Histone deacetylase inhibitor treatment downregulates VLA-4 adhesion in hematopoietic stem cells and acute myeloid leukemia blast cells

Ulrich Mahlknecht and Christiane Schönbein

Saarland University Medical Center, Department of Immunotherapy and Gene Therapy, Homburg/Saar; Department of Hematology/Oncology, University of Heidelberg Medical Center, Heidelberg, Germany

ABSTRACT

The $\alpha4\beta1$ integrin very late activation antigen-4 (VLA-4) is an $\alpha4$ (CD49d)/$\beta1$ (CD29) heterodimer. It plays a key role in the adhesion of both hematopoietic progenitor cells and leukemic blast cells to bone marrow stromal cells which express the vascular cell adhesion molecule-1 (VCAM-1) or produce fibronectin. VLA-4 expression has been associated with bone-marrow minimal residual disease, which causes relapse after chemotherapy in patients with acute myelogenous leukemia. Conversely, the absence of VLA-4 reduces bone marrow retention of both hematopoietic progenitor and leukemic blast cells. We report on the downregulation of VLA-4/CD49d for various acute myelogenous leukemia cells lines, on primary cells from patients with acute myelogenous leukemia, and on hematopoietic stem cells and peripheral blood mononuclear cells from healthy donors on treatment with the histone deacetylase inhibitors suberoylanilide hydroxamic acid and valproic acid, which is associated with decreased adhesion to mesenchymal stromal cells. These findings suggest that HDAC-inhibitor treatment may on the one hand impair stem cell homing, while on the other it may improve peripheral blood stem cell mobilization and significantly help to reduce minimal residual disease from acute myelogenous leukemia.

Key words: homing, adhesion, histone deacetylase, stem cell transplantation, minimal residual disease.

Citation: Mahlknecht U and Schönbein C. Histone deacetylase inhibitor treatment downregulates VLA-4 adhesion in hematopoietic stem cells and acute myeloid leukemia blast cells. Haematologica 2008 Mar; 93(3):443-446. doi: 10.3324/haematol.11796

©2008 Ferrata Storti Foundation. This is an open-access paper.

Introduction

Homing describes the co-ordinated migration of hematopoietic progenitor cells through the blood and across the vascular endothelium to the bone marrow niches. This migration process requires rolling, extravasation and adhesion between stem cells, stromal cells and the extracellular matrix, and depends on the presence of various factors. These include chemokine stromal-derived factor (SDF)-1,[1] the chemokine receptor CXCR4 and the stem cell factor (SCF),[2] the activation of a number of cell surface molecules, for example the lymphocyte function-associated antigen (LFA)-1 (CD11a/CD18), the very late antigens VLA-4 (CD49d/CD29) and VLA-5 (CD49e/CD29),[3-5] the hyaluronic acid receptor CD44,[6] and the membrane type (MT) 1-metalloproteinase (MMP) activation and secretion of MMP2/9,[7,8] which are essentially all necessary for successful hematopoietic stem cell engraftment after myeloablative chemotherapy.

By contrast, decreased expression of the very late activation antigen 4 (VLA-4) is one of the requirements for successful *mobilization* of hematopoietic progenitor cells from the bone marrow.[9] In this context, VLA-4 has been said to be involved in the retention of both normal hematopoietic progenitor cells and leukemic blast cells within the bone marrow.[10,11] Accordingly, both the inducible ablation of alpha4 integrins in adult mice, as well as *in vivo* treatment of mice with antibodies to VLA-4, resulted in the efficient mobilization of $CD34^+$ hematopoietic stem cells from the bone marrow and their release into the peripheral blood.[12,13] VLA-4 is known to function not only as an adhesion receptor, but also as a bi-directional signaling molecule for both incoming and outgoing signals in its close interaction with hematopoietic and microenvironmental cells or with soluble signaling factors such as cytokines and chemokines.[14] It has been suggested that VLA-4, which is being found abundantly on leukemic cells, induces resistance to cytostatic agents through its interaction with fibronectin on bone marrow stromal cells. This throws new light on the molecular pathogenesis of bone marrow minimal residual disease after chemotherapy in patients with acute myelogenous leukemia. At the same

Acknowledgments: we thank Rainer Saffrich for help with microscopy and Imke Bokelmann for technical assistance.
Funding: supported by the German National Science Foundation (Deutsche Forschungsgemeinschaft, Ma 2057/2-4).
Manuscript received May 31, 2007. Manuscript accepted October 30, 2007.
Correspondence: Ulrich Mahlknecht, Saarland University Medical Center Department of Immunotherapy and Gene Therapy, Kirrberger Straße, Bldg. 4.3, D-66424 Homburg/Saar, Germany. E-mail: ulrich.mahlknecht@uks.eu

time, VLA-4 has not only been demonstrated to increase adhesion of leukemic cells to bone marrow stromal cells, but also to protect leukemic cells from drug-induced apoptosis via the phosphatidylinositol-3-kinase/AKT/Bcl2 signaling pathway, which is activated by the interaction of VLA-4 and fibronectin. Accordingly, in a minimal residual disease mouse model, a 100% survival rate was achieved when VLA-4-specific antibodies were combined with cytosine arabinoside, while cytosine arabinoside alone only slightly prolonged survival. Also, it has been reported that the 5 year overall survival was 100% in VLA-4$^-$ patients compared with 44.4% in VLA-4$^+$ patients.[11] The interaction between VLA-4 on leukemic cells and fibronectin on stromal cells is, therefore, a crucial event in the mediation of bone marrow minimal residual disease and AML prognosis.

We report in the downregulation of VLA-4/CD49d for various AML cell lines, on primary cells from AML patients, and on hematopoietic stem cells and peripheral blood mononuclear cells from healthy donors on treatment with SAHA and VPA, which is associated with decreased adhesion to mesenchymal stromal cells. These findings have a huge impact on current and future treatment approaches which involve stem cell harvesting, transplantation, and the prevention of minimal residual disease following treatment of AML under concurrent HDAC-inhibitor treatment.

Design and Methods

Cell culture

Human peripheral blood mononuclear cells (PBMCs), bone marrow derived CD34$^+$ hematopoietic cells from healthy volunteers and leukemic blast cells from AML patients were separated over a density gradient (Ficoll). Hematopoietic progenitor cells, PBMCs, leukemic blast cells, the human acute myeloid leukemia cell lines KG-1A and HEL were cultured in RPMI medium supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin.

Exposure to HDAC-inhibitors

Hematopoietic progenitor cells, KG1a cells, HEL cells, and primary AML cells were exposed to SAHA (0.5; 1 and 2 μM) and VPA (0.5; 1 and 2 mM) for 48 hrs. and four days respectively. SAHA treatment of KG1A cells was carried out for a maximum of four days. PBMCs were exposed to SAHA (0.5; 1 and 2 μM) for 48 hrs. The *in vitro* doses used correspond to the lowest concentrations used in patients in clinical studies. The doses were calculated for serum concentrations that are ideally reached in a patient with a body weight of 70 kg and a body height of 1.70 m.

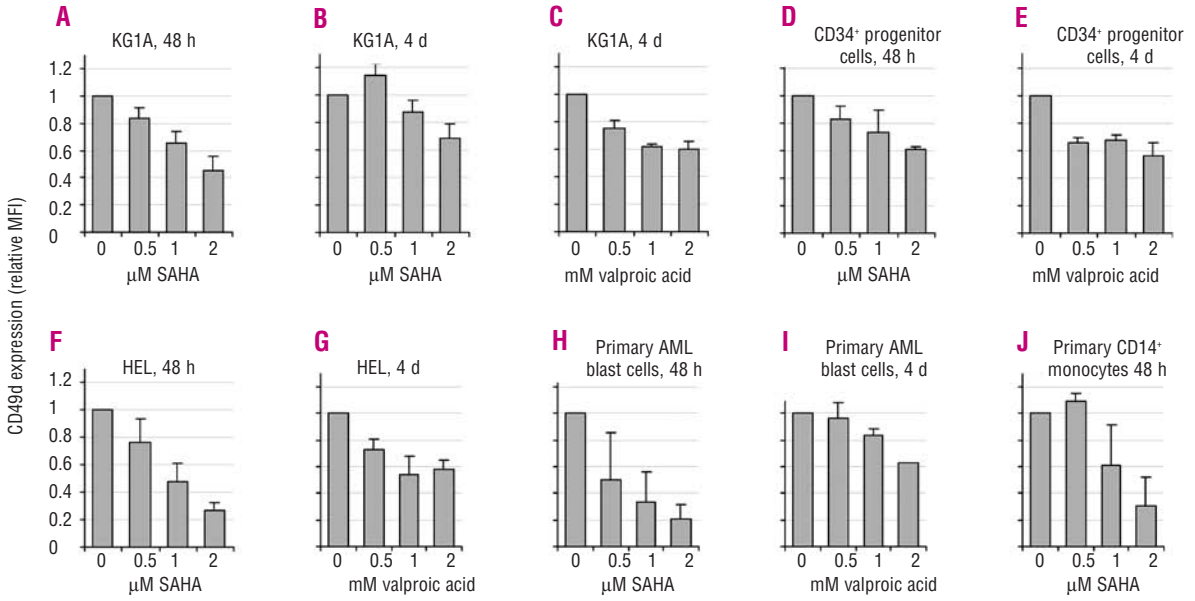

Figure 1. Effect of HDAC-inhibitor treatment on VLA-4 cell surface expression. (A-B) KG1A cells were treated with SAHA for 48 hrs or four days respectively. CD49d cell surface expression was decreased by up to 60%. (C) KG1A cells were treated with VPA for four days and a maximum downregulation of CD49 by 40% was observed. (D-E) A similar reduction to that seen in KG1A cells was observed in human CD34$^+$ hematopoietic progenitor cells from healthy donors. However, (F-G) in the acute myeloid leukemia cell line HEL, (H-I) as well as in primary blast cells from AML patients, HDAC-inhibitor treatment downregulated CD49d surface expression by up to 80%. (J) Similarly, on primary CD14$^+$ monocytes from healthy donors, SAHA treatment reduced CD49d expression by almost 80%. Cells were treated for either 48 hrs. or for four days. Only non-apoptotic cells were assessed by FACS analysis. The data are presented as relative geometric MFI (mean fluorescence intensity). Relative MFIs were determined in relation to the negative control (untreated cells). Averages and standard deviations have been calculated from 3–5 independent experiments.

Flow cytometry analysis

Cells were stained with phycoerythrin-cyanin 5 (PC-5) conjugated anti-CD34 and fluorescein isothyocyanate (FITC)-conjugated and phycoerythrin (PE)-conjugated anti-CD44, anti-CD18, anti-CD49e, anti-CD49d, anti-CD11a, anti-CD29 and anti-CD14 respectively. Up to 50,000 events were collected on a FACScan (Becton Dickinson Immunocytometry Systems, Mountain View, CA) using the CellQuest software.

Adhesion assay

KG1A cells were exposed to SAHA for 48 hrs. Cells were stained with PKH-26 (Sigma) according to the supplier's instructions and with propidium iodine (PI). PKH-26^+/PI^- cells were then sorted using an automatic cell-depositing unit on a fluorescence-activated cell sorting (FACS)–Vantage-SE flow cytometry system (Becton Dickinson, San Jose, CA). These cells were then allowed to rest for one hour. at 37°C and 5% CO_2 before 0.75×10^5 KG1A cells were added to the layer of confluent MSCs. Cells were then spun down at 210 g for one minute. to ensure a direct interaction between MSCs and KG1A cells and allowed to adhere for one hour. Cells were then washed four times in supplemented medium and were then shaken at 100 rpm for one minute. to get rid of non-adhering and weakly adhering cells. The remaining adhering cells were then counted by light microscopy.

Real-time polymerase chain reaction

Total RNA was prepared using RNeasy Mini Kit (Qiagen). Up to 10 µg of total RNA were applied in the High-capacity cDNA archive kit (ABI) for the generation of cDNA. TaqMan Gene Expression Assays (Applied Biosystems) were used to analyze CD49d mRNA expression according to the manufacturer's instructions. The thermal cycling conditions were 95 °C for two minutes, followed by 40 cycles at 95°C for 15 secs. and 60°C for one minute. Real-time polymerase chain reaction was performed as triplicates in 96 well plates using ABI Prism 7700 Sequence Detector (Applied Biosystems). Results were analyzed with the ABI Prism 7700 sequence detection system software (Applied Biosystems). Each reaction was normalized by the cycle threshold (Ct) of *gapdh* cDNA expression. Relative expression was calculated with the ΔΔCt method.

Results and Discussion

KG1A cells were treated either with the HDAC-inhibitor SAHA for 48 hrs. or with VPA for four days. The expression of a number of adhesion molecules was then assessed. These are known to be essential during

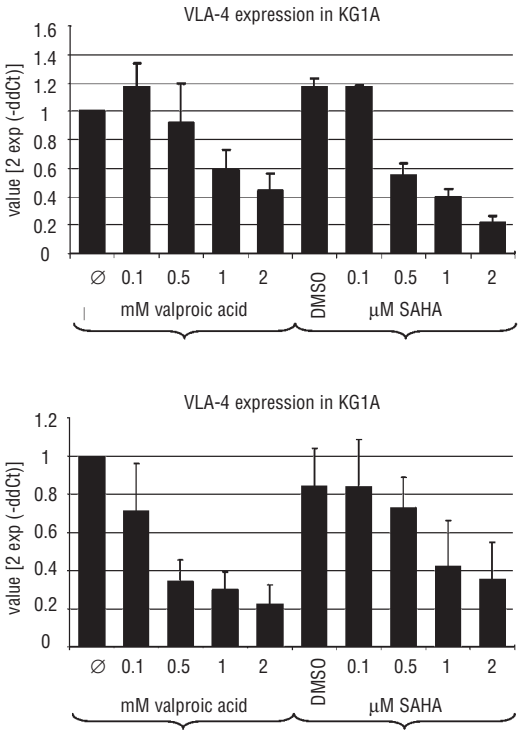

Figure 2. HDAC inhibitor treatment downregulates VLA-4 mRNA expression levels. Levels of mRNA were measured after 48 hours cultivation by real time PCR. Analysis of mRNA levels was performed in KG1A or HEL cells subsequent to treatment with either valproic acid at concentrations ranging from 0.1–2.0 mM or with SAHA at concentrations ranging from 0.1–2.0 µM. The relative amounts of mRNA were normalized to gapdh.

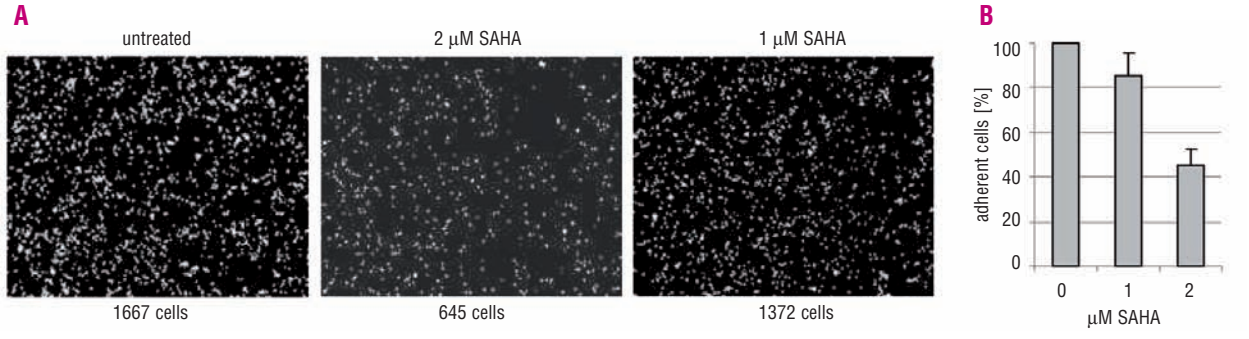

Figure 3. Effect of HDAC-inhibitor treatment on KG1A cell adhesiveness. KG1A cells were exposed to SAHA for 48 hrs. Cells were then stained with PKH-26 and PI as described and allowed to rest on a MSC layer for one hour. After vigorous shaking and several washing steps, adherent cells were counted by fluorescence microscopy. (A) Shows the adherent fluorescent cells. Results are representative of 3 experiments. (B) Shows the averages and standard deviations from 3 experiments. Results were normalized in relation to untreated adherent cells.

hematopoietic stem cell homing and engraftment, in hematopoietic stem cell and AML leukemic blast cell retention in the bone marrow, as one condition that favors MRD and as limiting factors in peripheral blood hematopoietic stem cell harvesting.[7,8,15]

The surface density of the VLA-4 α4 integrin subunit CD49d was downregulated on all cell types that have been assessed. On primary leukemic blast cells from 22 AML patients (AML M0, n=2; AML M1, n=5; AML M2, n=8; AML M4, n=5; AML M5 n=2) a downregulation of CD49d by 80% was observed, while on the AML cell lines KG1A and HEL and on healthy donor monocytes a decrease of up to 70% was seen. On $CD34^+$ hematopoietic progenitor cells, the CD49d surface density was downregulated by approximately 60% (Figure 1A-J), which was also confirmed by real-time PCR (Figure 2) and correlated inversely with increasing histone acetylation levels. In addition, cell surface expression of CD11a, a subunit of the LFA-1 receptor, was also markedly decreased, while SAHA treatment did not affect the expression of CD18, the second subunit of LFA-1, of CD49e or CD29 (VLA-5). These effects were seen in all myeloid cell lines tested (including HEL, KG1A, HL-60 and primary cells from AML patients), but were not observed in lymphoid cells. Unexpectedly, SAHA treatment increased the surface density of the hyaluronic acid receptor CD44 (*data not shown*). To further investigate whether the observed downregulation of the adhesion molecules did in fact have an impact on cellular adhesion itself, we carried out functional adhesion assays on KG1A cells in the presence or absence of SAHA. Untreated KG1A cells were found to strongly adhere to the MSC layer while adhesion was reduced by approximately 60% in the presence of 2 μM SAHA (Figure 3 a,b).

Since SAHA and VPA downregulate the surface expression of VLA-4, hematopoietic stem cell migration and homing to the bone marrow are likely to be severely impaired as a consequence of HDAC inhibitor treatment. On the other hand, optimal stem cell mobilization requires a significant downregulation of CD49d. The addition of HDAC-inhibitor treatment might, therefore, complement currently available hematopoietic stem cell mobilization protocols. In addition, we expect that the downregulation of VLA-4 on the surface of leukemic blast cells by Histone-Deacetylase inhibitors such as SAHA or VPA is likely to diminish leukemic blast cell anchorage within the bone marrow niches. This would considerably reduce bone-marrow minimal residual disease (MRD) after chemotherapy in patients with acute myelogenous leukemia (AML). This remains, however, to be further evaluated in the context of clinical studies.

To summarize, these data suggest that HDAC-inhibitor treatment is likely to be useful during hematopoietic stem cell mobilization and in the prevention of minimal residual disease from AML, while it is likely to be disadvantageous during engraftment after stem cell transplantation.

Authorship and Disclosures

UM designed the project and wrote the manuscript; CS carried out the experiments. The authors reported no potential conflicts of interest.

References

1. Imamura R, Miyamoto T, Yoshimoto G, Kamezaki K, Ishikawa F, Henzan H, et al. Mobilization of human lymphoid progenitors after treatment with granulocyte colony-stimulating factor. J Immunol 2005; 175:2647-54.
2. Lapidot T, Dar A, Kollet O. How do stem cells find their way home? Blood 2005; 106:1901-10.
3. Kollet O, Spiegel A, Peled A, Petit I, Byk T, Hershkoviz R, et al. Rapid and efficient homing of human CD34(+)CD38(-/low)CXCR4(+) stem and progenitor cells to the bone marrow and spleen of NOD/SCID and NOD/SCID/B2m (null) mice. Blood 2001;97:3283-91.
4. Peled A, Kollet O, Ponomaryov T, Petit I, Franitza S, Grabovsky V, et al. The chemokine SDF-1 activates the integrins LFA-1, VLA-4, and VLA-5 on immature human CD34(+) cells: role in transendothelial/stromal migration and engraftment of NOD/SCID mice. Blood 2000;95:3289-96.
5. van der Loo JC, Xiao X, McMillin D, Hashino K, Kato I, Williams DA. VLA-5 is expressed by mouse and human long-term repopulating hematopoietic cells and mediates adhesion to extracellular matrix protein fibronectin. J Clin Invest 1998; 102:1051-61.
6. Avigdor A, Goichberg P, Shivtiel S, Dar A, Peled A, Samira S, et al. CD44 and hyaluronic acid cooperate with SDF-1 in the trafficking of human CD34+ stem/progenitor cells to bone marrow. Blood 2004; 103:2981-9.
7. Kosugi H, Towatari M, Hatano S, Kitamura K, Kiyoi H, Kinoshita T, et al. Histone deacetylase inhibitors are the potent inducer/enhancer of differentiation in acute myeloid leukemia: a new approach to anti-leukemia therapy. Leukemia 1999; 13:1316-24.
8. Drummond DC, Noble CO, Kirpotin DB, Guo Z, Scott GK, Benz CC. Clinical development of histone deacetylase inhibitors as anticancer agents. Annu Rev Pharmacol Toxicol 2005;45:495-528.
9. Fu S, Liesveld J. Mobilization of hematopoietic stem cells. Blood Rev 2000;14:205-18.
10. Oostendorp RA, Dormer P. VLA-4-mediated interactions between normal human hematopoietic progenitors and stromal cells. Leuk Lymphoma 1997;24:423-35.
11. Matsunaga T, Takemoto N, Sato T, Takimoto R, Tanaka I, Fujimi A, et al. Interaction between leukemic-cell VLA-4 and stromal fibronectin is a decisive factor for minimal residual disease of acute myelogenous leukemia. Nat Med 2003;9:1158-65.
12. Scott LM, Priestley GV, Papayannopoulou T. Deletion of a4 integrins from adult hematopoietic cells reveals roles in homeostasis, regeneration, and homing. Mol Cell Biol 2003;23:9349-60.
13. Hernandez PA, Gorlin RJ, Lukens JN, Taniuchi S, Bohinjec J, Francois F, et al. Mutations in the chemokine receptor gene CXCR4 are associated with WHIM syndrome, a combined immunodeficiency disease. Nat Genet 2003; 34:70-4.
14. Hynes RO. Integrins: bidirectional, allosteric signaling machines. Cell 2002; 110:673-87.
15. Hart C, Drewel D, Mueller G, Grassinger J, Zaiss M, Kunz-Schughart LA, et al. Expression and function of homing-essential molecules and enhanced in vivo homing ability of human peripheral blood-derived hematopoietic progenitor cells after stimulation with stem cell factor. Stem Cells 2004;22:580-9.