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 most common 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 censors of genes encoding transcription factors, we investigated whether the poor RA sensitivity of nonpromyelocytic AMLs could reflect an active, HDAC-dependent repression of RA transcription signaling pathway.

Materials and Methods

Cell Cultures. Bone marrow and/or peripheral blood were obtained from 23 informed, newly diagnosed AML patients. Cases were classified as AML-M2 or AML-M4 according to the French-American-British classification and showed an initial percentage of circulating blasts >80% (17). Cyto genetic analysis and RT-PCR to rule out the presence of the APL-associated fusion genes were performed according to standard methods as described (18–20).

The 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-MHA-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 before the addition of RA (Sigma) and during the RA treatment.

Cell Proliferation and Differentiation. Cell proliferation and differentiation were evaluated and quantified by direct cell counting (trypsin 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 trypsin 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 (21, 22).

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 Axioplan fluorescence microscope (Zeiss s.p.a.). Immunoblot analysis of total cell homogenates (60 μg) was performed using an anti-acetylated H4 antibody (Upstate Biotechnology) as described (22).

Immunoreactivity was determined using the ECL method (Amersham). An anti-acetylated H4 antibody (Upstate Biotechnology) was used to detect acetylated H4. The signal was quantified by densitometry (Fuji LAS-1000). The data are given as the mean ± SEM of three independent experiments.

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The authors contributed equally to the work.
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 internal control and for normalization of reactions. 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 Ficol-Hyphaque-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 deoxynucleotide 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'-AAGCT TGGTG ACCGC ACCAT GTTGT ATGGA TG3'- and antisense, 5'-AGCCC TTACA TCCCT CACAG-3' for RARβ; sense, 5'-CGGGA AGCTG GTGAT CAATGG-3', and antisense, 5'-GGCAG TGATG GCATG GACTG-3', for GAPDH (used as an internal control). PCR products were electrophoresed on a 1.2% agarose gel. Blots were hybridized with end-labeled 35S primers using [32P]ATP and 3T4 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 [3H]putrescine (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 gene PML/RARA, CBFB/MYH11, DEK/CAN, BCR/ABL, and MLL rearrangements.

We next evaluated the capacity of RA and TSA to activate transcription of endogenous RA target genes. Four AML cases were analyzed for the effects of TSA and RA on the expression of the RA target genes RARα and RARβ (27). Expression of RARα and RARβ

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>FAB¢</th>
<th>Karyotype</th>
<th>Differentiation* (5-day cultures)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M4</td>
<td>Inv(16)(p13q22)</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>2</td>
<td>M4EO</td>
<td>del(20)(q13)</td>
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<td>3</td>
<td>M2</td>
<td>Complex aberrations‡</td>
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<td>del(20)(q13)</td>
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<td>M2</td>
<td>del(4)(q21q26)</td>
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<td>t(8;21)(q22q22)²</td>
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<td>NN</td>
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<tr>
<td>23</td>
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<td>NN</td>
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</tbody>
</table>

¢ FAB, French-American-British classification.

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

‡ 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, CBFβ/MYH11, DEK/CAN, BCR/ABL, and MLL rearrangements.

4,4,4X.ter1(11ins11111:3)(p21q21)(t:17(q12p12);der8)(8(3);12(q7);8(3);12(q7);8(3);12(q7);8(3);12(q7);8(3);12(q7);8(3);12(q7);8(3);12(q7));17.

46.XY/46.XY(t:8;21)(q22q22)/46 idem del(9)(q13q22)/45.XX,der(1)ins(1;3)(p21;q?)t(1;17)(q21;p12);der8)(8(3);12(q7);8(3);12(q7);8(3);12(q7);8(3);12(q7);8(3);12(q7);8(3);12(q7);8(3);12(q7);8(3);12(q7)).

We next evaluated the capacity of RA and TSA to activate transcription of endogenous RA target genes. Four AML cases were analyzed for the effects of TSA and RA on the expression of the RA target genes RARα and RARβ (27). Expression of RARα and RARβ...
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 is a gene transcriptionally regulated by retinoids and recently shown to be a marker of RA-induced differentiation in APL cells (8, 22). 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 leukaemogenesis, 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
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) 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 βRARE2-tk-Luc transcriptional response to physiological concentrations of RA (1–10 nM; Fig. 4b).

The calculated EC50 values (concentrations that give 50% efficacy) for βRARE2-tk-Luc transactivation potency of RA as sole agent and RA + TSA were 240 and 9 nM, respectively. In Kasumi-1 cells, TSA greatly increased the effects of RA treatment on acetylation of histones H3 and H4 and type II TGase activity (data not shown and Fig. 4c). These findings, together with the results obtained in blasts from two AML patients (AML patients 14 and 19) expressing the AML1/ETO fusion (Figs. 1–4 and data not shown), strongly suggest that RA signaling is repressed in AML1/ETO-expressing cells and that this repression can be relieved by inhibition of HDAC activity. Thus, AML1/ETO might act as a transcriptional repressor of the RA pathway.

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 U937).
AML/ETO-transfected U937 cells. In the absence of AML1/ETO expression, (Fig. 4e, U937-MT, U937-AE −Zn, and U937 Mock), the activity of the βRARE$_{2}$-tk-Luc promoter was induced ~50-fold by RA and potentiatiated 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$_{2}$-tk-Luc. Notably, TSA treatment relieved transcriptional repression by AML1/ETO (Fig. 4f). 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$_{2}$-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) antibody, 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$_{2}$-tk-Luc activity (Fig. 4e; a slight increase in βRARE$_{2}$-tk-Luc activity was seen after ETO expression), and (b) induction of AML1/ETO expression by Zn$^{2+}$ treatment (in the U937-AE clone) or by transient transfection (into the parental U937 cells) strongly repressed RA-induced βRARE$_{2}$-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 EVII), 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, thereby suggesting that TSA might represent a useful agent for transcriptional/differentiation therapy in AMLs.

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References


Table 2 Combined effect of RA + TSA on growth and differentiation of Kasumi-1 cells.

<table>
<thead>
<tr>
<th>Treatment</th>
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<th>NBT (%)</th>
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<td>(4 days)</td>
<td>(× 10$^3$)</td>
<td>(AU)</td>
<td>G2 S G3</td>
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<tr>
<td>Control</td>
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<td>11.8</td>
<td>8.1</td>
</tr>
<tr>
<td>TSA (50 ng/ml)</td>
<td>11.4</td>
<td>10.7</td>
<td>18.9</td>
</tr>
<tr>
<td>RA (1 μM)</td>
<td>9.4</td>
<td>15.5</td>
<td>22.7</td>
</tr>
<tr>
<td>RA + TSA</td>
<td>6.8</td>
<td>24.8</td>
<td>62.5</td>
</tr>
</tbody>
</table>

* AU, arbitrary units.

AML DIFFERENTIATION BY HDAC TARGETED TREATMENT


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