azaD/NA [2.2-fold]) did not promote the expansion of CD34+CD90+ cells relative to 5azaD/TSA or VPA-expanded CB cells.

These data indicate that among the CMAs examined, VPA and 5azaD/TSA best expand CD34+CD90+ cells. We previously demonstrated that CD34+CD90+ cells exclusively retain *in vivo* hematopoietic repopulation potential [16], but the repopulation capacity of VPA- or 5azaD/VPA-expanded cells is not known.

Functional Potency of CMA-Expanded CB Grafts

To determine the correlation between the expansion of CD34+CD90+ cells and their *in vitro* functional potential, CFC and CAFC assays were performed using CB cells that were expanded using various CMAs. Figure 1E depicts the