Role of Promyelocytic Leukemia (PML) Protein in Tumor Suppression

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Abstract

The promyelocytic leukemia (PML) gene encodes a putative tumor suppressor gene involved in the control of apoptosis, which is fused to the retinoic acid receptor α (RARα) gene in the vast majority of acute promyelocytic leukemia (APL) patients as a consequence of chromosomal translocations. The PMLRARα oncoprotein is thought to antagonize the function of PML through its ability to heterodimerize with and delocalize PML from the nuclear body. In APL, this may be facilitated by the reduction to heterozygosity of the normal PML allele. To determine whether PML acts as a tumor suppressor in vivo and what the consequences of deregulated programmed cell death in leukemia and epithelial cancer pathogenesis are, we crossed $PML^{-/-}$ mice with human cathepsin G (hCG)-PMLRARα or mammary tumor virus (MMTV)/neu transgenic mice (TM), models of leukemia and breast cancer, respectively. The progressive reduction of the dose of PML resulted in a dramatic increase in the incidence of leukemia, and in an acceleration of leukemia onset in PMLRARα TM. By contrast, PML inactivation did not affect neu-induced tumorigenesis. In hemopoietic cells from PMLRARα TM, PML inactivation resulted in impaired response to differentiating agents such as RA and vitamin D_3 as well as in a marked survival advantage upon proapoptotic stimuli. These results demonstrate that: (a) PML acts in vivo as a tumor suppressor by rendering the cells resistant to proapoptotic and differentiating stimuli; (b) PML haploinsufficiency and the functional impairment of PML by PMLRARα are critical events in APL pathogenesis; and (c) aberrant control of programmed cell death plays a differential role in solid tumor and leukemia pathogenesis.

Key words: promyelocytic leukemia protein • acute promyelocytic leukemia • leukemogenesis • apoptosis • transgenic mice

Introduction

Acute promyelocytic leukemia (APL),[1] a distinct subtype of acute myelogenous leukemia (AML), accounts for >10% of all AMLs and is characterized by: (a) the accumulation of leukemic cells with promyelocytic features in the bone marrow (BM); (b) the invariable association with chromosomal translocations involving the retinoic acid receptor α (RARα) locus in chromosome 17; and (c) the exquisite sensitivity of the APL blasts to the differentiating action of the RA (1–3). In the vast majority of cases, RARα fuses to the promyelocytic leukemia (PML) gene as a consequence of a reciprocal and balanced translocation between chromosomes 15 and 17 (t[15;17]; reference 2). PML belongs to a family of proteins characterized by the presence of the RING-B-box-coiled-coil (RBCC) motif (4), which consists of a C_3HC_4 zinc finger (RING finger) and one or two additional Cys-rich regions (B-boxes) followed by a predicted leucine coiled-coil region. The PML coiled-coil domain is responsible for the formation of stable PML and PMLRARα homo- and heterodimers (5). PML and PMLRARα heterodimerization results in the delocalization of PML from discrete speckled nuclear structures, the nuclear bodies (NBs). As a consequence, in APL cells, PML acquires an aberrant microspeckled nuclear

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[1]*Abbreviations used in this paper:* APL, acute promyelocytic leukemia; BM, bone marrow; hCG, human cathepsin G; LFS, leukemia-free survival; MMTV, mammary tumor virus; NB, nuclear body; PML, promyelocytic leukemia; RA, retinoic acid; RXR, retinoid X receptor; TFS, tumor-free survival; TM, transgenic mice; TUNEL, terminal deoxynucleotidyl transferase–mediated uridine triphosphate end labeling; VDR, vitamin D receptor; WT, wild-type.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/2001/02/521/09 $5.00
Volume 193, Number 4, February 19, 2001 521–529
http://www.jem.org/cgi/content/full/193/4/521

localization pattern (6). This observation has led to the hypothesis that the function of PML is deregulated in the presence of the PMLRARα fusion oncoprotein (1, 2, 6).

In vitro experiments have shown that PML can act as a tumor suppressor (6–9). PML can inhibit transformation induced by neu (c-erbB2, ERBB2), Ha-ras, mutant p53, Ha-ras plus c-myc in NIH3T3, and rat fibroblasts (7–9). In fibroblasts, PML is involved in the regulation of p53-dependent senescence upon oncogenic transformation (10). PML also plays a role in multiple apoptotic pathways (11). $PML^{-/-}$ mice and cells are protected from multiple caspase-dependent apoptotic signals such as Fas, TNF, ceramide, IFNs, and ionizing radiation (11). $PML^{-/-}$ mice developed a greater number of skin papillomas when topically challenged with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and dimethybenzanthracene (DMBA [12]). In a second model, DMBA was injected into the salivary glands, which lead to a greater number of B and T lymphomas in $PML^{-/-}$ mice than the wild-type (WT) controls (12). However, the fact that $PML^{-/-}$ mice succumbed to infections severely compromised the long-term assessment of tumor incidence, whereas the incidence of spontaneous tumors in the $PML^{-/-}$ cohort was not increased during the first year of life (12). Moreover, the fact that $PML^{-/-}$ mice develop tumors only when challenged with carcinogens casts doubts on the relevance of PML in tumor suppression. Therefore, it remains to be determined whether, in vivo, PML would act as a tumor suppressor in a relevant disease context and, if that is the case, by which mechanisms PML would antagonize tumorigenesis. Furthermore, in APL the dose of PML is reduced to heterozygosity in view of the fact that one allele is involved in the chromosomal translocation. If indeed PML acts as a tumor suppressor in vivo, this may dramatically enhance the oncogenic potential of PMLRARα, even more so if the oncoprotein can act as a dominant negative on PML function. To address these questions we studied whether, and through which mechanisms, PML would antagonize PMLRARα–induced leukemogenesis and neu-induced breast tumorigenesis in human cathepsin G (hCG)-PMLRARα and mammary tumor virus (MMTV)/neu transgenic mice (TM). We here report that PML acts in vivo as a tumor suppressor through control of programmed cell death and cellular differentiation.

Materials and Methods

Generation and Genotyping of Mutant Mice. The generation of the $PML^{-/-}$ mutants and hCG-PMLRARα TM has been described elsewhere (12, 13). $PML^{-/-}$ mice were mated with hCG-PMLRARα TM to generate hCG-PMLRARα/PML mutants of six different genotypes. Similarly, we crossed TM harboring the neu protooncogene under the control of the MMTV promoter (The Jackson Laboratory [14]) with $PML^{-/-}$ mice. PML mutants were identified by Southern blot analysis of tail DNA using a 1-kb ApaI mouse PML fragment (probe A), as described previously (12). MMTV/neu TM were identified also by Southern blot analysis using a neu cDNA-specific probe (14). To distinguish hCG-PMLRARα heterozygous (+/−) from hCG-PMLRARα homozygous (+/+) mice, tail DNA was digested with EcoRI, hybridized with a 250-bp HindIII-XbaI hCG genomic fragment (probe CT), and cohybridized with a murine 2.3-kb BamHI p53 cDNA probe. The genotype of the TM was determined using a PhosphorImager Densitometer (Bio-Rad Laboratories) based on the comparative analysis of the intensity of p53 and hCG-PMLRARα signals.

Follow Up of Mutant Mice. hCG-PMLRARα/PML mutants were bled twice a month from the tail, and white blood cells (WBCs), hemoglobin, and platelet counts were determined using a Technicon H2 automated counter. The differential counts of the peripheral blood (PB) were performed microscopically on Wright-Giemsa stained smears. Diagnosis of leukemia was made on the basis of the following concomitant criteria: (a) presence of blasts/promyelocytes (>1%) in the PB; (b) leukocytosis (WBCs > $30 \times 10^3/\mu l$); and (c) anemia (hemoglobin < 10 g/dl) and/or thrombocytopenia (platelets < $500 \times 10^3/\mu l$). The MMTV/neu/PML mutants were inspected closely on a weekly basis for the appearance of mammary tumors and/or a decline in overall body condition. Animals were killed just before their natural demise or when discomfort due to the tumor mass was observed. Postmortem analysis was performed in all tissues, including mammary glands, lungs, BM, spleen, liver, and lymph nodes.

Flow Cytometry. The immunophenotype of PB, BM, and spleen cells from leukemic hCG-PMLRARα/PML mutants was determined using the following fluorochrome-conjugated mAbs: CD11b, Gr1, c-kit, Sca1, CD3, and CD45R/B220. Fluorochrome conjugated isotypic Abs of irrelevant specificity were used as controls. All Abs were obtained from BD PharMingen. Samples were analyzed on a FACScan™ flow cytometer using CellQuest™ software (Becton Dickinson).

In Vitro Methylcellulose Colony Assay. 5×10^4 BM cells obtained from 2–3-mo-old sex-age–matched mice of various genotypes were cultured in methylcellulose and CFU-GM were scored at day 7 as described previously (12). Three independent experiments, each in triplicate, were carried out using one mouse per genotype. RA (Sigma-Aldrich) in DMSO (Sigma-Aldrich) was added at a final concentration of 100 nM, 1,25-dihydroxyvitamin D_3 (vitamin D_3; Sigma-Aldrich) in ethanol was added at a final concentration of 1 nM, and the anti-Fas mAb (clone Jo2; BD PharMingen) in PBS was added at a final concentration of 100 ng/ml. Negative controls included the incubation with the respective vehicles and, for the experiments with anti-Fas mAb, the addition of an irrelevant immunoglobulin of the same class (BD PharMingen).

Assessment of Myeloid Differentiation and Apoptosis in Liquid Cultures. 2×10^6 BM cells from 2–3-mo-old sex-age–matched mice of various genotypes were cultured in DMEM supplemented with 30% of FCS, 20 ng/ml GM-CSF, and 50 ng/ml G-CSF. RA, vitamin D_3, anti-Fas mAb, or their respective vehicles, were added at the above mentioned concentrations. Myeloid differentiation was assessed by determining the number of $CD11b^+$ cells after 72 h of incubation with RA or vitamin D_3 by flow cytometry. The percentage of apoptotic cells after 24 h of incubation with the anti-Fas mAb was determined by staining the cells with an anti–annexin V mAb and propidium iodide (PI) in combination using the Apoptosis Detection Kit II (BD PharMingen).

In Vivo Detection of Apoptosis and Immunohistochemistry. The number of apoptotic cells in breast tumor paraffin sections (5 μm) from MMTV/neu TM was scored by in situ terminal deoxynucleotidyl transferase–mediated uridine triphosphate end labeling (TUNEL) as described previously (15). Immune detection of PML and Ki67 in frozen and paraffin breast tumor sections respectively were performed as described (12, 16).

Statistical Analysis. Statistical analysis was carried out using the SPSS software (SPSS). Leukemia-free survival (LFS) and tumor-free survival (TFS) analyses (dated from birth to diagnosis) was based on Kaplan-Meyer estimation and groups were compared by log-rank test. Differences in disease incidence were evaluated using the Fisher's exact test. The comparison between the number of hemopoietic colonies, the percentage of $CD11b^+$, of $Ki67^+$, and of apoptotic cells between the groups was performed by the Mann-Whitney U test. All quoted *P* values are two-sided, and confidence intervals refer to 95% boundaries.

Results and Discussion

TM expressing the PMLRARα fusion gene under the control of the hCG-PMLRARα develop a form of leukemia that closely resembles the human APL (13). However, only 10–15% of these mice develop the disease after a long latency period (>12 mo; reference 13), indicating that PMLRARα is necessary, but not sufficient, to cause full-blown leukemia. To determine whether PML inactivation would accelerate leukemia onset and/or penetrance, we crossed hCG-PMLRARα TM with $PML^{-/-}$ mice. Compared with hCG-$PMLRAR\alpha^{+/-}PML^{+/+}$ mice, hCG-$PMLRAR\alpha^{+/-}PML^{+/-}$ or $PML^{-/-}$ mutants presented a significant decrease in the LFS (mean LFS ± SD in hCG-$PMLRAR\alpha^{+/-}$/$PML^{+/+}$ mice: 686.4 ± 35.5 d; hCG-$PMLRAR\alpha^{+/-}PML^{+/-}$ mice: 498.9 ± 31.3 d [$P = 0.003$]; hCG-$PMLRAR\alpha^{+/-}PML^{-/-}$ mice: 434.4 ± 30.6 d [$P < 0.0001$]; Fig. 1 a). Moreover, the incidence of

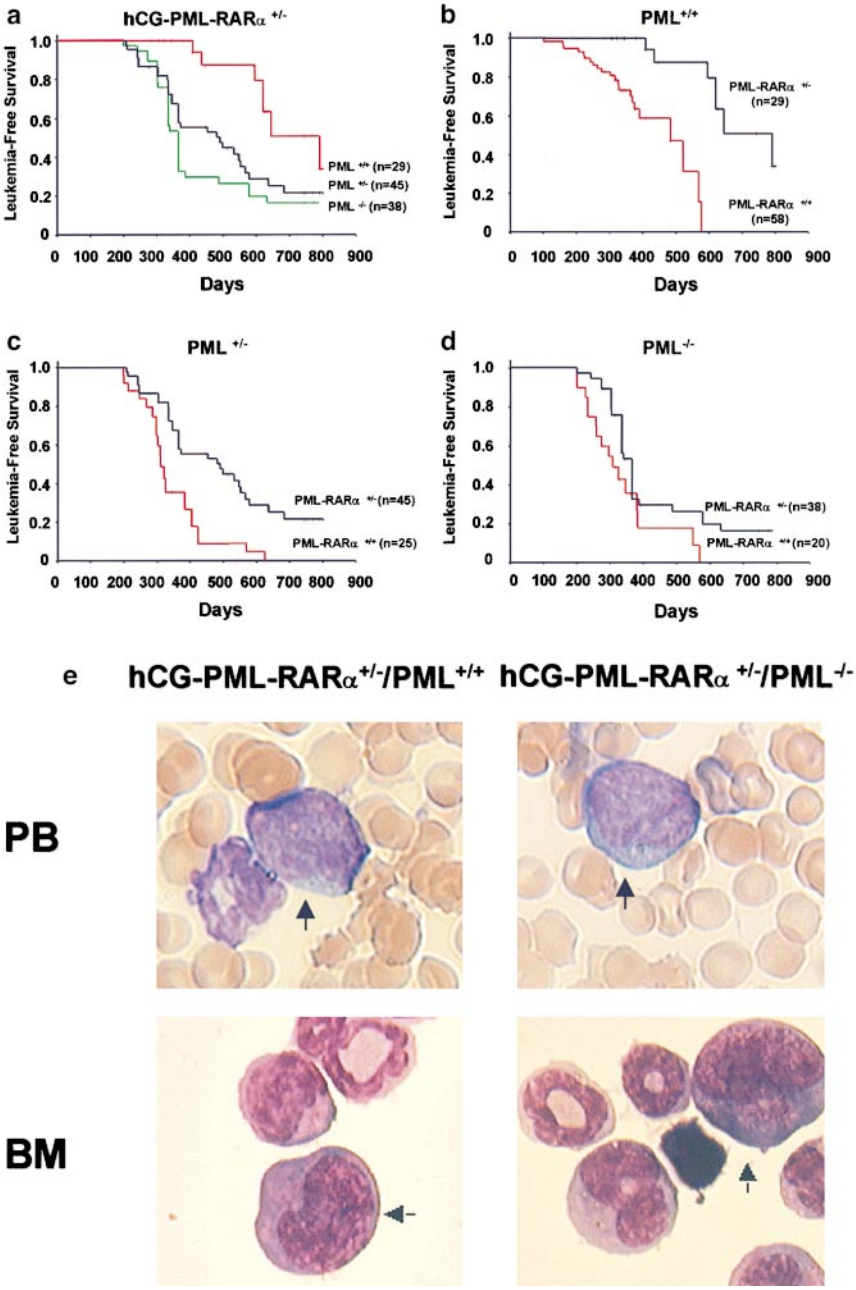

Figure 1. PML inactivation leads to an increase in frequency and an earlier leukemia onset in hCG-PMLRARα TM. (a–d) hCG-PML-RARα TM were mated with mice in which the PML gene was disrupted by homologous recombination. The various genotypes and the numbers of mice analyzed are indicated. LFS (in days) was significantly shorter and leukemia frequency higher in hCG-$PMLRAR\alpha^{+/-}PML^{-/-}$ and hCG-$PMLRAR\alpha^{+/-}PML^{+/-}$ mice compared with hCG-$PMLRAR\alpha^{+/-}PML^{+/+}$ mice (a). The leukemia frequency was also increased in hCG-$PMLRAR\alpha^{+/+}$ mice compared with hCG-$PMLRAR\alpha^{+/-}$ mice either in $PML^{+/+}$ (b), $PML^{+/-}$ (c), or $PML^{-/-}$ (d) backgrounds. (e) Leukemic cells in all the studied groups exhibited similar features. PB smears and BM cytospin preparations from leukemic hCG-$PMLRAR\alpha^{+/-}PML^{-/-}$ and hCG-$PMLRAR\alpha^{+/-}PML^{+/+}$ mice were stained with Wright-Giemsa stain. The arrows indicate the leukemic cells. Original magnification: ×1,000.

leukemia in the first year of life was of 12.5% in hCG-PMLRARα$^{+/-}$PML$^{+/+}$ mice, whereas in the hCG-PMLRARα$^{+/-}$PML$^{+/-}$ and hCG-PMLRARα$^{+/-}$PML$^{-/-}$ mice it was of 31.1% ($P < 0.001$) and 53.1% ($P < 0.001$), respectively (Fig. 1 a). The difference in the LFS between hCG-PMLRARα$^{+/-}$PML$^{+/-}$ and hCG-PMLRARα$^{+/-}$PML$^{-/-}$ mice was not statistically significant ($P = 0.21$). On the contrary, the incidence of leukemia was significantly higher in the hCG-PMLRARα$^{+/-}$PML$^{-/-}$ mice ($P = 0.003$). Thus, PML inactivation dramatically contributes to APL leukemogenesis and the inactivation of one PML allele has already a striking effect on the incidence and latency of the disease.

To test if the increase in the dose of the PMLRARα oncoprotein would have similar effects, we compared the LFS and the incidence of leukemia between hCG-PMLRARα$^{+/-}$ and hCG-PMLRARα$^{+/+}$ TM (Fig. 1, b–d). LFS ± SD of the hCG-PMLRARα$^{+/+}$PML$^{+/+}$, hCG-PMLRARα$^{+/+}$PML$^{+/-}$, and hCG-PMLRARα$^{+/+}$PML$^{-/-}$ mutants was of 439.4 ± 22.6, 335 ± 19.5, and 315 ± 19.5 d, respectively (Fig. 1, b–d). Therefore, regardless of the PML dose, the LFS was significantly shorter in the hCG-PMLRARα$^{+/+}$ TM compared with the hCG-PMLRARα$^{+/-}$ TM. Accordingly, the incidence of leukemia in the first year of life was significantly higher in the hCG-PMLRARα$^{+/+}$ compared with the hCG-PMLRARα$^{+/-}$ TM in all the PML subgroups (25.8, 52, and 60% in the PML$^{+/+}$, PML$^{+/-}$, and PML$^{-/-}$ subgroups, respectively). The differences in the LFS and leukemia incidence were not significant between the hCG-PMLRARα$^{+/+}$PML$^{+/-}$ and the hCG-PMLRARα$^{+/+}$PML$^{-/-}$ mice. The inactivation of PML did not change the morphology (Fig. 1 e) or the immunophenotypic features of the leukemic cells (data not shown) in hCG-PMLRARα$^{+/-}$ or hCG-PMLRARα$^{+/+}$ TM. In addition, the hematological parameters of the peripheral blood at the disease onset were similar between the six analyzed groups (data not shown). The fact that the increase in the PML-RARα dose further accelerated leukemia onset and penetrance even in a PML$^{-/-}$ background suggests that the oncoprotein is not solely affecting the PML pathway and is in agreement with the notion that the fusion protein can also interfere with the RARα/retinoid X receptor (RXR) pathway.

As_2O_3, a chemical used in traditional Chinese medicine, induces complete remission in about 90% of t(15;17) APL (17). This drug causes the degradation of PMLRARα and PML proteins, as well as the reconstitution of the normal PML-NB pattern (18–20). In vitro studies demonstrated that As_2O_3 also induces the relocalization of PML into the PML-NB, suggesting that the antileukemic and proapoptotic action of As_2O_3 may depend on PML expression (18). Therefore, we tested whether the inactivation of PML would impair the in vivo response to As_2O_3. We treated leukemic hCG-PMLRARα$^{+/-}$PML$^{+/+}$ ($n = 6$) and hCG-PMLRARα$^{+/-}$PML$^{-/-}$ ($n = 3$) with 2.5 μg of As_2O_3 (Sigma-Aldrich) per gram of body weight daily, intraperitoneally for 3 wk, a regimen previously shown to be effective in the treatment of leukemia in hCG-PMLRARα TM (20). Regardless of the presence of PML, As_2O_3 induced disappearance of circulating leukemic blasts, normalization of the hemoglobin levels and platelet counts, as well as the reduction of promyelocytes in the BM to <5%, thus demonstrating that As_2O_3 can induce remission in both hCG-PMLRARα$^{+/-}$PML$^{+/+}$ and hCG-PMLRARα$^{+/-}$PML$^{-/-}$ leukemic mice. Furthermore, there was no significant difference in LFS between PML$^{-/-}$ and PML$^{+/+}$ leukemic TM treated with As_2O_3 (PML$^{+/+}$: 36.7 d, 95% confidence interval (C.I.): 29.5–43.9 d; PML$^{-/-}$: 42 d 95% C.I.: 30.9–53 d). In both groups, death was preceded by disease relapse and the mice did not receive a second course of treatment. The fact that the antileukemic activity of As_2O_3 is independent of the presence of PML corroborates the study by Wang et al. demonstrating that this drug inhibits growth and induces apoptosis in both PML$^{-/-}$ and PML$^{+/+}$ murine embryonic fibroblasts and hematopoietic progenitors (21).

We next tested whether PML would play a role in antagonizing neu-induced breast tumorigenesis by analyzing the effect of PML inactivation in MMTV/neu TM (14). In NIH3T3 cells, PML can antagonize oncogenesis by neu and in fibroblasts it controls cellular senescence induced by oncogenic Ras (7, 10). neu oncogenic activation can occur through point mutations in the transmembrane domain, deletion of the extracellular domain, or overexpression (22–26). Overexpression and amplification of the neu protooncogene have been implicated in human breast cancer pathogenesis (25). Overexpression of neu protein may in fact be the primary mechanism contributing to human breast cancer, as neu activating mutations were not identified in primary cancer biopsy samples (26). MMTV/neu TM develop mammary tumors after a long latency (14; Fig. 2 a). Surprisingly, PML inactivation did not modify the frequency, the latency, the size of breast tumors, or the frequency of metastatic involvement in MMTV/neu TM (Fig. 2 a). The mean TFS ± SD in PML$^{+/+}$ group was of 568 ± 35 d; in the PML$^{+/-}$ mice: 567 ± 36 d; and in the PML$^{-/-}$ mice: 590 ± 26.8 d ($P = 0.94$). All together, these results demonstrate that in vivo PML plays a selective tumor suppressive role antagonizing leukemogenesis by PMLRARα, but not neu-induced mammary tumorigenesis.

PML can control cellular differentiation upon stimuli such as RA (1, 12, 27), programmed cell death, as well as cellular proliferation and senescence (10–12). Therefore, we evaluated through which mechanisms PML would antagonize oncogenesis and if and to which extent these processes would be affected in the two TM models of neoplasia. In the breast tumors from MMTV/neu TM, apoptosis was virtually undetectable in both PML$^{+/+}$ and PML$^{-/-}$ backgrounds, as analyzed by TUNEL (<1 in 400 cells; $n = 3$ per genotype; Fig. 2 b). By contrast, cells from breast tumor samples from the MMTV/neu/PML$^{+/+}$ and MMTV/neu/PML$^{-/-}$ mice were found in active proliferation, but, surprisingly, no differences were detected in the percentage of Ki67^{+} cells between the two PML genotypes ($n = 3$ per genotype; mean ± SD: 30.5% ± 7.6 [PML$^{+/+}$] and 27% ±

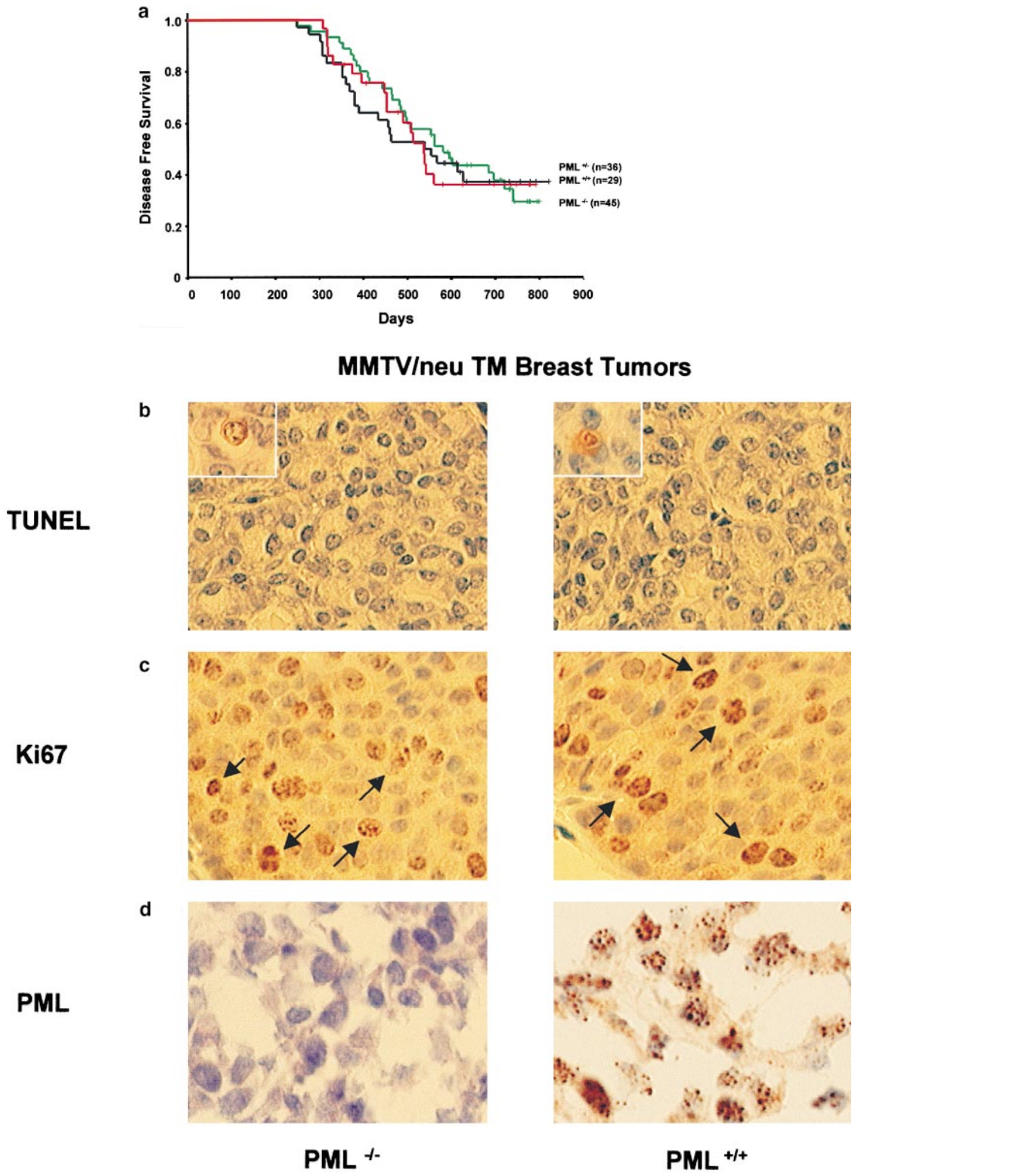

Figure 2. PML inactivation does not affect tumorigenesis in MMTV/neu TM. (a) MMTV/neu TM were mated with $PML^{-/-}$ mice and their progenies monitored for the incidence of breast tumors. The various genotypes and the numbers of mice analyzed are indicated ($PML^{+/+}$, red line; $PML^{+/-}$, black line; $PML^{-/-}$, green line). A similar frequency and TFS was observed in the MMTV/neu TM regardless of the PML genotype. (b and c) Assessment of apoptosis (TUNEL) and proliferation (Ki67 staining) in breast tumors from MMTV/neu/$PML^{+/+}$ and MMTV/neu/$PML^{-/-}$ mice. Apoptotic cells are virtually absent in these tumors (<1/400 cells scored in both genotypes: see indents) whereas numerous proliferating cells are detected in comparable numbers regardless of the PML background. Arrows show examples of positively stained cells. (d) PML is expressed in breast tumors from MMTV/neu TM. Immunostaining with an anti-PML Ab of breast tumor frozen sections from MMTV/neu TM shows the characteristic PML nuclear speckled pattern. Original magnification: ×400.

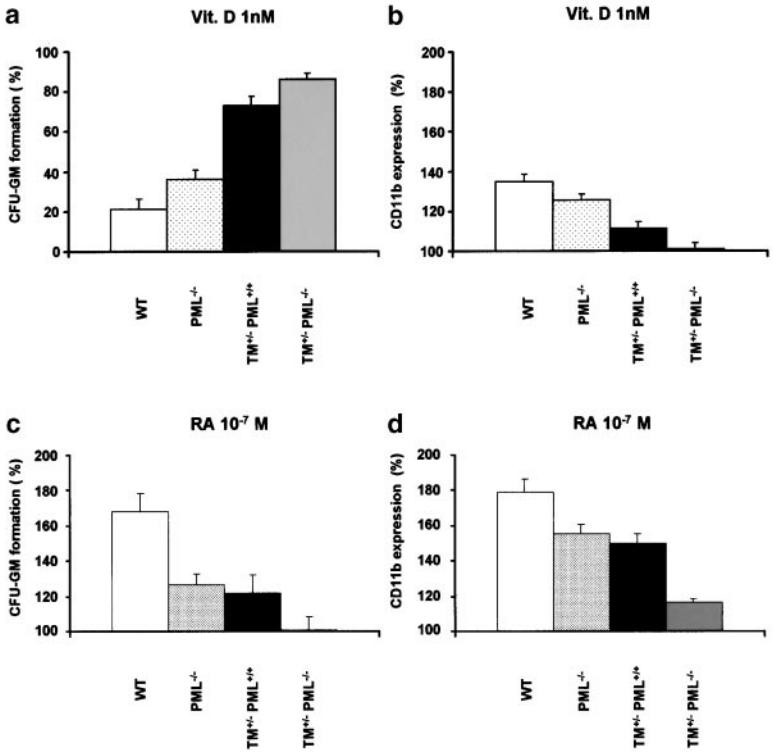

Figure 3. PML inactivation impairs the growth inhibitory and differentiating activities of vitamin D_3 (Vit. D) and RA in BM cells from hCG-PMLRAR$\alpha^{+/-}$ TM. Hemopoietic cells from WT (white bars), PML$^{-/-}$ (dotted bar), hCG-PMLRAR$\alpha^{+/-}$PML$^{+/+}$ (black bar), and hCG-PMLRAR$\alpha^{+/-}$PML$^{-/-}$ (gray bar) mice were cultured in semisolid media (a and c) or in liquid media (b and d) in the presence or absence of vitamin D_3 (10^{-9} M) or RA (10^{-7} M). The number of myeloid colonies (CFU-GM) formed in methylcellulose (a and c) and the expression of CD11b in liquid cultures (b and d) were analyzed after 7 and 3 d, respectively. The results are reported as percentages observed in treated samples relative to controls. For either a, b, c, or d, one experiment performed in triplicate, out of three independent experiments with similar results, is shown (Materials and Methods).

4.8 [PML$^{-/-}$] Ki67^{+} cells; Fig. 2 b). This is in spite of the fact that PML is expressed at high levels not only in breast epithelium, but also in breast tumor samples ($n = 3$ per genotype; Fig. 2 b, and not shown). Thus, surprisingly, in a tumor model in which increased cellular proliferation appears to be the main pathogenic event, PML inactivation does not enhance the main oncogenic effect of neu, in agreement with the fact that incidence latency and tumor burden are unaffected in a PML$^{-/-}$ background.

By contrast, PML heterozygosity or complete PML inactivation dramatically accelerated leukemogenesis in hCG-PMLRARα TM. Therefore, we investigated whether this would affect the ability of hemopoietic cells, before leukemia onset, to respond to growth inhibitory, differentiating, and proapoptotic stimuli. At first, we studied the effects of vitamin D_3 and RA on the ability of BM hemopoietic precursors from the various mutants to form myeloid colonies in a methylcellulose assay and mature myeloid cells in liquid cultures. In these ex vivo assays, vitamin D_3 is a potent growth inhibitory molecule and a modest differentiating agent (28, 29), whereas RA is an effective inducer of myeloid terminal differentiation (1, 12, 27). In the absence of vitamin D_3 or RA and in standard cytokine concentrations (see Materials and Methods), hemopoietic precursors from the various mutant mice generated similar numbers of erythroid and myeloid colonies

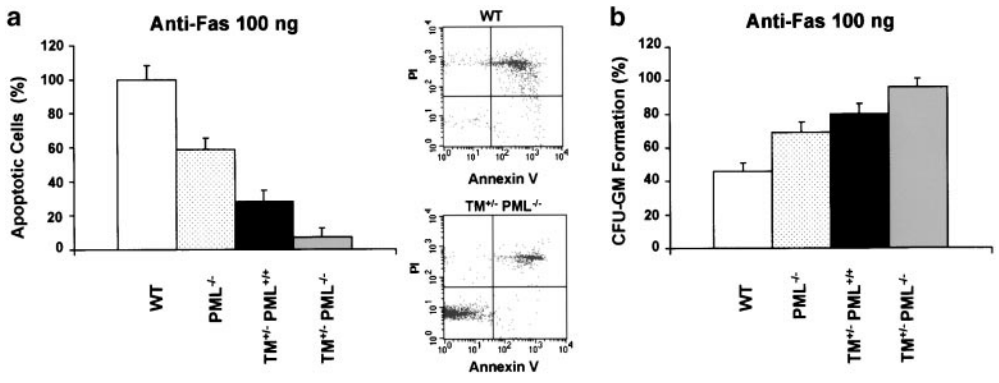

Figure 4. PML inactivation enhances the protection from Fas-induced apoptosis in BM cells from hCG-PMLRAR$\alpha^{+/-}$ TM. BM cells from mice of indicated genotypes were cultured in liquid media (a) or semisolid media (b) in the presence or absence of 100 ng of anti-Fas Ab. (a) The number of apoptotic cells in the liquid culture assay was determined after 24 h by staining with propidium iodide (PI) and annexin V. Histogram bars represent the percentage of apoptotic cells induced by anti-Fas treatment. The mean percentage of apoptotic cells in WT cultures is presented as 100%. Illustrative dot plots from flow cytometric analyses of Fas-stimulated BM cultures from mice of indicated genotypes are shown in the right panel. (b) CFU-GM from in vitro methylcellulose colony assays were scored at day 7. The results are reported as the percentage of myeloid colonies observed in treated samples relative to controls. For both a and b, one experiment performed in triplicate, out of three independent experiments with similar results, is shown (Materials and Methods).

(data not shown). As expected, in BM cells from WT mice, vitamin D_3 (10^{-9} M) and RA (10^{-7} M) caused a decrease and an increase in the number of CFU-GM colonies, respectively. The growth inhibitory activity of vitamin D_3 was already impaired in hemopoietic precursors from PMLRARα$^{+/-}$PML$^{+/+}$ mice. Compared with BM cells from WT mice, BM cells from PMLRARα$^{+/-}$PML$^{+/+}$ generated, in fact, a significantly higher number of CFU-GM colonies ($P < 0.001$) in the methylcellulose assay. The ability of vitamin D_3 to induce terminal myeloid differentiation (CD11b$^+$ cells) in the liquid BM cultures was also markedly reduced ($P = 0.04$; Fig. 3, a and b). Similarly, PML inactivation resulted in impaired response to vitamin D_3 (Fig. 3, a and b). Moreover, the inactivation of PML in BM cells from PMLRARα TM resulted in a further enhancement of the unresponsiveness to vitamin D_3 in both the in vitro colony ($P = 0.01$) and in the liquid culture ($P = 0.009$) assays (Fig. 3, a and b).

The differentiating effect of RA was significantly reduced in BM cells from PML$^{-/-}$ and PMLRARα$^{+/-}$ PML$^{+/+}$ mice ($P = 0.001$ and $P < 0.001$, respectively; Fig. 3, c and d). Furthermore, when treated with RA, BM cells from PMLRARα$^{+/-}$PML$^{-/-}$ mice generated a significantly lower ($P = 0.001$) number of CFU-GM colonies compared with BM cells from PMLRARα$^{+/-}$PML$^{+/+}$ mice (Fig. 3 c). Similar effects were noticed in the liquid BM culture assay where RA increased the percentage of CD11b$^+$ cells in culture from WT mice, whereas this effect was greatly reduced in cells from PML$^{-/-}$ ($P = 0.001$) and in PMLRARα$^{+/-}$PML$^{+/+}$ mice ($P = 0.001$; Fig. 3 d). In complete agreement with the results obtained in the colony assay, the inactivation of PML caused a further significant impairment in the ability of PMLRARα cells to respond to RA in the liquid BM culture assay ($P = 0.007$; Fig. 3 d). Thus, the inactivation of the PML pathway and/or the presence of PMLRARα block the response of myeloid hemopoietic progenitors to the growth inhibitory and differentiating ability of vitamin D_3 and RA.

PML inactivation results in unresponsiveness to many apoptotic stimuli including Fas (11). Therefore, we used the response of BM cells to an activating anti-Fas mAb as a read out of the possible survival advantage observed in the BM cells from the various mutant mice before leukemia occurrence (see Materials and Methods). Under these experimental conditions, in WT BM cells cultures, 73.7 ± 11.6% of the cells underwent apoptosis upon anti-Fas treatment. Compared with the WT, the percentage of apoptotic cells upon Fas stimulation was significantly reduced in the PML$^{-/-}$ ($P < 0.001$) and in the PMLRARα$^{+/-}$ PML$^{+/+}$ ($P < 0.001$) mutants (Fig. 4 a). PML inactivation in PMLRARα TM resulted in a significant further decrease in the number of apoptotic cells ($P = 0.004$). As expected, the anti-Fas mAb also induced a decrease in the number of CFU-GM colonies from WT BM cells in in vitro colony assays. Once again, compared with WT cells, BM cells from PML$^{-/-}$ ($P = 0.02$) and PMLRARα$^{+/-}$ PML$^{+/+}$ ($P < 0.001$) mutants showed a marked protection from Fas-induced apoptosis. Moreover, the number of CFU-GM colonies was significantly higher in the PML-RARα PML$^{-/-}$ mice ($P = 0.006$).

In summary, the results reported here conclusively demonstrate in vivo and in relevant disease contexts that: (a) PML acts as a tissue specific tumor suppressor by rendering the cells sensitive to proapoptotic and differentiating stimuli. (b) PML haploinsufficiency and its functional impairment by PMLRARα are critical events in pathogenesis of APL-like leukemia in mice, thus indicating that the loss of one PML allele, as a consequence of the t(15;17), may be a critical event in human APL. (c) The acquisition of survival advantage is a critical event in the multistep process toward APL leukemogenesis. (d) Although PML can control senescence and cell proliferation and in vitro tumorigenesis by neu (7, 10), in vivo PML inactivation does not play a significant role in neu-induced breast tumorigenesis.

PML may regulate RARα/RXR transcription function by participating in this complex as a ligand-dependent transcriptional coactivator (27). By contrast, the mechanisms by which PML may regulate vitamin D response are yet unclear. Vitamin D receptor (VDR) bind DNA predominantly in its heterodimeric form with RXR (30). Binding of the ligand to the VDR leads to the recruitment of coactivators such as steroid receptor coactivator (SRC)/p160 and the vitamin D receptor interacting protein (DRIP; also known as thyroid hormone receptor–associated protein [TRAP], activator recuited cofactor [ARC], and negative regulator of activated transcription [NAT]) coactivator complex (31). PML can be part of the DRIP complex associated with RARα/RXR (27). Thus, it is tempting to speculate that PML may participate in VDR/RXR complex as well. As PMLRARα can heterodimerize with and sequester PML (5, 32), both a reduction in the dose of PML or an increase in the dose of PMLRARα could result in a striking increase in the dominant negative action of the oncoprotein on PMLRARα/RXR and PML VDR/RXR pathways (27).

In an accompanying study in this issue by Kogan et al. (33), leukemogenesis in a PMLRARα transgenic model is accelerated by coexpression of the BCL-2 protooncogene, which lends to the hemopoietic progenitors a survival advantage. PML has been implicated in p53-dependent and -independent pathways for apoptosis (6, 11). As a result, Pml$^{-/-}$ cells are resistant to multiple proapoptotic stimuli such as Fas, DNA damage, ceramide, TNF, and IFN. Thus, PMLRARα can act as antiapoptotic oncogene possibly through its ability to antagonize the function of PML. In agreement with this tenet, PML inactivation exacerbates the antiapoptotic activity of PMLRARα and accelerates leukemogenesis. Thus, protection from apoptosis may be a key event in APL pathogenesis.

We thank M. Jiao and L. Freedman for materials, help, and advice.

P.P. Pandolfi is a Scholar of the Leukemia & Lymphoma Society of America (formerly known as the Leukemia Society of America). E.M. Rego was a recipient of a Fundacão de Amparo a Pesquisa do Estado de São Paulo (FAPESP) fellowship. This work is supported by the National Cancer Institute (CA08748) and National Institutes of Health (CA71692 and CA74031 to P.P. Pandolfi).

Submitted: 23 August 2000
Revised: 4 December 2000
Accepted: 14 December 2000

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