In vivo analysis of the role of aberrant histone deacetylase recruitment and RAR\(\alpha\) blockade in the pathogenesis of acute promyelocytic leukemia

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The promyelocytic leukemia–retinoic acid receptor \(\alpha\) (PML–RAR\(\alpha\)) protein of acute promyelocytic leukemia (APL) is oncogenic in vivo. It has been hypothesized that the ability of PML–RAR\(\alpha\) to inhibit RAR\(\alpha\) function through PML-dependent aberrant recruitment of histone deacetylases (HDACs) and chromatin remodeling is the key initiating event for leukemogenesis. To elucidate the role of HDAC in this process, we have generated HDAC1–RAR\(\alpha\) fusion proteins and tested their activity and oncogenicity in vitro and in vivo in transgenic mice (TM). In parallel, we studied the in vivo leukemogenic potential of dominant negative (DN) and truncated RAR\(\alpha\) mutants, as well as that of PML–RAR\(\alpha\) mutants that are insensitive to retinoic acid. Surprisingly, although HDAC1–RAR\(\alpha\) did act as a bona fide DN RAR\(\alpha\) mutant in cellular in vitro and in cell culture, this fusion protein, as well as other DN RAR\(\alpha\) mutants, did not cause a block in myeloid differentiation in vivo in TM and were not leukemogenic. Comparative analysis of these TM and of TM/PML\(^{-}\) and p53\(^{-}\) compound mutants lends support to a model by which the RAR\(\alpha\) and PML blockade is necessary, but not sufficient, for leukemogenesis and the PML domain of the fusion protein provides unique functions that are required for leukemia initiation.

Acute promyelocytic leukemia (APL) is characterized by nonrandom reciprocal translocations that always involve the retinoic acid receptor \(\alpha\) (RAR\(\alpha\)) gene on chromosome 17. RAR\(\alpha\) fuses to the promyelocytic leukemia (PML) gene in the vast majority of APL cases (1, 2). These chromosomal translocations generate X–RAR\(\alpha\) and RAR\(\alpha\)–X fusion proteins. X–RAR\(\alpha\) fusion proteins are oncogenic in vivo (2–6).

APL is characterized by a distinctive block of differentiation at the promyelocytic stage of myeloid development and by unique sensitivity to retinoic acid (RA) treatment (1, 2). RAR\(\alpha\) binds to retinoic acid response elements (RARE) as a heterodimer with RXR\(\alpha\) (1). In the absence of RA, the RAR\(\alpha\)/RXR\(\alpha\) heterodimer inhibits transcription through recruitment of histone deacetylases (HDACs; e.g., HDAC1), nuclear receptor corepressors such as N-CoR or SMRT, and DNA methyltrasferases (DNMT) (7). In the presence of a physiological concentration of RA (10\(^{-8}\) M), the RAR\(\alpha\)/RXR\(\alpha\) heterodimer dissociates from the HDAC complex and recruits transcriptional coactivators (8). In contrast, at physiological RA concentration, PML–RAR\(\alpha\) protein acts as a dominant negative (DN) RAR\(\alpha\) by forming aberrant complexes with HDAC and DNMT through the PML moiety of the fusion protein (4, 8–11). At a pharmacological dose of RA, PML–RAR\(\alpha\) releases the HDAC complex and activates transcription, thus mimicking RAR\(\alpha\). Point mutations have been reported in the RAR\(\alpha\) ligand–binding domain of PML–RAR\(\alpha\) in cases with acquired resistance to RA (12). Collectively, these data suggest that aberrant recruitment of HDAC to RARE represents...
RESULTS AND DISCUSSION

To determine whether aberrant HDAC-dependent transcriptional repression is necessary and sufficient for leukemogenesis, we generated transgenic mice harboring the following: (a) DN RARα mutants along with their PML–RARα counterpart and (b) an artificial HDAC–RARα fusion protein along with its enzymatically inactive counterpart. We also studied in vivo an RARα truncated mutant corresponding to the moiety of RARα invariably shared by all the APL fusion proteins (1, 2) (Fig. 1A).

RARαE carries a glycine (G) to glutamate (E) substitution at amino acid 303 in the RARαE domain that is responsible for ligand binding. This mutation leads to RA resistance and in vivo photocopies the RARα KO phenotype (13). RARαE harbors a leucine (L) to proline (P) substitution at amino acid 398 in domain E; and PML–RARα harbors the same mutation found in RARαE (14). This mutation leads to RA-insensitive transcriptional repression (14).

HDAC1–RARα expresses the full-length HDAC1 coding sequence fused to RARα. HDAC1 is part of the aberrant PML–RARα transcription (4, 9, 10). mHDAC1–RARα carries a point mutation that abrogates HDAC1 enzymatic activity (histidine to phenylalanine at HDAC1 amino acid 199) (15). ΔRARα carries a deletion that removes domain A from RARα. This deletion is identical to the one observed in the X-RARα fusion proteins and removes a domain responsible for transcriptional activation function (1, 16). These constructs were cloned in the human cathepsin-G (hCG) minigene (3, 4) and used to generate transgenic lines (Fig. 1 B and C).

We assessed whether HDAC1–RARα displayed the distinctive features of the X-RARα fusion proteins. We found that HDAC1–RARα can homodimerize and heterodimerize with RXRα within the cell (Fig. 2, A and B). HDAC1–RARα can effectively bind to the DR5 consensus sequence. Electromobility shift analysis (EMSA) produced a single HDAC1–RARα protein DNA complex, whereas HDAC1–RARα with RXRα formed two complexes (Fig. 2 C). These bands were abolished by the addition of an excess of unlabeled DR5 and super shifted with specific antibodies (Fig. 2 C). These data demonstrate that HDAC1–RARα forms homodimeric and, and more efficiently, heterodimers that are able to bind to the DR5 consensus sequence, as previously demonstrated in the case of other APL fusion proteins (17, 18).

Next, we investigated whether HDAC1–RARα acts as a transcriptional repressor. Vectors expressing RARα, PML–RARα, PLZF–RARα, HDAC1–RARα, mHDAC1–RARα, and HDAC1 were transfected into 293T cells together with RARβ-luc, a luciferase reporter construct containing the RARβ-responsive promoter region of hCG. Luciferase assays demonstrated that HDAC1–RARα acted as a potent transcriptional repressor (Fig. 3 A). As expected as the result of disruption of HDAC1 enzymatic activity, mHDAC1–RARα showed a much weaker transcriptional repression. HDAC1–RARα, PLZF–RARα, and PML–RARα repressed transcription equally well in the presence of RA, whereas mHDAC1–RARα did not (Fig. 3 A). HDAC1–RARα, therefore, acts as an aberrant transcriptional repressor and this property depends on the HDAC1 enzymatic activity.
Figure 2. Biochemical properties of HDAC1-RARα. (A) HDAC1-RARα homo- and heterodimerize in vivo. 293T cells were transfected as indicated. Immunoprecipitation (IP) was performed with the anti-Flag antibody and Western blots with the anti-Xpress antibody (top). The blot was stripped and rehybridized with anti-RXRα antibody (bottom). Arrows indicate specific bands; (B) HDAC1-RARα heterodimerizes with RXRα within the cell. 293T cells were transfected as indicated. IP was performed with anti-Flag and immunoblot blot analysis was performed with an anti-RXRα antibody (top). The blots were stripped and rehybridized with anti-Flag antibody (bottom). Flag-RARα was used as a positive control. (C) HDAC1-RARα homodimers and HDAC1-RARα/RXRα bind to DR5 in vitro (top). In vitro translated proteins were incubated with 32P-labeled DR5 probe as indicated and resolved by electrophoresis. Competition and bandshift experiments were performed as indicated (bottom). Arrows indicate specific protein–DNA complexes. HR: HDAC1-RARα homodimer, het: heterodimer, *, supershifted band with anti-RXRα antibody; **, nonspecific bands.

Chromatin immunoprecipitation (ChIP) experiments on the promoter of the cytoplasmic retinoic acid binding protein II (CRABP-II) gene revealed that HDAC1-RARα inhibited acetylation of histone H3 (Fig. 3 B). HDAC1 and HDAC1–RARα both inhibited histone H3 and H4 acetylation by the bromodomain of the p300 protein (19). This inhibition was partially abrogated with mHDAC1–RARα (Fig. 3 C). Thus, HDAC1–RARα displays HDAC activity.

Because both PML-RARα and PLZF-RARα block TGFβ1 and vitamin D₃-induced cellular differentiation of U937 cells (20, 21), we tested whether constitutive expression of HDAC1–RARα affected cellular differentiation upon TGFβ1 and vitamin D₃ treatment. We found a significant reduction in the induction of the myeloid marker CD11b in cells transfected with MIGR1-PLZF–RARα (P = 0.01, calculated by the Student’s t test) and MIGR1-HDAC1–RARα (P = 0.02, calculated by the Student’s t test), whereas MIGR1-mHDAC1–RARα and MIGR1-HDAC1 exerted no significant effect on myeloid differentiation (Fig. 3 D).

Collectively, these data suggest that HDAC1–RARα shares many of the features of the X-RARα protein, including its ability to act as a transcriptional repressor of RARα through HDAC activity.

We derived six ΔRARα, six RARαE, four RARαM, five PML–RARαM, three HDAC1–RARα, and three mHDAC1–RARα hCG-transgenic lines (Figs. 1 B and 4 A) (3, 4). The transgene was invariably expressed (Fig. 1 C). Leukemia was observed in three PML–RARαM transgenic lines. Latency was 8–9 mo (Fig. 4 A), in agreement with what we observed in PML–RARα transgenic lines (3). Strikingly, only 1 of the RARαE transgenic lines out of the 19 lines expressing DN RARα mutants (ΔRARα, RARαM, RARαE, and HDAC1–RARα) developed leukemia after a long latency (18–19 mo) and at low penetrance (Fig. 4, A–C).

Morphological analysis of the leukemic bone marrows and spleens revealed the presence of blasts with promyelocytic features. Flow cytometric analysis with Mac-1, Gr-1, c-kit, B220, CD3, and Ter119 cell surface markers of the confirmed diagnosis of APL (Fig. 4, B and C, and not depicted). RARαE-induced leukemias were transplatable in secondary recipients and leukemic mice showed no response to RA treatment as compared with PML–RARα leukemic mice (Fig. 4 D) (mean survival time: 10.4 d; 95% confidence interval = 1.9–18.9 d vs. mean survival time: 44.3 d; 95% confidence interval = 36.7–51.9 d) (22).

The RARα gene is invariably involved in the APL-associated chromosomal translocations (1, 2). Therefore, alteration of RARα pathway has been thought to play a central role in APL pathogenesis. Indeed, RA inhibits the proliferation of hematopoietic precursors and promotes the terminal granulocytic differentiation of granulocyte/monocyte progenitors and multipotent erythroid/monocytic cells. Vitamin A deficiency, unligated RARα, RARα antagonist, or DN RARα can block myeloid differentiation (23). Moreover, the X–RARα fusion proteins can block differentiation when overexpressed in myeloid leukemia cell lines
such as U937 cells and interference with PML function seems not to be required for this function (20, 21). These observations support the notion that DN blockade of the RARα pathway is crucial for APL leukemogenesis. Our in vivo genetic analysis challenges this notion, allowing us to reach three major conclusions.

Figure 3. Biological properties of HDAC1-RARα. (A) HDAC1-RARα is a transcriptional repressor. Luciferase assay in transfected 293 cells. (black bars) RA-treated cells; (white bars) untreated cells. Luciferase activities were expressed relative to the value of lysates transfected with the reporter alone. (B) HDAC1-RARα binds to RARE and deacetylates histone H3. (top) ChIP assay on lysate of transiently transfected 293T cells with the indicated antibodies. PCR was performed with RARE specific primers. (middle) PCR analysis performed with RARE-specific primers on the cell lysates was used for ChIP assay. (bottom) The intensity of the bands was determined by densitometry. The value obtained from the lysate transfected with the empty vector is expressed as 1. (C) HDAC1-RARα represses the acetylation of histone H3 and H4 induced by BrHAT. 293T cells were cotransfected as indicated and the lysate was immunoblotted. The same membrane was hybridized and stripped in series with the indicated antibodies. The arrows indicate transfected Flag-tagged proteins (bottom). The ratio of acetylated/total histone levels was assessed by densitometry and provided by the histogram at the bottom. The value obtained from the lysate transfected with pCMV alone is expressed arbitrarily as 100%. The value for acetylated H3/total H3 and acetylated H4/total H4 is provided. (D) HDAC1-RARα inhibits the differentiation of U937. Cells were retrovirally transduced as indicated. Transduced cells were isolated by cell sorting and cultured with or without 2 ng/ml of TGFβ1 in addition to 500 ng/ml of vitamin D3 for 96 h. Expression of CD11b was detected by flow cytometry. (black bars) percentage of treated cells expressing CD11b; (white bars) untreated cells. After treatment, CD11b expression is significantly reduced in PLZF-RARα (P = 0.01) and HDAC1-RARα (P = 0.02). Error bars indicate standard deviations.
The first major conclusion is that HDAC1-dependent DN blockade of RARα function is neither sufficient to cause leukemia nor to block myeloid differentiation in vivo. The fact that only PML–RARα and PML–RARαM4 (which retain the X moiety), but none of the other DN RARα mutants triggered leukemia in multiple transgenic lines demonstrates that inhibition of RARα per se is not sufficient to initiate leukemogenesis. Our experiments do not rule out that HDAC-chimeric constructs other than HDAC1–RARα may display a leukemogenic effect. Indeed, corepressors do not solely recruit HDAC1, but also other types/classes of histone deacetylases, and PML interacts with both HDAC1

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**Figure 4. Characteristics of leukemias induced by PML–RARαM4 and RARαE.** (A) Schematic representation of transgenic lines and respective leukemia incidence. (B) Peripheral blood (PB) and bone marrow cells (BM) from representative leukemic RARαE, PML–RARαM4, and PML–RARα transgenic mice stained with the Wright-Giemsa stain (×1,000). Note the presence of leukemic blasts in both BM and PB. (C) Flow cytometric analysis of BM cells from representative leukemic RARαE, PML–RARαM4, and PML–RARα transgenic mice. Anti–Mac-1 and c-kit antibodies were used. (green line) Isotypic control. The percentages of positive cells are given in the respective histograms. (D) Leukemia induced by RARαE is resistant to RA treatment in vivo. Percentage of leukemic cells present in the PB of two RARαE transgenic mice (blue) and three nude mice (red) transplanted with leukemic cells obtained from RARαE transgenic mice. The horizontal axis indicates the length of treatment in days. Crosses indicate the time of death of each animal. RA had no impact on the percentage of leukemic cells present in the PB.
and 2. However, prior observations support this conclusion as PLZF-RARα, transgenic mice develop leukemia, but not a block of myeloid differentiation, whereas RARα−/− mice display a normal myeloid differentiation (4, 24).

The second major conclusion is that only one out of the six RARαE lines developed APL after a long latency (1.5 yr) with very low incidence. This observation strongly suggests that blockade of RARα function is necessary, but not sufficient, for leukemogenesis. Interestingly, these leukemias were resistant to RA, demonstrating that RARαE functions in these leukemic cells as an RA-insensitive receptor.

The third major conclusion is that PML moiety is important in leukemogenesis not solely because it permits aberrant recruitment of HDAC1 and HDAC2, DNMT or homodimerization (11, 18, 25, 26) but also because it interferes with the tumor suppressive function of the wild-type PML gene product. Indeed, only PML-RARα and PML-RARαM1 lead to a DN disruption of the PML-NB both in vitro and in vivo (unpublished data). The critical role of PML functional inactivation is further underscored by the fact that APL is dramatically accelerated in M4 lead to a DN disruption of the PML-NB both in vitro and in vivo (unpublished data). The critical role of PML functional inactivation is further underscored by the fact that APL is dramatically accelerated in vivo by the fact that APL is dramatically accelerated in PML-RARα/PML−/− mice (27). In addition, through the PML/X moiety, the fusion protein acquires aberrant gain-of-function properties (e.g., aberrant DNA binding activity) (28).

Indeed, it has been shown that PML-RARα homodimer binds specific DNA sites that are not preferentially recognized by the RARα/RXRα heterodimer, thus suggesting the possibility that X-RARα may exert oncogenic functions that are not derived from its DN activity against the RARα/RXRα heterodimer (28, 29). This is supported by the fact that neither RARαM1 nor RARαE triggered leukemia even in the absence of PML: MR8–RARαM1/PML−/− and MR8–RARαE/PML−/− mice did not develop leukemia during a 12-mo follow up (unpublished data and Kogan, S., personal communication). Interestingly, RARαM1 did not trigger leukemia in the absence of p53, either; MR8–RARαM1H4/p53−/− compound mutants succumbed to lymphoma with incidence and onset similar to p53 null mice (Kogan, S., personal communication).

We propose a model by which the combined inactivation of the X and RARα pathways are both required, but not sufficient, for tumor initiation. PML-RARα is bestowed with additional PML-dependent functional gains that critically contribute toward full-blown transformation. Nevertheless, additional genetic abnormalities are required for leukemogenesis even in the presence of the full-length oncogenic fusion protein, as strongly suggested by the long leukemia latency observed in any of the X-RARα transgenic models and the recurrent chromosomal abnormalities that the leukemic blasts from these models invariably harbor at presentation (30, 31).

On the basis of this model, it remains to be explained why RA and HDAC inhibitors are effective in APL treatment. In this respect, it is tempting to speculate that the blockade of the RARα pathway, while not sufficient for leukemia initiation, may be necessary for leukemia maintenance.

MATERIALS AND METHODS

Cells and expression vectors. Cells were obtained from the American Type Culture Collection. Vitamin D3 was obtained from Sigma-Aldrich and TGFβ1 was obtained from PeproTech. Plasmids expressing RARα, RXRα, RARαM1, PML–RARα, PML–RARαM1, PLZF–RARα, HDAC1–FLAG (provided by P. A. Marks and V. Richon, Memorial Sloan Kettering Cancer Center, New York, NY), His–BrHAT (provided by A. Tomita, Nagoya University, Nagoya, Aichi, Japan), and RARαE have been described previously (4, 13, 14, 19, 32). pSG5–HDAC1–RARα carries the full-length HDAC1 gene fused in frame with the full-length RARα. Mutant HDAC1–RARα (mHDAC1–RARα) was generated by site-directed mutagenesis. pSG5–ΔRARα was generated by PCR. pCMV–PML–RARα, pCMV–HDAC1–RARα, pCMV–mHDAC1–RARα, and pCMV–HDAC1 are pCMV–Tag2B (FLAG-tagged) derivative (Stratagene). pCDNA3.1/His–HDAC1–RARα, pCDNA3.1/His–RXRα, pCDNA3.1/His–RARαE, and pCDNA3.1/His–RARαM1 were obtained by cloning the respective cDNAs into pCDNA3.1/His–C (Invitrogen). To generate retroviral constructs, Flag–tagged RARα, PLZF–RARα, HDAC1–RARα, and HDAC1 were cloned into pMIGR1. The sequence of each vector was confirmed sequencing.

Transgenic mice. RARα mutants were cloned into the Sall site of the hCG minigene vector (3, 4). All constructs were sequenced. Egg injection was performed as described previously (3, 4). The mouse studies were approved and overseen by the Institutional Animal Care and Use Committee.

Antibodies, immunoprecipitations, and Western blot analyses. We used the antibodies specific for: RARα (C-20) and RXRα (D-20) (Santa Cruz Biotechnology, Inc.), histone H3, H4, acetylated histone H3 and H4 (Upstate Biotechnology); PML (Chemicon International), M2 anti-Flag (Sigma–Aldrich), and anti-Xpress (Invitrogen).

Gel shift assay. RARα, RXRα, and HDAC1–RARα proteins were generated in vitro by TNT Coupled Reticulocyte Lysate Systems (Promega). Protein synthesis was confirmed by Western blot. CD11b was quantified by FACScan (BD Biosciences). Expression of mutant RARα proteins was determined by RT–nested PCR. Total RNA was extracted from mouse bone marrow with TRIZol (Invitrogen) and treated with DNases I. RT was performed using 2 μg of total RNA with SuperScript First-Strand Synthesis System (Invitrogen). 1 μl of cDNA was used for nested PCR.

Chromatin immunoprecipitation (ChIP) assay. We used the ChIP assay kit (Upstate Biotechnology).

Retroviral transduction and flow cytometry analysis of U937 cells. Recombinant retroviruses were used to transduce U937 cells by spinoculation for three consecutive days. GFP-positive cells were sorted with MoFlo (DakoCytomation). Expression of mutant RARα was confirmed by Western blot. CD11b was quantified by FACSscan (BD Biosciences). These experiments were repeated five times. The unpaired Student’s t test was used to compare CD11b expression between cells transduced with the MIGR1 vector and the ones transduced with MIGR1 vectors expressing PLZF–RARα, PML–RARα, HDAC1–RARα, and mHDAC1–RARα.

Southern blot analysis. Southern blots were done as described using probes for the hCG, p62(001) and PLZF genes (3, 4).
Follow up of transgenic mice. Mice were monitored and diagnosed with leukemia as described previously (24, 27). Diagnosis was confirmed by morphological and flow cytometric analysis of bone marrow cells with Mac-1 (CD11b), Gr-1, c-kit (CD117), Sca-1, B220, CD3, and Ter119 antibodies (BD Biosciences).

Bone marrow transplants in nude mice and ATRA treatment. Leukemic cells were obtained from bone marrow and spleens of leukemic RARα ETM. 4–8-wk-old Nu/J Hh 1nu nude mice were injected with 2 × 10^6 leukemic cells intravenously. Transplanted nude mice (NM) were bled once a week. The leukemic TM and the NM that developed leukemia after transplantation received intraperitoneal injections of 1.5 μg/g of RA daily (22).

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