Histone Deacetylase-targeted Treatment Restores Retinoic Acid Signaling and Differentiation in Acute Myeloid Leukemia

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Abstract

Histone deacetylase (HDAC)-dependent transcriptional repression of the retinoic acid (RA)-signaling pathway underlies the differentiation block of acute promyelocytic leukemia. RA treatment relieves transcriptional repression and triggers differentiation of acute promyelocytic leukemia blasts, leading to disease remission. We report that transcriptional repression of RA signaling is a common mechanism in acute myeloid leukemias (AMLs). HDAC inhibitors restored RA-dependent transcriptional activation and triggered terminal differentiation of primary blasts from 23 AML patients. Accordingly, we show that AML1/ETO, the commonest AML-associated fusion protein, is an HDAC-dependent repressor of RA signaling. These findings relate alteration of the RA pathway to myeloid leukemogenesis and underscore the potential of transcriptional/differentiation therapy in AML.

Introduction

The RA-signaling pathway is involved in the regulation of hematopoietic myeloid differentiation and is altered in APL, resulting in blockage of the differentiation of leukemic blasts (1–5). The APL-specific RARα fusion proteins PML/RARα and PLZF/RARα bind to and constitutively repress promoters of RA target genes via aberrant recruitment of multi-subunit complexes containing HDAC activities. HDACs induce deacetylation of nucleosomal core histone tails, leading to a chromatin conformation that correlates with gene silencing (6, 7). Consistent with a model in which HDACs are crucial molecular targets of APLs, treatment with HDAC inhibitors and/or RA reverts the blockage of differentiation of APL blasts in vitro and induces disease remission in vivo by inhibiting or releasing, respectively, the HDAC-RARα fusion repressor complex (8–11).

APL accounts for ~5–10% of AMLs (12). All other AML subtypes express RARs but are not sensitive to RA action on differentiation (13, 14). It is not known whether such unresponsiveness is a consequence of the leukemic state or whether it reflects an active repression of the RA-signaling pathway. Abrupt histone acetylation also results from chromosomal rearrangements associated to other AML subsets. In t(8;21) AML, for example, a stable association of AML1/ETO

4 U937-AML1/ETO (U937 AE) cells were obtained by electroporation into U937 cells of an HA-tagged AML1/ETO cDNA generated and subcloned into the Zn2+-inducible mouse MT-1 promoter as described (15, 21). Different neomycin-selected clones were screened for AML1/ETO expression before and after Zn2+ treatment using an anti-HA antibody (BabCO). The U937 MT-HA-AE clone 16 was selected because of its almost undetectable expression of AML1/ETO prior to Zn2+ treatment and high and persistent (up to 96 h) expression after Zn2+ treatment.

AML blasts and cell lines Kasumi-1 (AML-M2), U937 (AML-M5), and U937-AE were maintained in RPMI 1640 supplemented with 50 units/ml penicillin, 50 µg/ml streptomycin, and 10% FCS at 37°C in a humidified atmosphere containing 5% CO2. AML blasts were treated with the HDAC inhibitors TSA (WAKO Chemicals) or sodium phenylbutyrate (Sigma) for 1 h treatment.

Cell Proliferation and Differentiation. Cell proliferation and differentiation were evaluated and quantified by direct cell counting (trypan blue dye exclusion method) using a hemocytometer chamber, light microscopy morphological examination of Wright-Giemsa-stained cytospins, NBT dye reduction assay (at least 500 morphologically intact cells per experimental condition were counted and corrected for viability, measured by trypan blue exclusion method), cell cycle analysis of cells stained with propidium iodide, and direct immunofluorescence staining of CD11b cell surface myeloid-specific antigen (Coulter Epics XL flow cytometer; Beckman Coulter) as described previously.

Analysis of Acetylated H3 and H4 Histones. AML blasts were fixed in 100% methanol at room temperature for 5 min, followed by acetone for 2 min at −20°C, and incubated with anti-acetylated histone H3 antibody (Upstate Biotechnology) according to the manufacturer’s protocol. Immunofluorescence was detected using a Zeiss Axiosplan fluoromicroscope (Zeiss s.p.a.). Immunoblot analysis of total cell homogenates (60 µg) was performed using an anti-acetylated H4 antibody (Upstate Biotechnology) as described.

Transcriptional Assays. AML blasts were transiently transfectioned with the pBARE-tk-Luc, the TRE2-tk-Luc, or the RARβpr-Luc reporter (23, 24) by electroporation using a

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4 The abbreviations used are: RA, retinoic acid; APL, acute promyelocytic leukemia; RARα, retinoic acid receptor α; HDAC, histone deacetyase; AML, acute myeloid leukemia; RT-PCR, reverse transcription-PCR; NBT, nitroblue tetrazolium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TGFα, transforming growth factor alpha.

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Gene-Pulser II apparatus (Bio-Rad), pCDNA3 expression vector (mock) or pCDNA3s containing ETO, HA-AML1, or HA-AML1/ETO cDNAs (15, 25) were cotransfected with the above-described reporters in U937 cells. The plasmid encoding β-galactosidase (pSV-βgal) was cotransfected and used as an internal control and for normalization of transfections. Six h after electroporation, cells were treated for 16 h, lysed, and then assayed using the Luciferase Assay Kit (Promega) on a luminometer (Berthold).

### RNA Preparation and RT-PCR Analysis
Total RNA was extracted from Ficoll-Hypaque-isolated AML patient blasts as described (18). One microgram of total RNA was heated at 65°C for 10 min and used as a template for first-strand cDNA (cDNA) synthesis using SuperScript II RNase H Reverse Transcriptase and random hexamers as primers (Life Technologies). cDNAs were amplified for a total of 30 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s. Each PCR reaction contained 2 μl of the cDNA, 0.2 mM deoxyribonucleotide triphosphates, 1.5 mM MgCl2, and 2.5 units of PLATINUM Taq DNA Polymerase (Life Technologies). The RARα and AML1/ETO primers used in the reactions have been described previously (18, 26). The following oligonucleotides were used for RARβ and GAPDH transcripts: sense, 5′-AACGTT TGGCT ACCGG ACCAT GTTTG ACTGT ATGGA TG-3′; antisense, 3′-GAPDH primers using [32P]ATP and T4 kinase (Life Technologies). Radioactivities were detected using a Fuji BAS1800 scanner (Raytest) and analyzed by Advanced Image Data Analyzer (Raytest).

### TGase Activity Assay
The TGase activity assay was performed on total homogenate as described previously (22) by measuring the incorporation of [14C]Hypotaurine (12.6 Ci/mmol; Amersham Corp.) into casein in duplicate or triplicate cultures.

### Results and Discussion
Primary blasts from the peripheral blood and/or bone marrow of 23 newly diagnosed (non-APL) AML patients were included in this study. The morphologic and genetic features of AML blasts are shown in Table 1. None of the blasts expressed the APL-associated fusion proteins, as determined by RT-PCR using specific primers for the PML/RARα, CBFB/MYH11, DEK/CAN, BCR/ABL, and MLL rearrangements.

To investigate whether the effect of TSA correlated with modifications of histone acetylation in AML blasts, we evaluated the status of acetylation of histone H3 or H4 by immunofluorescence and Western blotting analysis, respectively, using specific antibodies. In untreated AML blasts, acetylated forms of histone H3 were almost undetectable, whereas they became detectable after treatment with RA or TSA used as single agents. Combination treatment of RA + TSA further increased acetylation of histone H3, as shown by the marked staining of the nucleus (Fig. 2a). Immunoblot analysis using an anti-acetylated histone H4 antibody (Fig. 2b) showed that combined treatment with RA and TSA or sodium phenylbutyrate (another inhibitor of HDACs already used in clinical trials), strongly induced acetylation of histone H4. Together, these results suggest that myeloid differentiation induced by RA + TSA might be the consequence of a combined effect at the level of RA target promoters: transcriptional derepression by TSA and transcriptional activation by RA.

Therefore, we investigated whether TSA affects RA-induced activation of RA target genes in AML blasts. In a first set of experiments, we evaluated the capacity of TSA to modify the RA response of RA target promoters transiently transfected into AML blasts. For this purpose, we used two reporter constructs: (a) RARβpr-Luc, which contains the entire promoter region (~5 kb to ~155 kb) of RARβ cloned 5′ to the luciferase reporter gene; and (b) RARE−tk-Luc, which contains three copies of the βRARE direct repeat sequence (DR5) of the RARβ promoter, cloned 5′ to the thymidine kinase promoter and the luciferase gene. RARβ is a natural RA target gene. Its induction has been proposed as a general mechanism by which retinoids inhibit growth and induce differentiation in cancer cells (27).

<table>
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<tr>
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<td>Complex aberrations⁴</td>
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* FAB, French-American-British classification.

b Differentiation (as evaluated by morphologic criteria): +, 10–20%; ++, 20–40%; +++, 50–80%; ++++, 80–100% more mature metamyelocytes and granulocytes than control cultures.

⁴ NN, normal karyotype. All cases with no detectable aberrations by conventional karyotyping were also negative by RT-PCR and Southern blot for the fusion genes PML/RARA, CBFB/MYH11, DEK/CAN, BCR/ABL, and MLL rearrangements.
was evaluated by semiquantitative RT-PCR analysis performed on total RNA prepared from AML blasts before or after treatment for 16 h with RA, TSA, or RA + TSA. Results revealed no modification of the levels of RARα and RARβ mRNA transcripts by TSA, slight up-regulation by RA alone, and significant up-regulation by RA + TSA in two AML-M2 cell lines, HL-60 and Kasumi-1 (data not shown). We then measured cytosolic type II TGase activity in four AML cases, two of which are shown in Fig. 3d. Type II TGase activity was strongly up-regulated after 3 days of treatment with RA + TSA (Fig. 3d). Therefore, it appears that TSA potentiates the RA induction of endogenous RA target genes RARα, RARβ, and type II TGase, strongly suggesting that the promoters of RA-responsive genes are repressed in AML blasts via mechanisms involving HDAC activities.

Transcriptional activation of RA target genes by RA + TSA correlates with the correction of a crucial component of the leukemic phenotype, e.g., the differentiation block. Thus, transcriptional repression of RA target genes in AMLs might be part of the same mechanism(s) that lead to transformation. Because different genetic events, the majority of which are still unknown, underlie myeloid leukemogenesis, repression of RA target genes might be a common event in AMLs. To investigate this possibility, we evaluated the ability of TSA to confer sensitivity to RA-induced differentiation in cells expressing the AML-associated fusion protein AML1/ETO. It has been shown that AML1/ETO represses transcription of AML1 target genes and myeloid differentiation by an aberrant recruitment of a complex containing HDAC activity (5, 15, 16).

We therefore evaluated the biological response to RA and TSA of the Kasumi-1 cell line, derived from an AML-M2 patient with the t(8;21) translocation and expressing the AML1/ETO fusion product. In this cell line, the level of AML1/ETO expression is comparable with that detectable in blasts from t(8;21) AML patients (see cases AML 14 and AML 19 in Fig. 4a). Four days of in vitro treatment with RA or TSA as single agents induced a modest effect on the growth
antibodies. By immunoblot analysis using anti-HA and anti-ETO antibodies, U937-AE and U937 transfected cells were quantified for expression levels of AML1, ETO, and AML1/ETO in type II TGase activity in Kasumi-1 cells.

Fig. 3. Inhibition of HDAC activities affects RA signaling on RA-responsive promoters and RA target genes in AML blasts. a, TSA cooperates with RA to activate βRARE3-tk-Luc reporter transiently transfected in primary blasts from AML patients. The numbers correspond to AML cases described in Table 1. Filled and hatched columns indicate luciferase activities in the absence or presence of RA (1 μM), respectively. In primary AML blasts, TSA (100 ng/ml) potentiates RA (1 μM) induction of RARA and RARβ mRNA expression (b and c) and type II TGase activity (d). GPDH, glyceraldehyde-3-phosphate dehydrogenase. Bars, SD.

and differentiation properties of Kasumi-1 cells. Combined RA + TSA treatments, instead, caused growth arrest, accumulation of cells in the G1 phase of the cell cycle, and increased NBT positivity and expression of differentiation marker CD11b (Table 2). In addition, flow-cytometric analysis of propidium iodide-stained cells revealed only a moderate increase in the frequency of apoptosis (5–10%) after treatment with RA (1 μM). RA in combination with TSA (100 ng/ml) showed slight up-regulation (~1.2-fold) by pharmacological doses of RA (1 μM) as single agent and strong up-regulation (15-fold) by the combined treatment with TSA (50–100 ng/ml; Fig. 4b). In contrast, in the presence of similar concentrations of TSA, RA treatment did not significantly affect the activity of the TRE2-tk-Luc reporter gene (a synthetic reporter containing the palindromic sequence TRE, which mediates both RA and thyroid hormone transactivation; Refs. 27, 28). A ligand-dependent assay showed that doses of TSA of 100 ng/ml also restored βRARE3-tk-Luc transactivation potency of RA as sole agent and strong up-regulation (15-fold) by pharmacological doses of TSA of 100 ng/ml.

We next evaluated the RA transcriptional response in Kasumi-1 cells. Analysis of the transfected βRARE3-tk-Luc promoter showed slight up-regulation (~1.2-fold) by pharmacological doses of RA (1 μM) as single agent and strong up-regulation (15-fold) by the combined treatment with TSA (50–100 ng/ml; Fig. 4b). In contrast, in the presence of similar concentrations of TSA, RA treatment did not significantly affect the activity of the TRE2-tk-Luc reporter gene (a synthetic reporter containing the palindromic sequence TRE, which mediates both RA and thyroid hormone transactivation; Refs. 27, 28). A ligand-dependent assay showed that doses of TSA of 100 ng/ml also restored βRARE3-tk-Luc transactivation potency of RA as sole agent and strong up-regulation (15-fold) by pharmacological doses of TSA of 100 ng/ml.

To investigate this possibility more directly, we measured the effects of AML1/ETO expression on RA response using U937-AE cells, which express the AML1/ETO cDNA under a Zn2+-inducible promoter, or U937 cells transiently transfected with the HA-AML1/ETO cDNA (see Fig. 4d for Western blotting analysis of U937-AE or

Fig. 4. Expression of AML1/ETO, the AML-associated fusion protein, induces transcriptional repression of RA signaling that is reverted by inhibiting HDAC activities. a, expression levels of AML1/ETO mRNA in cell lines and AML blasts (patients 14 and 19) were detected by RT-PCR using specific primers as described (26). b, TSA cooperates with RA to activate βRARE3-tk-Luc reporter, but not the synthetic TRE2-tk-Luc transiently transfected in the AML Kasumi-1 cell line, which expresses the AML1/ETO fusion product. Filled and hatched columns indicate luciferase activities in the absence or presence of RA (1 μM), respectively. A RA dose-dependent assay indicates that 100 ng/ml TSA restores the activation of βRARE3-tk-Luc reporter by physiological RA concentrations (1–10 nM) in transfected Kasumi-1 cells. c, TSA cooperates with RA to induce type II TGase activity in Kasumi-1 cells. d, expression levels of AML1, ETO, and AML1/ETO in U937-AE and U937 transfected cells were quantified by immunoblot analysis using anti-HA and anti-ETO antibodies. e, effect of 1 μM RA, 100 ng/ml TSA and RA + TSA in induction of the reporter βRARE3-tk-Luc transiently transfected into U937, U937-MT-1, or the U937-AE clone expressing AML1/ETO under the control of the Zn2+-inducible mouse MT-1 promoter. Open and hatched columns indicate luciferase activities in the absence or presence of Zn2+ (100 μM), respectively.
AML1/ETO-transfected U937 cells. In the absence of AML1/ETO expression, (Fig. 4e, U937-MT, U937-Æ –Zn, and U937 Mock), the activity of the βRARE<sub>2</sub>-tk-Luc promoter was induced ~50-fold by RA and potentiated by TSA by a factor of 10. Strikingly, the induction of AML1/ETO expression in these cells led to a marked reduction (~75%) of the RA-induced transactivation of the βRARE<sub>2</sub>-tk-Luc. Notably, TSA treatment relieved transcriptional repression by AML1/ETO (Fig. 4e). Thus, it appears that in myeloid cells the β-RARE element, which is present in promoters of RA target genes, including myeloid genes (1), is activated by the combined RA + TSA treatment and is repressed by AML1/ETO expression.

To evaluate the specificity of the effect of AML1/ETO on the RA-signaling pathway, we next analyzed the effects of AML1, ETO, and AML1/ETO on RA-dependent transactivation. To this end, we performed cotransfection experiments in U937 cells using the expression vectors for ETO, AML1B, or AML1/ETO and the βRARE<sub>2</sub>-tk-Luc promoter. Relative AML1, ETO, and AML1/ETO expression levels in U937-AE and U937 transfected cells were measured by immunoblotting analysis using an anti-HA (recognizing HA-AML1 and HA-AML1/ETO) or an anti-ETO (recognizing overexpressed ETO, but not the AML1/ETO fusion) antibody,<sup>5</sup> as shown in Fig. 4d (for a representative experiment). Results showed that (a) overexpression of ETO or AML1 did not repress RA- or RA + TSA-induced βRARE<sub>2</sub>-tk-Luc activity (Fig. 4e); a slight increase in βRARE<sub>2</sub>-tk-Luc activity was seen after ETO expression), and (b) induction of AML1/ETO expression by Zn<sup>2+</sup> treatment (in the U937-AE clone) or by transient transfection (into the parental U937 cells) strongly repressed RA-induced βRARE<sub>2</sub>-tk-Luc activity (Fig. 4e). Taken together, these results suggest that AML1/ETO (but not AML1 or ETO), when expressed in myeloid cells, acts as a specific transcriptional repressor of the RA pathway.

Indirect evidence suggest that common mechanisms underlie myeloid leukemogenesis: (a) The genes involved in the AML-associated translocations invariably encode transcription factors (e.g., AML1, CBFB, RARα, MLL, p300, CBP, HOX genes, and EVI1), which are physiologically involved in hematopoietic differentiation and cooperatively regulate promoters or enhancers present on myeloid specific genes (1, 5, 12). (b) Accumulation of undifferentiated precursors is a prominent feature of the myeloid leukemia phenotype, and ectopic expression of fusion proteins in hematopoietic precursors induces blockage of differentiation and leukemia in animal models (10, 29–32). (c) Aberrant recruitment of HDAC complexes is crucial to the activity of the AML-specific fusion proteins PML/RARα, PLZF/RARα, and AML1/ETO (8–10, 15, 16), suggesting that modification of the chromatin structure in the target promoters of fusion proteins represents an important mechanism of leukemogenesis. Thus, it is conceivable that different genetic alterations may result in common patterns of deregulated gene expression, leading to blockage of differentiation and favoring myeloid leukemogenesis. Here we show that in AMLs, regardless of their underlying genetic alteration, the RA-signaling pathway is constitutively repressed through an HDAC-dependent mechanism and that inhibition of HDAC activities restores the RA-differentiation response. These results indicate that repression of the RA-signaling pathway is a general pathogenetic event in AMLs and that HDACs are common targets for AMLs, highlighting the possibility of transcriptional/differentiation therapy in AMLs other than APL.

Butyrate has been used as single agent in the treatment of an AML patient resistant to conventional chemotherapy, and was shown to trigger terminal differentiation of leukemic blasts in vivo (33). The usage of butyrates and their derivatives as HDAC inhibitors, however, is limited by their poor specificity, as well as by their transient and reversible activities. Indeed, high drug plasma concentrations must be sustained to obtain a biological effect (11). In contrast, TSA is a highly specific, stable, and potent HDAC inhibitor. We recently demonstrated that “in vivo” modulation of gene transcription by micromolar concentrations of TSA is not toxic in adult mice and does not perturb mouse embryonic or postnatal development,<sup>6</sup> thereby suggesting that TSA might represent a useful agent for transcriptional/differentiation therapy in AMLs.

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References


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