Two Critical Hits for Promyelocytic Leukemia

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Summary

Acute promyelocytic leukemia (APL) is associated with chromosomal translocations that always involve the RARα gene, which variably fuses to one of several distinct loci, including PML or PLZF (X genes). Due to the reciprocity of the translocation, X-RARα and RARα-X fusion proteins coexist in APL blasts. PLZF-RARα transgenic mice (TM) develop leukemia that lacks the differentiation block at the promyelocytic stage that characterizes APL. We generated TM expressing RARα-PLZF and PLZF-RARα in their promyelocytes. RARα-PLZF TM do not develop leukemia. However, PLZF-RARα/RARα-PLZF double TM develop leukemia with classic APL features. We demonstrate that RARα-PLZF can interfere with PLZF transcriptional repression and that this is critical for APL pathogenesis, since leukemias in PLZF−/−/PLZF-RARα mutants and in PLZF-RARα/RARα-PLZF TM are indistinguishable. Thus, both products of a cancer-associated translocation are crucial in determining the distinctive features of the disease.

Introduction

Oncogenesis in either solid tumors or hemopoietic malignancies is commonly regarded as a multistep process. This tenet entails that multiple genetic events progressively allow the premalignant cell to acquire full neoplastic potential and, subsequently, in the case of solid tumors, the acquisition of metastatic potential. This concept is at the basis of an active search for genes, commonly referred to as tumor modifiers, that play a role in accelerating tumorigenesis. Genes whose aberrant activity or inactivation ultimately leads to full-blown transformation are commonly referred to as oncogenes or tumor suppressor genes. In such a model for neoplastic transformation, various genetic events are required in order to render a cell of a specific histological type malignant and invasive in a cumulative/additive model toward cancer. However, in the clinically manifest tumor these multiple genetic events coexist, and it is, therefore, difficult to assess their relative contribution to tumorogenesis. What is also unclear is whether two or more hits are not only concomitantly required to make the clonal population fully malignant, but are also a prerequisite for the tumor to acquire its distinctive and recognizable morphological and clinical features.

Leukemias are ideal model systems for addressing these issues for two reasons: (1) they have been recognized and classified as clinical entities on the basis of morphological criteria, because the accumulating blast population is arrested at, and morphologically reminiscent of, one of the differentiating steps through which the hemopoietic progenitor cell undergoes in order to reach functional maturity. This introduces a further element of analysis, which is the definition of the ability of a leukemia-associated aberrant gene product to perturb/block hemopoietic differentiation. While impaired cellular differentiation probably underlies the pathogenesis of any cancer, in other tumor types this aspect is not often precisely recognizable. (2) Leukemic cells harbor specific chromosomal translocations that are often reciprocal, thus leading to the generation of two distinct aberrant fusion genes whose products coexist in the leukemic cell (reviewed in Melo et al., 1993; Look, 1997; He et al., 1999; Melnick and Licht, 1999). This brings us to the following question: do these aberrant gene products participate in leukemogenesis and in determining response to therapy, and if so, how? Acute promyelocytic leukemia (APL), the M3 subtype of acute myeloid leukemia (AML; reviewed in Warrell et al., 1993; He et al., 1999; Melnick and Licht, 1999), is a unique model system to use to address these questions, because of its distinctive features: (1) the accumulation in the bone marrow (BM) of tumor cells that are arrested at the promyelocytic stage of myeloid differentiation; (2) the invariable association with specific reciprocal and balanced translocations involving the retinoic acid receptor α (RARα) gene on chromosome 17, and the promyelocytic leukemia (PML), promyelocytic leukemia zinc finger (PLZF), nucleophosmin (NPM), nuclear mitotic apparatus (NuMA), and signal transducer and activator of transcription 5B (STAT 5B) genes (X genes) on chromosomes 15q22, 11q23, 5q32, 11q13, and 17q11, respectively; and (3) the exquisite sensitivity of APL blasts to the differentiating action of all-trans-retinoic acid (RA). RA overcomes the block of differentiation at the promyelocytic stage, leading to complete, albeit transient, disease remission—the reason for which APL has become the paradigm for cancer “differentiation therapy.” Strikingly, however, unlike other APL, leukemia associated with the t(11;17)/PLZF-RARα shows a distinctly worse prognosis, with little or no response to RA treatment (Licht et al., 1995).

The various APL specific translocations result in the generation of X-RARα and RARα-X fusion genes and the co-expression in the leukemic blasts of their chimeric products, which are identical in their RARα moieties (He et al., 1999; Melnick and Licht, 1999). X-RARα are able
to form heterodimeric complexes with their respective X protein. Similarly, the RARα portion retained in X-RARα is able to mediate heterodimerization with RXR, as well as DNA and ligand binding through the RARα RA and DNA binding domains (He et al., 1999; Melnick and Licht, 1999). Therefore, X-RARα have the ability to potentially interfere with both X and RAR/RXR pathways.

By contrast, very little is known on the biochemical and biological role of the various RARα-X fusion proteins.

We and others have previously reported that PML-RARα transgenic mice (TM) develop leukemia with striking APL features, including the promyelocytic block of myeloid differentiation (Brown et al., 1997; Grisolano et al., 1997; He et al., 1997), while PLZF-RARα TM develop myeloid leukemia that is reminiscent of chronic myelogenous leukemia (CML) (He et al., 1998), a different form of human myeloid leukemia that completely lacks the distinctive differentiation block at the promyelocytic stage that characterizes APL. Here, we have generated RARα-PLZF TM that we have intercrossed with PLZF-RARα TM to reconstruct the dual complexity of t(11;17) APL.

We show that these two genetics events are concomitantly required to recreate the disease in its uniqueness: while one of the two events is oncogenic, neither event by itself can cause a disease that can be recognized as APL. However, the combined action of both molecules results in leukemia with classic APL features.

We demonstrate biochemically and genetically that RARα-PLZF acts through its ability to functionally interfere with PLZF. RARα-PLZF epitomizes a novel and distinct function in cancer etiopathogenesis. These aberrant proteins are not oncogenic per se, nor can they accelerate tumor onset unlike classic tumor modifiers. However, they are required to determine the phenotypic and clinical characteristics of the disease in its recognizable form.

**Results**

**Generation of RARα-PLZF TM**

We generated TM in which the expression of the RARα-PLZF chimeric cDNA was under the control of a myeloid-promyelocytic-specific human cathepsin-G (hCG) minigene (Figure 1A) (He et al., 1997; He et al., 1998). The RARα-PLZF cDNA was subcloned into hCG gene exon 1 (Figure 1A). Seven mice carrying the hCG-RARα-PLZF transgene were identified (Figure 1B), harboring 2 to 36 copies of the transgene in a head-to-tail and/or tail-to-tail arrangement as evaluated by Southern blot analysis (Figures 1A and 1B; Experimental Procedures). Five of the seven TM transmitted the transgene to the germ line. Utilizing a nested reverse transcriptase–polymerase chain reaction (RT-PCR) approach that distinguishes the unprocessed gene from the processed cDNA (Figure 1A), we demonstrated the expression of the RARα-PLZF fusion gene in the BM cells of all five founders’ progeny (Figure 1C). Northern blot and Western blot analyses on BM samples obtained from RARα-PLZF TM and PLZF-RARα/PLZF double TM confirmed the expression of an RARα-PLZF fusion transcript/protein (Figure 1C; 3D). These TM lines were further expanded and more than 200 mice from the five lines that expressed the RARα-PLZF transgene were followed up over a 30-month period.

**Aberrant Hematopoiesis in RARα-PLZF TM**

All five RARα-PLZF lines displayed a slow and progressive accumulation of myeloid cells in the BM and spleen (Figure 2A). The infiltrating cells retained the ability to...
not affected (data not shown). After the first year of life, approximately 20% of the mice developed overt splenomegaly (Figure 2B), but leukemia was never observed. Flow-cytometric analysis confirmed: (1) the myeloid nature of the cells accumulated in the BM and spleen of RARα-PLZF TM as revealed by the increase in Gr-1 (a marker expressed in various mature stages of granulocytic differentiation) and Mac-1 (a marker for mature monocytes/macrophages and granulocytes) positive cells (Figure 2C); and (2) that the accumulating cellular population retained the ability to terminally differentiate (Figure 2C). Thus, RARα-PLZF can perturb myelopoiesis, but it is not sufficient for inducing a block in promyelocytic differentiation or leukemogenesis.

APL in PLZF-RARα/PLZF-RARα Double TM

We next intercrossed PLZF-RARα TM from two independent transgenic lines with RARα-PLZF mice from three independent lines to obtain double TM that were analyzed on a comparative basis (Figure 3A). PLZF-RARα TM develop myeloid leukemia with CML-like hematological features at 100% of penetrance after 6 months of age (He et al., 1998). Surprisingly, although hemopoiesis in RARα-PLZF TM is characterized by the hyperplasia of the myeloid compartment, leukemia onset was unaffected in double TM. Leukemia still occurred after 6 months with 100% penetrance (data not shown). Thus, RARα-PLZF does not function as a classic tumor modifier.

By contrast, the comparative analysis of the morphological and hematological features of the leukemia in double and single PLZF-RARα TM revealed striking differences (Table 1; Figures 3B and 3C). Leukemia in PLZF-RARα mice was characterized by leukocytosis in the PB, and the infiltration in all organs by myeloid leukemic cells that fully retained the capacity to terminally mature, as revealed by differential counts on PB, BM, and spleen (Table 1; Figure 3B), but leukemia in double TM mice was characterized by a dramatic accumulation of immature blasts and of cells blocked at the promyelocytic stage of differentiation (more than 50% of promyelocytic stage of differentiation) and Mac-1 (a marker for mature monocytes/macrophages and granulocytes) positive cells (Figure 2C); and (2) that the accumulating cellular population retained the ability to terminally differentiate (Figure 2C). Thus, RARα-PLZF can perturb myelopoiesis, but it is not sufficient for inducing a block in promyelocytic differentiation or leukemogenesis.

Figure 2. Aberrant Hematopoiesis in RARα-PLZF TM
(A) Wright-Giemsa-stained BM and spleen cells from RARα-PLZF TM and WT littermates. Differential counts and morphological analysis showed a marked increase in myeloid components at various stages of maturation in both BM and spleen of the TM. Magnification: ×1000.
(B) Splenomegaly in RARα-PLZF TM. The ratio between the spleen and body weight in the diseased RARα-PLZF TM was 0.85 ± 0.06 (n = 9); WT mice, 0.30 ± 0.01 (n = 30).
(C) Flow cytometrical analysis of BM and spleen (SP) cells from RARα-PLZF TM and WT littermates. This analysis (n = 9) confirmed the accumulation of myeloid cells of various degree of maturation (Gr-1, Mac-1, and Gr1/Mac1 positive) in both the BM and spleen of RARα-PLZF TM.

terminally differentiate toward mature granulocytes, as shown by the fact that the differential counts in the BM and spleen did not reveal the consistent accumulation of a specific immature cellular population, but instead revealed progressive hyperplasia of the myeloid/granulocytic compartment in the absence of a block of differentiation (ratio of myeloid to lymphoid in BM: 4.99 ± 2.7 in RARα-PLZF TM [n = 9] versus 2.28 ± 0.4 in wild type [WT; n = 30]) (Figure 2A). The peripheral blood (PB) was
Figure 3. Characterization of PLZF-RARα/PML-RARα-PLZF Double TM

(A) The interbreeding strategy and the respective phenotypes of the various transgenic lines are indicated.

(B) Wright-Giemsa-stained PB and BM cells from PLZF-RARα and double leukemic mice. Magnification: ×400 for PB; ×1000 for BM. The number of WBC in PB is dramatically increased in the PLZF-RARα leukemic mice, while mildly increased in double TM. In the PLZF-RARα, leukemia myeloid cells at various stages of maturation replace the normal BM populations. In the double leukemia, a homogeneous population of cells showing promyelocytic features infiltrates the BM.

(C) Flow cytometric analysis on BM cells and splenocytes from PLZF-RARα, double leukemic mice, and control mice using antibodies against the cell surface markers Gr-1, Mac-1, c-Kit, and CD34. The green line shows the isotypic control for each sample. For each antibody, the percentage of positive cells is given in the respective histogram.

(D) Expression of PLZF-RARα and RARα-PLZF fusion proteins in double TM. Cell lysate was prepared from BM cells of double TM with leukemia (lane 1, a representative TM obtained by intercrossing line 5814 with line 6014; lane 2, a representative TM obtained by intercrossing line 6062 with line 5998) and WT mouse. 100 μg of protein were separated on 8% or 12% SDS-PAGE minigels and run in parallel for PLZF-RARα or RARα-PLZF, respectively. PLZF-RARα and RARα-PLZF were detected utilizing anti-RARα (RPα[F]) and anti-PLZF antibodies, respectively. β-actin revealed the amount of protein loaded.
Table 1. Automated and Differential Counts of PB and BM from WT, PLZF-RARα, and Double Transgenic Mice with Leukemia

<table>
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<tr>
<th>Mice</th>
<th>Hb (g/dl)</th>
<th>Plt (10^9/l)</th>
<th>WBC (10^9/l)</th>
<th>Myeloid (%)</th>
<th>Pro + Blast (%)</th>
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<tr>
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<td>1239</td>
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</tr>
<tr>
<td>PLZF-RARα</td>
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<td>2.9</td>
<td>1239</td>
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</tr>
<tr>
<td>Double TM</td>
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<td>2.9</td>
<td>1239</td>
<td>6.2</td>
<td>0.3</td>
</tr>
</tbody>
</table>

The data reported here are means ± SD and refer to 20 mice from each genotype at diagnosis for PB, 7 WT and double TM, and 12 PLZF-RARα TM sacrificed at diagnosis for BM. Statistical analysis (t test) showed significant difference between WT and leukemia in either or double TM in all parameters except Seg + Band (%).

Seg: Band: segmented and band nuclear granulocytes; Meta: metamyelocytes and myelocytes; Pro + Blast: promyelocytes and blasts.

In comparing leukemia in PLZF-RARα double TM to differentiate after exposure to RA at the same concentration (10^-6 M) utilized to trigger the differentiation of human APL blasts and in the presence of growth factors (spleen cell conditioned media [SCCM]; see Experimental Procedures) that are known to potentiate RA-induced differentiation in t(11;17) APL (Jansen et al., 1998). Terminal differentiation was analyzed by nitroblue tetrazolium (NBT) test, and morphological differential counts scoring the percent of terminally differentiated granulocytes. RA-induced differentiation in leukemic cells from double TM was markedly reduced (Figure 4D). Thus, RARα-PLZF contributed both in vivo and in vitro to rendering leukemic cells from double TM further unresponsive to RA in full agreement with the clinical refractoriness to RA observed in human t(11;17) APL.

A Transcriptional Role for RARα-PLZF

RARα-PLZF retains seven out of the nine Krüppel-type zinc fingers that constitute the PLZF DNA binding domain (Figure 5A) (He et al., 1999; Melnick and Licht, 1999). However, the N-terminal PLZF moiety that contains the transcriptional repression BTB/POZ domain, capable of negatively regulating transcription through recruitment of histone deacetylases, is replaced in RARα-PLZF by one of the transacting domains from RARα (A domain) (Figure 5A) (Nagpal et al., 1999; Chambon, 1996). We therefore compared the DNA binding and transcriptional activity of RARα-PLZF and PLZF. Electrophoresis mobility shift assays (EMSAs) were performed utilizing an oligonucleotide corresponding to the PLZF binding site identified in the LexAop (Sitterlin et
A DNA-protein complex was detected with whole cell lysate from 293T cells transfected with either PLZF-RARα-PLZF or PLZF (Figure 5B). The specificity of the LexAop-RARα-PLZF or LexAop-PLZF complexes was demonstrated as they were completely competed away by an excess of unlabeled LexAop oligo (Figure 5B). Anti-PLZF and anti-FLAG antibodies (for RARα-PLZF) supershifted these complexes, demonstrating that they contain PLZF and RARα-PLZF, respectively. We next transfected 293T cells with a luciferase reporter construct harboring the PLZF responsive element from the LexAop, and PLZF or RARα-PLZF expression vectors, and compared the ability of these proteins to repress transcription. The basal activity of the reporter construct driven by an HSV-TK heterologous promoter was repressed by PLZF in a dose-dependent manner (Figure 5C). RARα-PLZF completely lost the transcriptional repressive ability of PLZF (Figure 5C). Thus, RARα-PLZF may interfere with PLZF function at the transcriptional level through its ability to interact with PLZF responsive elements.

The Functional Inactivation of PLZF Is a Critical Event in APL Pathogenesis

To test genetically whether RARα-PLZF acts as a dominant negative inhibitor of PLZF function, we utilized mice in which we had inactivated PLZF by homologous recombination (Barna et al., 2000). We intercrossed PLZF-RARα TM with PLZF+/− mice to obtain PLZF-RARα TM in the three distinct PLZF+/-, PLZF+/− and PLZF−/− backgrounds (n = 20 per group), and characterized, on a comparative basis, leukemogenesis and hemopoiesis in littermates obtained from these crosses (Figure 6A). PLZF−/− mice did not display defects in myeloid hemopoiesis as evaluated by flow cytometric analysis, WBC count, and differential counts performed in BM, PB, and spleen in a 1-year follow up (not shown). PLZF-RARα/PLZF+/− mice obtained from these intercrosses developed “CML”-like leukemia, indistinguishable from those observed in our original PLZF-RARα TM (Tables 1 and 2; Figures 3 and 6; He et al., 1998). Strikingly, however, leukemia in PLZF-RARα/PLZF+/− mice displayed identical features to those in PLZF-RARα/PLZF−/− double TM, such as a dramatic accumulation of blast/promyelocytic cells in the BM and spleen and the absence of leukocytosis, as demonstrated by morphological and flow cytometric analysis. Infiltrating cells were c-Kit, Gr-1, Mac-1 positive, and displayed promyelocytic features (PLZF-RARα/PLZF−/− mice: c-Kit+, 34.17% ± 18.1%; Gr-1−/Mac-1−, 59.4% ± 20.1%; PLZF-RARα/PLZF+/− mice: c-Kit+, 15.2% ± 3.8%; Gr-1−/Mac-1−, 76.1% ± 12.8%; data are means ± standard deviation [SD] from n = 3 mice for each group) (Table 2; Figures 6B and 6C). Moreover, leukemias in PLZF-RARα/PLZF−/− mice displayed morphological and flow cytometric features

Figure 4. RARα-PLZF Controls Proliferation, Survival, and Differentiation of Leukemic Blasts

(A) RARα-PLZF increased the proliferative potential of leukemic cells. [3H]-thymidine incorporation assays were performed on BM cells from sex-age matched leukemic PLZF-RARα, double TM, and WT littermates after a 24 hr incubation with [3H]-thymidine. 1 × 10⁶/ml BM cells were incubated in DMEM medium supplemented with 20% FBS, with or without 2% SCCM (GF+ and GF−). Means ± SD from triplicate values of one of three representative experiments are shown.

(B) RARα-PLZF enhances the formation of leukemic colonies in methylcellulose colony forming assays. Colony formation assays were performed on BM cells from sex-age matched leukemic PLZF-RARα, double TM, and WT littermates. 3 × 10⁴ cells were plated per dish in 0.9% methylcellulose medium in triplicate. CFU-GM and leukemic clusters were scored at day 7. CFU-GM includes CFU-G, M, and GM. Colony numbers (means ± SD from triplicates) are from one of two experiments with similar results.

(C) RARα-PLZF protects leukemic cells from apoptosis. BM cells were incubated in DMEM medium, supplemented with 20% FBS and 2% SCCM, and apoptosis scored by Annexin V and TUNEL staining analyses after 48 hr of culture (GF+). Apoptosis upon growth factor withdrawal was measured by culturing the cells with SCCM for 3 days as mentioned above and for 2 additional days in the absence of factors (GF−). The percentages of viable cells (Annexin V−/PI−) (left panels; mean ± SD from three experiments), and apoptosis as detected by in situ TUNEL staining (right panels; percent of positive cells: GF+: double TM, 5.2% ± 2%; single TM, 8.8% ± 1.6%; GF−: double TM, 34% ± 4%; single TM, 80% ± 7.8%; means ± SD of one of three experiments with similar results) are shown.

(D) Differential response to RA in leukemic cells from double TM. BM cells from PLZF-RARα and double TM with leukemia were incubated with or without 10⁻⁶ M RA in the presence of 2% SCCM. Cytospins from BM cells after a 4-day culture were stained with Wright-Giemsa. Percentage (means ± SD from two experiments) of mature (segmented) granulocytes and representative morphology are shown.
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Figure 6. APL in PLZF-RARα/PLZF-α/ mice

(A) The interbreeding strategy and the respective phenotypes of the transgenic and knock-out lines are indicated.
(B) Flow cytometry analysis on BM cells from PLZF-RARα/PLZF-α/ leukemic mice using a c-Kit antibody. The green line shows the isotypic control for each sample. The percentage of positive cells is given in the respective histogram.
(C) Morphological characterization of PLZF-RARα/PLZF-α/ leukemic mice. Cytospins of BM cells from representative leukemic mice were stained with Wright-Giemsa. In the PLZF-RARα/PLZF-α/ leukemia, myeloid cells at various stages of maturation replace the normal BM cells.

Discussion

Tumor Modifiers and Tumor Metamorphoses

We have demonstrated that two genetic events that occur simultaneously in human APL as a consequence of a reciprocal chromosomal translocation are both necessary, in combination, to cause the disease in its distinctive form. Neither event is sufficient per se for the pathogenesis of a disease that would resemble APL. Leukemia in double TM displayed features of classic APL, closer to human APL than the leukemia observed in PML-RARα TM (Grisolano et al., 1997; He et al., 1997). Several examples of multistep carcinogenesis have been described and elucidated (Kinzler and Vogelstein, 1998). These multiple events are required for full-blown malignant transformation, and each event would be se-
Table 2. Automated and Differential Counts of PB and BM from PLZF-RARα/PLZF mice with leukemia

<table>
<thead>
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<th>Mice with Leukemia</th>
<th>PB</th>
<th>BM</th>
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<td></td>
<td>Hb (g/dl)</td>
<td>Plt (10 9/l)</td>
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<tr>
<td>PLZF-RARα/PLZF</td>
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<td>PLZF-RARα/PLZF</td>
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The data reported here are means ± SD and refer to nine and six for PLZF-RARα/PLZF and PLZF-RARα/PLZF mice at diagnosis, respectively. The statistical analysis (t test): a p < 0.01; b p < 0.05.

Selected because it favors cellular proliferation, survival, and tumor invasiveness. Mutations in strong tumor determinants, such as proto-oncogenes or tumor suppressors, would have catastrophic consequences inevitably leading to tumorigenesis; while mutations in weaker determinants not able to transform on their own, the tumor modifiers, would accelerate the oncogenic process. In this model, neoplastic transformation is regarded as an additive/quantitative process, as if there were a threshold value for neoplastic transformation that has to be reached by adding multiple transforming units. This simple model is challenged by what is observed modeling t(11;17) APL in the mouse. In this case, one oncogenic event, PLZF-RARα, already leads to leukemia at complete penetrance. In the aforementioned model for transformation, the PLZF-RARα provide several transforming units that allow for the easy passage of the threshold value through the accumulation of additional genetic events in the 6-month latency period. However, the leukemia triggered by PLZF-RARα is not APL. RARα-PLZF is, therefore, a critical determinant of the leukemia phenotype. In this process, RARα-PLZF acts neither as a classic oncogene, since its activity is not leukemogenic per se, nor as a tumor modifier: RARα-PLZF metamorphoses a CML-like phenotype into a leukemia similar to human APL, without affecting tumor onset. The most relevant implication of these findings is that they genetically demonstrate the qualitative nature of the neoplastic process, an aspect that can be summarized by the algorithm: (phenotype A, CML-like leukemia [PLZF-RARα]) + phenotype B, myeloid hyperplasia [RARα-PLZF = phenotype C, APL [PLZF-RARα + RARα-PLZF]]. Phenotype C cannot simply be regarded as the mere addition of the phenotypes observed in A and B. APL in double TM is the qualitatively novel biological outcome of two concomitant aberrant activities on distinctive pathways.

Furthermore, because PLZF-RARα does not require RARα-PLZF for leukemogenesis, the formation of an aberrant RARα-PLZF fusion gene would not necessarily be selected as a subsequent additional hit throughout tumor progression. Indeed, in APL, PLZF-RARα and RARα-PLZF are formed simultaneously as a consequence of a single reciprocal translocational event, and the majority of t(11;17) APL patients are found to express the RARα-PLZF fusion gene, thus underscoring the relevance of the coexistence of these two products (Licht et al., 1995; Licht et al., 1996; Grimwade et al., 1997). It remains to be seen whether PLZF-RARα by itself could cause a CML-like leukemia in humans as well, when not co-expressed with RARα-PLZF.

By contrast only 70% of t(15;17) APL expresses the reciprocal product (Alcalay et al., 1992; Chang et al., 1992; Grimwade et al., 1996). This is consistent with the phenotype observed in PML-RARα TM. In fact, PML-RARα can cause APL-like leukemia in mice in absence of RARα-PML (Grisolano et al., 1997; He et al., 1997). However, the long latency period (more than 1 year) and the low penetrance (10%–20% of TM) of these APL-like leukemias suggest that additional events are required for full-blow transformation. When expressed, RARα-PML could therefore act as a classic tumor modifier required for the acceleration of the leukemogenic process. In fact, RARα-PML does not cause leukemia in
The Multiple Biological Functions of RARα-PLZF in APL Pathogenesis

When co-expressed with PLZF-RARα, RARα-PLZF can induce a block in myeloid/promyelocytic differentiation, increase cellular proliferation, and enhance cell survival. Each of these functions is obviously critical for leukemogenesis. However, while the last two functions are compatible with the myeloid hyperplasia observed in RARα-PLZF TM, the block of differentiation is not observed in RARα-PLZF TM. Two possibilities can be entertained to reconcile this apparent paradox: (1) PLZF-RARα and RARα-PLZF could interfere with two distinct molecular pathways (see below), whose combined deregulation would then lead to impaired myeloid differentiation; or (2) an excessive proliferation rate of early myeloid progenitors triggered by the combined activities of PLZF-RARα and RARα-PLZF could indirectly cause a block in promyelocytic differentiation, in agreement with the notion that proliferation and differentiation of hematopoietic precursors are tightly and inversely interrelated (Liu et al., 1996; Cheng et al., 2000).

RARα-PLZF can also modulate response to RA. t(11;17) APL patients do not respond to RA treatment (Licht et al., 1995). Leukemia in PLZF-RARα TM also responds poorly to RA treatment (He et al., 1998). However, RA can induce partial response and prolong survival in PLZF-RARα leukemic TM. Furthermore, suprapharmacological doses of RA (4-fold greater than the one administered to human APL) can induce complete albeit transient remission in PLZF-RARα leukemic TM as well (He et al., 1998). The further RA unresponsiveness conferred by RARα-PLZF to the leukemic blasts both in vitro and in vivo not only explains the marked resistance to RA treatment observed in human t(11;17) APL, but also provides a further mechanism for the differentiation block observed in leukemia from double TM, in view of the important physiological role played by RA in controlling normal myelopoiesis (Gudas et al., 1994; Chambron, 1996). By contrast, no apparent difference was observed in RA sensitivity or clinical outcomes in t(15;17) APL patients who do, or do not, harbor the RARα-PML fusion transcript (Grimwade et al., 1996; Li et al., 1997), or in PML-RARα/RARα-PML double TM (Pollock et al., 1999).

A Two Critical Hits for t(11;17) APL

PLZF-RARα can act as a dominant negative transcriptional repressor of RARα, through aberrant nuclear co-repressor/HDAC associations (Grignani et al., 1998; Guídez et al., 1998; He et al., 1998; Lin et al., 1998). PLZF-RARα can form, via its PLZF moiety, co-repressor complexes that are insensitive to RA. PLZF-RARα can also interfere with the function of the remaining normal RARα allele and other nuclear receptors through its ability to homodimerize and heterodimerize with and sequester RXR within the co-repressor/HDAC complex (Minucci et al., 2000; Saloni and Pandolfi, 2000). PLZF-RARα can also physically interact with PLZF, possibly affecting its function as well (He et al., 1999; Melnick and Licht, 1999). The fact that RARα-PLZF can act as an aberrant version of PLZF at the transcriptional level, and that its presence or the inactivation of PLZF cause an APL-like phenotype in PLZF-RARα TM demonstrates the importance of the blockade of PLZF function for APL pathogenesis. On the other hand, these findings imply that if PLZF-RARα does interfere with the PLZF pathway, this does not result in abrogation of PLZF function. The concomitant interference of PLZF-RARα and RARα-PLZF with the RARα/RXR pathways, respectively, is therefore, necessary, albeit not sufficient, for APL pathogenesis.

Experimental Procedures

Construction of the Transgene

The human RARα-PLZF cDNA (1 kb) was cloned into the artificial Sall site of the hCG minigene expression vector, which was introduced one base pair (bp) after the hCG ATG, which was previously mutagenized. hCG minigene drives the expression of the fusion cDNA, specifically in early stages of myeloid cell differentiation (He et al., 1997; He et al., 1998). A BamHI-NotI fragment from this construct was purified for egg injection as previously described (He et al., 1997; He et al., 1998).

DNA, RNA, and Western Blot Analyses

Genomic tail DNA was digested with EcoRI or HindIII and hybridized with probe CT, a 250-bp HindIII-XbaI genomic fragment, and probe HS1, a 1-Kb hCG genomic fragment. A murine 2.3-Kb BamHI p53 cDNA probe was used to normalize for DNA loading and determine the transgene copy number. Total RNA was prepared from mouse BM using Trizol reagents (Gibco BRL). Nested RT-PCR was performed using the primers shown in Figure 1A, which span cathepsin G intron 1. For Northern blot analysis, denatured total RNA (20 μg) was hybridized with the human RARα-PLZF Hapl fragment as a probe (RP probe, see Figure 1A) following standard procedures. For Western blot analysis, cell lysate was prepared from BM cells of leukemic mice and WT mice in E1A buffer as described (He et al., 1997; He et al., 1998). Total protein was then separated on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) minigels in parallel for PLZF-RARα or RARα-PLZF, respectively. PLZF-RARα was detected with the RARα/P anti-RARα (kindly provided by P. Chambon) and RARα/-PLZF with an anti-PLZF antibody (Barna et al., 2000). The amount of protein loaded was normalized by reblotting the membranes with a β-actin antibody.

Follow-Up of TM

Hematopoiesis in all five RARα-PLZF lines was studied over a 30-month follow-up period. One group of mice was bled on a monthly basis, together with age-matched littermate controls (60 TM and 30 control mice). Automated and differential counts, as well as morphological analysis of PB cells, were performed on each sample. In a second group, four TM and four WT controls were sacrificed every 2 months within the first year of life, and eight TM and four controls were sacrificed every 2 months after the first year of life for gross and microscopic examination of all organs, and in particular PB, BM, spleen, liver, lymph nodes, and thymus. To obtain double TM, we intercrossed two lines of PLZF-RARα (L5814 and L6002) with three lines of RARα-PLZF (L5998, L6006 and L6014), Double TM harboring PLZF-RARα, RARα-PLZF, and the single PLZF-RARα TM (over 100 for each genotype) were studied on a comparative basis as described above. To obtain PLZF-RARα TM in various PLZF backgrounds, we intercrossed two PLZF-RARα TM lines (L5814 and L6002) with one PLZF TM line (Barna et al., 2000), and PLZF-RARα/ PLZF + , PLZF-RARα/PLZF + , and PLZF-RARα/PLZF + littermates (20 for each genotype) were followed up on a comparative basis.

Hematology, Histopathology, and Immunohistochemistry

Mice were bled from the tail. WBC, hemoglobin (Hb) and platelet (Plt) in PB were monitored utilizing an automated counter (Technicon...
H2). Differential counts of PB and BM were performed microscopically on Wright-Giemsa–stained cytospin preparations. Hematoxylin and eosin, myeloperoxidase (Dako), B220/CD45R, and CD3 antibodies (PharMingen) were used for immunohistochemical staining on BM and spleen sections as described (He et al., 1997).

The diagnosis of leukemia was made on the basis of the following concomitant criteria: (1) WBC > 30,000/μl; (2) Hb < 10 g/dl; and (3) Platelets < 80,000/μl. Alternatively, when leukocytosis was absent the diagnosis was also made based on: (1) Hb < 6 g/dl and Platelets < 500,000/μl; (2) Hb < 3 g/dl; or (3) Platelets < 300,000/μl. All these criteria were fulfilled in two consecutive weekly tests.

Flow Cytometry
Cells were stained with fluorescein-conjugated mouse IgG or IgM (PharMingen), Gr-1, CD34, c-kit, Sca-1, and CD3 and CD4 as previously described (He et al., 1997; He et al., 1998). Fluorescein isothiocyanate or phycoerythrin conjugated isotypic antibodies were used as controls. All antibodies were obtained from PharMingen. Analysis was carried out using a FACSscan flow cytometer (Becton-Dickinson).

EMSA and Transcriptional Assays
PLZF/RA receptors were transiently expressed in 293T cells. Cellular extracts were incubated with a 32P-ATP-labeled 488-base pair probe, the 4x LexAop-derived sequence and electrophoresed on a 4% polyacrylamide gel as described. 293T cells were transiently transfected using Superfect reagents (Qiagen). The total amount of DNA transfected was 2 μg; the amount of expression vector (pSG5) was kept constant in each transfection. Luciferase activity was measured 24 hr after transfection. Results were normalized for β-galactosidase activity.

Proliferation, Apoptosis, and Clonogenic Assays
BM cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% fetal bovine serum (FBS) with or without 2% pokeweed mitogen-stimulated SCCM (StemCell Technologies, Vancouver; containing IL3 and GM-CSF). Aliquots from each culture were harvested after 20 hr and 40H-thymidine was added. After a further 24 hr incubation, incorporation of 3H-thymidine was scored after 5 days culture in the presence of SCCM followed by 2 additional days of culture without SCCM. Cellular apoptosis was analyzed by flow cytometry after propidium iodide (PI), an anti-Annexin V antibody (PharMingen) staining. Cytospin preparations were also stained using a kit for TUNEL analysis (Boehringer Mannheim). Apoptosis upon growth factor withdrawal was scored after 48 hr of culture. Apoptosis analysis was performed after 48 hr of culture. Apoptosis upon growth factor withdrawal was scored after 5 days culture in the presence of SCCM followed by 2 additional days of culture without SCCM. Cellular apoptosis was analyzed by flow cytometry after propidium iodide (PI), an anti-Annexin V antibody (PharMingen) staining. Cytospin preparations were also stained using a kit for TUNEL analysis (Boehringer Mannheim).

In Vivo RA Therapy
Preclinical trials with RA in PLZF–RARα−/RARα−→PLZF and PLZF–RARα− cells were carried out by administering RA per os daily at a dose of 1.5 μg/g of mouse body weight. RA treatment was initiated at the onset of leukemia. Response to RA was monitored by performing weekly differential and automated counts on PB, in order to monitor WBC, PI, and red cell counts, and hb values, and to score for the presence of blasts in the PB. Untreated control groups consisted of leukemic mice from the same lines, which were also bled once a week.

In Vitro Differentiation Assays with RA
Cells were cultured as for 40H-thymidine incorporation assays with or without RA (10−8 M and 10−7 M). At day 4, cells were harvested from cultures and applied for morphological analysis, differential count, and the NBT analyses as previously described (He et al., 1997).

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References


