Treatment with 5-Azacytidine Accelerates Acute Promyelocytic Leukemia Leukemogenesis in a Transgenic Mouse Model

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Abstract

A key oncogenic force in acute promyelocytic leukemia (APL) is the ability of the promyelocytic leukemia–retinoic acid receptor α (PML-RARA) oncoprotein to recruit transcriptional repressors and DNA methyltransferases at retinoic acid–responsive elements. Pharmacological doses of retinoic acid relieve transcriptional repression inducing terminal differentiation/apoptosis of the leukemic blasts. APL blasts often harbor additional recurrent chromosomal abnormalities, and significantly, APL prevalence is increased in Latino populations. These observations suggest that multiple genetic and environmental/dietary factors are likely implicated in APL. We tested whether dietary or targeted chemopreventive strategies relieving PML-RARA transcriptional repression would be effective in a transgenic mouse model. Surprisingly, we found that 1) treatment with a demethylating agent, 5-azacytidine, results in a striking acceleration of APL; 2) a high fat, low folate/choline–containing diet resulted in a substantial but nonsignificant APL acceleration; and 3) all-trans retinoic acid (ATRA) is ineffective in preventing leukemia and results in ATRA-resistant APL. Our findings have important clinical implications because ATRA is a drug of choice for APL treatment and indicate that global demethylation, whether through dietary manipulations or through the use of a pharmacologic agent such as 5-azacytidine, may have unintended and detrimental consequences in chemopreventive regimens.

Keywords

APL, 5-azacytidine, ATRA, Western diet, chemoprevention

Introduction

Cancer is driven by an initiating event that leads to the progressive accumulation of genetic mutations and of epigenetic changes that deregulate critical cancer genes.^{1,2} The retinoic acid receptor α (RARA) gene fuses to the promyelocytic leukemia (PML) gene in the vast majority of acute promyelocytic leukemia (APL) cases.³ The PML-RARA fusion protein initiates APL when expressed in the early myeloid compartment.^{4–6} The PML-RARA fusion protein not only forms aberrant complexes with histone deacetylases (HDAC) and DNA methyltransferases (DNMT) interfering with RARA transcription at multiple levels but also acts as a PML dominant-negative mutant.^{7–11} In addition, APL blasts often harbor recurrent chromosomal abnormalities, strongly suggesting that multiple genetic events cooperate over time to give rise to overt leukemia.^{12,13}

Mouse models of APL faithfully recapitulate this chain of genetic and epigenetic events. For instance, PML-RARA transgenic mice succumb to APL after a long latency period, suggesting that in addition to PML-RARA, several genetic events including recurrent karyological abnormalities are required for full-blown APL.^{14,15}

Epidemiological data point to a high rate of APL among Latino populations, which may suggest a genetic predisposition to and/or exposure to distinct dietary/environmental

factor(s).¹⁶ In this regard, it is noteworthy that numerous epidemiological and animal studies have demonstrated that a high-fat, low-folate/choline diet (i.e., “Western-style

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diet") is a cancer risk factor.^{17,18} However, to date, little is known on the direct molecular mechanisms by which environmental, dietary, or chemopreventive strategies may favor or antagonize leukemogenesis.

The experimental evidence indicating that PML-RARA transforms hematopoietic progenitors through the transcriptional repression of RARA target genes because of the ability of PML-RARA to recruit corepressors and HDAC onto RARA target genes led to the hypothesis that targeted agents that specifically relieve PML-RARA transcriptional repression would effectively chemoprevent APL. We reasoned that this strategy would be devoid of the toxic long-term effects of cytotoxic chemotherapy. Arsenic trioxide has striking anti-APL properties because of its ability to promote PML-RARA degradation. However, arsenic trioxide targets multiple cellular networks, and its long-term use poses significant hazards.³ Therefore, we tested the chemopreventive effects of targeted treatment aimed at relieving PML-RARA transcriptional repression in a well-established APL mouse model.⁶ We used all-trans retinoic acid (ATRA), the first drug of choice for APL both in induction and maintenance protocols. This drug is effective in APL because of the ability to target the RARA moiety of PML-RARA relieving transcriptional repression and converting PML-RARA into a transcriptional activator.³ We also used 5-azacytidine (5-AC), a DNA methylation inhibitor used for the treatment of myelodysplastic syndromes¹⁹ expected to interfere with aberrant DNA methylation induced by PML-RARA.

ATRA has been used with varying degrees of success in chemoprevention.²⁰ However, the long-term effects of ATRA and 5-AC on APL pathogenesis are currently unknown. In fact, point mutations within the RARA ligand-binding domain of PML-RARA occur in APL cases with acquired resistance to ATRA,^{21,22} while long-term treatment with 5-AC might result in genomic instability and lymphomagenesis in mice and humans.²³⁻²⁵ Moreover, to begin addressing the contribution of diet to APL leukemogenesis, we tested the long-term effects of the Western diet (WD), a high fat, low folate/choline-containing diet that is known to induce hypomethylation.²⁶ The key endpoint biomarker in these experiments was the occurrence of leukemia.

Results and Discussion

For these studies, we used a murine transgenic mouse model in which PML-RARA is expressed in myeloid cells under the MRP8 promoter. PML-RARA transgenic mice develop impaired myeloid differentiation early in life and APL, with a penetrance of 64% and a median latency of 8.5 months. APL that develops in these transgenic mice fully recapitulates the features of the human disease, including achieving remissions when treated with retinoic acid, which is the drug of choice in this disease. Moreover, these features are retained when preleukemic bone marrows are

transplanted in recipient mice.^{6,15} Thus, this mouse model of APL has attractive features to test preclinical chemopreventive and therapeutic strategies.

We generated cohorts of mice transplanted with bone marrow cells obtained from preleukemic APL transgenic mice to perform our chemoprevention studies. Results are summarized in Figure 1. Body weights and blood counts were comparable and within the normal range for all treatment groups until leukemia occurred (data not shown). Nonleukemic deaths, unless stated otherwise, were due to acute events secondary to manipulations that are required for venipuncture. We noted no significant drug-related toxic events.

Significantly, we observed 9 APL cases in the 5-AC treatment group. The mean onset of 5.9 months posttransplantation was strikingly accelerated as compared to the 10 months of the PBS injection group (Fig. 1B). There were 7 nonleukemic deaths in this group, 2 of which were due to sarcoma. We have also observed a substantial but nonsignificant APL acceleration in the WD group when compared to the control diet group ($P = 0.08$) (Fig. 1C).

Surprisingly, we observed no difference in leukemia-free survival between the ATRA and the control diet groups (Fig. 1D). In addition, 2 of the leukemias that arose in this group were proven to be insensitive to ATRA treatment (data not shown). We found no significant morphological and phenotypic differences between the APL that arose in the various treatment groups (Fig. 1F-H; note increased percentage of doubly positive Gr1 and Mac1 cells in F-H as compared to E, and data not shown).

To identify at the molecular level the determinants of the APL acceleration in 5-AC-treated mice, we examined the RNA expression profiles of the APL cases that arose in 5-AC-treated mice and in their control group. Among the top 100 most differentially expressed genes, we found several cancer genes down-regulated in the leukemias that arose during treatment with 5-AC such as DMTF1 (a cyclin D-binding protein that exerts a growth-suppressive function), FOXO1A (a tumor suppressor gene), Bach2 (a growth suppressor), DUSP1 (a down-regulator of multiple MAPK signaling pathways and protein kinases that play a role in cell cycle regulation), and sestrin 1 (a transcriptional target of the tumor suppressor p53). Among the genes up-regulated in the leukemias that arose during 5-AC treatment were Birc1e (a IAP inhibitor that antagonizes apoptosis), Sept9 (a proposed growth suppressor), and angiopoietin-like 4 (a gene with prometastatic functions) (Online Mendelian Inheritance in Man: <http://www.ncbi.nlm.nih.gov/entrez>) (Table 1 and Suppl. Table S1). When analyzed with the DAVID Bioinformatics Resource and Ingenuity Systems Pathway Analysis tools, these genes cluster in functional classes involved in the regulation of transcription, adaptive immune responses, and apoptosis. Therefore, it is tempting to speculate that these processes are involved with the mechanism(s) causing APL acceleration in 5-AC-treated

A

Treatment	Group size	Leukemic deaths	Non-leukemic deaths	Mice sacrificed at the study endpoint
Control Diet	17	4	4	9
Western Diet	17	8	5	4
ATRA	17	2	8	7
5-AC	16	9	7	0
IP Control	16	4	4	5
Total	83	27	31	25

B

□ Vehicle ○ 5-AC

C

○ Control Diet □ Western Diet

D

□ ATRA ○ Control

E **F** **G** **H**

Figure 1. Chronic treatment with 5-azacytidine (5-AC) increases acute promyelocytic leukemia (APL) leukemogenesis. **(A)** Summary of the mice census. Columns indicate the treatments administered, the cause of death, and the number of mice sacrificed at the study end point. **(B-D)** Kaplan-Meier representation of leukemia-free survival in the cohorts treated with 5-AC, the Western diet, and all-trans retinoic acid (ATRA). Each treatment group was compared with the control diet or vehicle. Circle and square symbols indicate the treatment administered. **(E)** Flow cytometry analysis of the peripheral blood of a representative control mouse 6 months after bone reconstitution with hMRP8-PML-RARA bone marrow. The percentage of positive Gr-1 and Mac-1 events is indicated in each quadrant. **(F-H)** Flow cytometry with Gr-1 and Mac-1 antibodies of the peripheral blood of a representative leukemic mouse treated with the Western diet, 5-AC, and ATRA. Note the observed increase of double-positive Mac-1/Gr-1-positive cells is diagnostic for APL.

Table 1. Differential Cancer Gene Expression in APL Leukemias that Arose in 5-AC Treated Mice

A			
Fold down-regulation 5-AC/CTRL	Gene Symbol	Gene Title	Gene Function
5.96	Dmt f1	Cyclin D binding Myb-like TF1	Growth suppressor
4.80	Foxo1	Forkhead Box 1	Tumor suppressor
3.82	Bach2	BTB and CNC homology 2	Growth suppressor
3.82	Dusp1	Dual specificity phosphatase 1	Growth suppressor
3.60	Macf1	Microtubule-actin factor 1	Microtubule dynamics
2.83	Sesn1	Sestrin 1	p53 responsive gene
2.50	Nolc1	Nucleolar phosphoprotein 1	nucleogenesis
2.08	Tbrg4	TGF-regulated gene 4	Growth suppressor
1.72	Btg2	B cell translocation gene 2	Anti-proliferative
B			
Fold up-regulation 5-5-AC/CTRL	Gene Symbol	Gene Title	Gene Function
6.6	Birc1e	IAP repeat containing 1e	Caspase inhibitor
6.1	Sept19	Septin 9	Growth suppressor
2.13	Angptl4	Angiopoietin-like 4	Metastasis
1.95	Cdc 25a	Cell division cycle 25 homolog A	Oncogene
1.90	Hnrpa2b1	Nuclear ribonucleoprotein	Splicing
1.90	Tfdp1	Transcription factor DP-1	E2F binding protein

A. Affymetrix arrays were used to analyze RNA samples extracted from leukemic mononucleated bone marrow obtained from 4 mice treated with 5-AC and 4 mice treated with saline. The table shows selected cancer genes that are down-regulated in leukemias in 5-azacytidine mice when compared to the ones that arose in control treated mice. The first of the columns indicates the ratio of relative gene expression in APL cases that arose in 5-AC treated mice vs the APLs of control treated mice (5-AC/CTRL). The gene symbol, title and function are indicated. **B.** Selected cancer genes up-regulated in APLs that arose in mice treated with 5-AC when compared to the ones developed by control treated mice. Columns indicate relative gene expression in the two groups of APLs (5-AC/CTRL). The gene symbol, title and function are indicated.

mice. However, the relevance of these gene alterations versus the effects of 5-AC on APL is yet to be established.

Our findings allow us to draw the following conclusions: 1) Chronic 5-AC treatment resulted in a striking acceleration of APL leukemogenesis. This result is surprising given the fact that the PML-RARA oncoprotein induces hypermethylation and transcriptional silencing at its target promoter sites and that this activity contributes to its leukemogenic potential.¹¹ DNA hypomethylation has been associated with chromosomal instability, reactivation of transposable elements, loss of imprinting, and activation of proto-oncogenes.²⁵ We speculate that 5-AC may cause genome-wide genetic and epigenetic effects that promote leukemogenesis. For example, recent reports show that genomic hypomethylation causes genomic instability and lymphomagenesis in mice.^{23,24} 2) Chemoprevention with ATRA did not prevent APL leukemogenesis and instead results in ATRA-resistant APL, a known complication of ATRA therapy.^{21,22} 3) WD induced a trend toward APL acceleration that, although did not reach statistical significance ($P = 0.08$), suggests that WD may represent a risk factor for APL leukemogenesis. Further, because the effects

of WD appear to parallel those by 5-AC, we may infer that these effects are due, in large measure, to its enhanced hypomethylating potential.

Our findings have in turn important clinical implications and suggest that further investigations should be considered to address the long-term effects of ATRA and 5-AC in APL and myelodysplastic syndromes. The study of the function of the genes differentially expressed between the 5-AC-treated and control mice may provide insights into the mechanisms underlying 5-AC-induced leukemogenesis.

Materials and Methods

Animals and treatment protocols. At 8 weeks of age, FVB/N mice underwent allogeneic bone marrow transplant with 10^7 viable preleukemic hMRP8-PML-RARA marrow cells obtained from 8-week-old donors.⁶ After hematopoietic reconstitution (about 8 weeks), mice were randomly assigned to treatment with standard AIN-76A diet (control diet) alone, control diet with ATRA (30 μ g/g of diet), or WD alone. The control diet standard provides 3.6 kcal/g, and its composition is as follows: fat (corn oil), 5%; calcium,

5 mg/g; vitamin D3, 1 IU/g; phosphorus, 4 mg/g; fiber, 5%; folic acid, 2 µg/g; methionine, 0.3%; choline bitartrate, 0.2%. The WD provides 4.5 kcal/g, and its composition is as follows: fat (corn oil), 20%; calcium, 0.5 mg/g; vitamin D3, 0.11 IU/g; phosphorus, 3.6 mg/g; fiber, 2%; folic acid, 0.23 µg/g; cysteine, 0.3%; choline bitartrate, 0.12%. Research Diet Inc. (Newark, NJ) formulated all diets. 5-AC (4-amino-1-β-D-ribofuranosyl-1,3,5-triazin-2(1H)-1), provided by the Chemopreventive Agent Development Research Group of the National Cancer Institute, was administered by weekly intraperitoneal injections at a concentration of 5 µg/g of body weight with PBS as a control. Each treatment arm consisted of 16 or 17 mice (Fig. 1A). Mice were evaluated clinically weekly and weighted biweekly. Hematopoiesis was monitored by tail vein venipuncture biweekly. Treatment continued until leukemia was diagnosed by venipuncture, by signs of distress, or until mice turned 18 months old. Postmortem analysis included necroscopy, detailed pathological examination, and flow cytometry analysis. Animal handling was carried out according to the guidelines of the Memorial Sloan-Kettering Cancer Center institutional animal care committees.

Follow-up of transgenic mice and flow cytometry. Mice were inspected weekly and weighted biweekly. Treatment continued until leukemia was diagnosed, signs of distress occurred, or mice turned 18 months old. To diagnose leukemia, mice were bled twice a month from the tail to determine white blood cells, hemoglobin, and platelet counts. APL was diagnosed on the basis of the following concomitant criteria: 1) presence of blasts/promyelocytes (>1%) in peripheral blood; 2) leukocytosis (white blood cells >30 × 10³/µL); and 3) anemia (hemoglobin <10 g/dL) and/or thrombocytopenia (platelets <500 × 10³/µL).²⁷ Diagnosis was confirmed by morphological and flow cytometric analyses of bone marrow cells with Mac-1 (CD11b), Gr-1, c-kit (CD117), Sca-1, B220, CD3, and Ter119 antibodies (BD Biosciences, Franklin Lakes, NJ).²⁸ Animal handling was carried out according to the guidelines of the Memorial Sloan-Kettering Cancer Center institutional animal care committees.

Affymetrix oligonucleotide microarray analysis. Affymetrix U74Av2 arrays (Santa Clara, CA) were used to analyze leukemic samples obtained from the bone marrow or spleen of 4 mice treated with 5-AC and 4 that arose from vehicle control-treated mice, following procedures already described.²⁹ The data were analyzed using the R statistical program and the Bioconductor microarray packages (Bioconductor Open Source Software for Bioinformatics). The raw data were quantitated using the GC-RMA algorithm to determine signal levels and the MAS5 algorithm to determine the absent/present flags. Genes that scored absent in

more than 75% of both groups were filtered from further analysis. We used a variant of the standard *t* test (the LIMMA method) to determine the genes, which had the lowest *P* values for the test and had a fold change difference of at least 1.5. Pathway analysis was performed with the DAVID Bioinformatics Resource (<http://david.abcc.ncifcrf.gov/home.jsp>) and Ingenuity Systems pathway analysis.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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References

1. Feinberg AP, Tycko B. The history of cancer epigenetics. *Nat Rev Cancer*. 2004;4(2):143-53.
2. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144(5):646-74.
3. Scaglioni PP, Pandolfi PP. The theory of APL revisited. *Curr Top Microbiol Immunol*. 2007;313:85-100.
4. He LZ, Tribioli C, Rivi R, *et al*. Acute leukemia with promyelocytic features in PML/RARalpha transgenic mice. *Proc Natl Acad Sci U S A*. 1997;94(10):5302-7.
5. Grisolan JL, Wesselschmidt RL, Pelicci PG, Ley TJ. Altered myeloid development and acute leukemia in transgenic mice expressing PML-RAR alpha under control of cathepsin G regulatory sequences. *Blood*. 1997;89(2):376-87.
6. Brown D, Kogan S, Lagasse E, *et al*. A PMLRARalpha transgene initiates murine acute promyelocytic leukemia. *Proc Natl Acad Sci U S A*. 1997;94(6):2551-6.
7. He LZ, Guidez F, Tribioli C, *et al*. Distinct interactions of PML-RARalpha and PLZF-RARalpha with co-repressors determine differential responses to RA in APL. *Nat Genet*. 1998;18(2):126-35.
8. Lin RJ, Evans RM. Acquisition of oncogenic potential by RAR chimeras in acute promyelocytic leukemia through formation of homodimers. *Mol Cell*. 2000;5(5):821-30.
9. Lin RJ, Nagy L, Inoue S, Shao W, Miller WH Jr, Evans RM. Role of the histone deacetylase complex in acute promyelocytic leukaemia. *Nature*. 1998;391(6669):811-4.
10. Grignani F, De Matteis S, Nervi C, *et al*. Fusion proteins of the retinoic acid receptor-alpha recruit histone deacetylase in promyelocytic leukaemia. *Nature*. 1998;391(6669):815-8.

11. Di Croce L, Raker VA, Corsaro M, *et al.* Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. *Science*. 2002;295(5557):1079-82.
12. Grimwade D, Howe K, Langabeer S, *et al.* Establishing the presence of the t(15;17) in suspected acute promyelocytic leukaemia: cytogenetic, molecular and PML immunofluorescence assessment of patients entered into the M.R.C. ATRA trial. M.R.C. Adult Leukaemia Working Party. *Br J Haematol*. 1996;94(3):557-73.
13. Berger R, Le Coniat M, Derre J, Vecchione D, Jonveaux P. Cytogenetic studies in acute promyelocytic leukemia: a survey of secondary chromosomal abnormalities. *Genes Chromosomes Cancer*. 1991;3(5):332-7.
14. Zimonjic DB, Pollock JL, Westervelt P, Popescu NC, Ley TJ. Acquired, nonrandom chromosomal abnormalities associated with the development of acute promyelocytic leukemia in transgenic mice. *Proc Natl Acad Sci U S A*. 2000;97(24):13306-11.
15. Le Beau MM, Bitts S, Davis EM, Kogan SC. Recurring chromosomal abnormalities in leukemia in PML-RARA transgenic mice parallel human acute promyelocytic leukemia. *Blood*. 2002;99(8):2985-91.
16. Douer D, Preston-Martin S, Chang E, Nichols PW, Watkins KJ, Levine AM. High frequency of acute promyelocytic leukemia among Latinos with acute myeloid leukemia. *Blood*. 1996;87(1):308-13.
17. Newmark HL, Yang K, Lipkin M, *et al.* A Western-style diet induces benign and malignant neoplasms in the colon of normal C57Bl/6 mice. *Carcinogenesis*. 2001;22(11):1871-5.
18. Xue L, Yang K, Newmark H, Leung D, Lipkin M. Epithelial cell hyperproliferation induced in the exocrine pancreas of mice by a Western-style diet. *J Natl Cancer Inst*. 1996;88(21):1586-90.
19. Silverman LR, Demakos EP, Peterson BL, *et al.* Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: a study of the cancer and leukemia group B. *J Clin Oncol*. 2002;20(10):2429-40.
20. Okuno M, Kojima S, Matsushima-Nishiwaki R, *et al.* Retinoids in cancer chemoprevention. *Curr Cancer Drug Targets*. 2004;4(3):285-98.
21. Cote S, Zhou D, Bianchini A, Nervi C, Gallagher RE, Miller WH Jr. Altered ligand binding and transcriptional regulation by mutations in the PML/RARalpha ligand-binding domain arising in retinoic acid-resistant patients with acute promyelocytic leukemia. *Blood*. 2000;96(9):3200-8.
22. Imaizumi M, Suzuki H, Yoshinari M, *et al.* Mutations in the E-domain of RAR portion of the PML/RAR chimeric gene may confer clinical resistance to all-trans retinoic acid in acute promyelocytic leukemia. *Blood*. 1998;92(2):374-82.
23. Braig M, Lee S, Loddenkemper C, *et al.* Oncogene-induced senescence as an initial barrier in lymphoma development. *Nature*. 2005;436(7051):660-5.
24. Gaudet F, Hodgson JG, Eden A, *et al.* Induction of tumors in mice by genomic hypomethylation. *Science*. 2003;300(5618):489-92.
25. Kopelovich L, Crowell JA, Fay JR. The epigenome as a target for cancer chemoprevention. *J Natl Cancer Inst*. 2003;95(23):1747-57.
26. Niculescu MD, Zeisel SH. Diet, methyl donors and DNA methylation: interactions between dietary folate, methionine and choline. *J Nutr*. 2002;132(8 Suppl):2333S-5S.
27. Rego EM, Wang ZG, Peruzzi D, He LZ, Cordon-Cardo C, Pandolfi PP. Role of promyelocytic leukemia (PML) protein in tumor suppression. *J Exp Med*. 2001;193(4):521-9.
28. Matsushita H, Scaglioni PP, Bhaumik M, *et al.* In vivo analysis of the role of aberrant histone deacetylase recruitment and RAR alpha blockade in the pathogenesis of acute promyelocytic leukemia. *J Exp Med*. 2006;203(4):821-8.
29. Talbot SG, Estilo C, Maghami E, *et al.* Gene expression profiling allows distinction between primary and metastatic squamous cell carcinomas in the lung. *Cancer Res*. 2005;65(8):3063-71.