Cooperation of Cytokine Signaling with Chimeric Transcription Factors in Leukemogenesis: PML-Retinoic Acid Receptor Alpha Blocks Growth Factor-Mediated Differentiation

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We utilized a mouse model of acute promyelocytic leukemia (APL) to investigate how aberrant activation of cytokine signaling pathways interacts with chimeric transcription factors to generate acute myeloid leukemia. Expression in mice of the APL-associated fusion, *PML-RAR*, initially has only modest effects on myelopoiesis. Whereas treatment of control animals with interleukin-3 (IL-3) resulted in expanded myelopoiesis without a block in differentiation, *PML-RAR* abrogated differentiation that normally characterizes the response to IL-3. Retroviral transduction of bone marrow with an IL-3-expressing retrovirus revealed that IL-3 and promyelocytic leukemia-retinoic acid receptor alpha (*PML-RAR* α) combined to generate a lethal leukemia-like syndrome in <21 days. We also observed that a constitutively activated mutant IL-3 receptor, β_c V449E, cooperated with *PML-RAR* α in leukemogenesis, whereas a different activated mutant, β_c I374N, did not. Analysis of additional mutations introduced into β_c V449E showed that, although tyrosine phosphorylation of β_c is necessary for cooperation, the Src homology 2 domain-containing transforming protein binding site is dispensable. Our results indicate that chimeric transcription factors can block the differentiative effects of growth factors. This combination can be potentially leukemogenic, but the particular manner in which these types of mutations interact determines the ability of such combinations to generate acute myeloid leukemia.

Numerous genetic abnormalities have been identified in human acute myeloid leukemia (AML) (3, 38). Alterations in transcription factors have been observed in particular morphological subtypes of AML. In addition, alterations in molecules that normally regulate cell behavior in response to external cues (hereafter referred to as “signaling molecules”) are common in AML, but these types of mutations are less closely associated with particular morphologies. In AML, chromosomal translocations commonly result in aberrant transcription factors, whereas small intragenic mutations can lead to abnormal transcription factors or mutant signaling molecules. Another type of alteration, chromosomal gain or loss, has been seen in some cases of AML, but the critical genes affected by these changes have not been definitively identified.

The number of genetic alterations required to cause human AML is not known and may vary with the type of leukemia. De novo leukemias with chromosomal translocations are believed to be genetically simpler than leukemias arising after myelodysplastic syndromes or cytotoxic chemotherapy, which are

often karyotypically complex. Of note, the proportion of AMLs with simple translocations declines with age whereas, conversely, AMLs with complex karyotypes increase (49). Differences in karyotype, epidemiology, and response to therapy indicate that AMLs are heterogeneous diseases. Therefore, although there may be common elements and molecular pathways that result in conversion of normal hematopoietic cells to AML, the types of genetic changes that in combination cause leukemia are likely to be heterogeneous.

We sought to identify a simple combination of genetic changes that is sufficient to cause leukemic transformation and to examine the manner in which these changes exert their cooperative effects. For this purpose, we utilized a transgenic mouse model of acute promyelocytic leukemia (APL) in which the *MRP8* promoter is used to express a *PML-RAR* fusion gene in myeloid cells.

The t(15;17)(q22;q12) results in the expression of a chimeric promyelocytic leukemia-retinoic acid receptor alpha (*PML-RAR* α) fusion protein that is responsible for nearly 99% of all human APLs (41, 50). There is evidence that, in fact, only a small number of genetic changes are required to cause APL. The t(15;17) is observed as the sole karyotypic change in 62% of cases (37), and the incidence of APL is constant over the human life span (56), suggesting one rate-limiting step. Although it is conceivable that the t(15;17) is fully sufficient to cause leukemia, there is evidence that additional mutations are required. In 38% of cases, the t(15;17) is accompanied by additional karyotypic changes (37), point mutations in genes

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encoding signaling molecules (such as FLT3) are common in APL (28, 33, 51, 58, 59), and expression of the PML-RAR α fusion protein in mice initially has only a modest impact on myelopoiesis (1, 15, 17).

With a median latency of 8.5 months, *MRP8 PML-RARA* transgenic mice developed AML with features of human APL (1). The transition from modest abnormalities of neutrophil maturation to acute leukemia was accompanied by the appearance of karyotypic abnormalities. Thus, this mouse model can serve to identify genetic changes that cooperate with PML-RAR α to cause acute leukemia.

We have previously reported that BCL-2 decreased the latency and increased the penetrance of leukemia in mice that express *PML-RARA*. However, the BCL-2-PML-RAR α combination was not sufficient for the leukemic phenotype: there was still a latency of >3 months, and recurring chromosomal abnormalities and complex karyotypes were a constant feature of the doubly transgenic leukemias (29, 37). In considering events able to complete leukemic transformation, we noted that preleukemic *PML-RARA/BCL2* doubly transgenic mice exhibited a marked impairment of neutrophil differentiation but that, prior to the onset of acute leukemia, the immature cells were not disseminated into nonhematopoietic tissues. These results suggested that AML reflects not only impaired differentiation but also relative autonomy from external cues. We therefore hypothesized that providing continuous growth factor stimulation to *PML-RARA*-expressing cells would be sufficient to cause a rapidly fatal leukemia. We also hypothesized that this model system could be used to elucidate the cellular and molecular mechanisms through which such cooperation between cytokine signaling and chimeric transcription factors occurs.

Our results show that PML-RAR α blocks the neutrophil differentiation that normally occurs when primary bone marrow cells are stimulated by growth factors and show that the resulting inexorable expansion of immature cells is rapidly fatal. Our results also indicate that the particular mix of signals generated by activated growth factor receptors determines the potential of such activation to contribute to acute leukemia. These findings suggest a model of normal myelopoiesis in which growth factor receptor activation generates a mix of signals that balance increased proliferation and survival with maintenance of maturation. Abrogation of the molecular events that normally ensure differentiation in the face of a growth stimulus is one pathway to AML.

MATERIALS AND METHODS

Mice. Mice were bred and maintained at the University of California at San Francisco, and their care was in accordance with University of California at San Francisco guidelines. *MRP8 PML-RARA* transgenic mice in the FVB/N background and control FVB/N mice were used for all experiments.

IL-3 injections. Transgenic and control mice were injected subcutaneously with interleukin-3 (IL-3) at a dose of 200 ng (10 μ g/kg) three times a day for 4 days. Mice were euthanized, and tissues were harvested 8 h after the last injection.

Differentiation assays. Bone marrow from FVB/N and *MRP8 PML-RARA* mice was harvested, depleted of red blood cells with Histopaque 1119 (Sigma) at 4°C according to the manufacturer's instructions, and cultured in Myelocult 5300 (Stem Cell Technologies) with 10% X63Ag8-mIL-3 conditioned medium (25), without or with all-trans-retinoic acid (ATRA; Sigma) at a concentration of 10^{-7} or 10^{-5} M.

Retroviral vectors. pMPZen-IL-3 is an IL-3-expressing retrovirus that does not express any additional gene (2). pRufNeo retroviral constructs with β_c wt, β_c V449E, and β_c I374N were described previously (22). We note that the amino acid numbering of β_c is per the National Center for Biotechnology Information reference sequence for CSF2RB (NP 000386). pRufNeo retroviral constructs β_c V449E/Y593F, β_c V449E/Y766F, and β_c V449E/Y6x6F (in which tyrosines 593, 628, 711, 766, 822, and 882 have all been mutated to phenylalanines) were generated by site-directed mutagenesis of the β_c V449E construct and were verified by sequencing.

Retroviral transductions. Donor mice were treated with 150 mg of 5-fluorouracil/kg, and marrow was harvested 5 days later. Marrow was prestimulated for 24 h in Dulbecco modified Eagle medium or Myelocult 5300 with 15% heat-inactivated fetal bovine serum, 5% X63Ag8-mIL-3 conditioned medium, 100 U of penicillin G/ml, 100 μ g of streptomycin/ml, 2 mM L-glutamine, 6 ng of mIL-3/ml, 10 ng of mIL-6/ml, and 100 ng of stem cell factor/ml. BOSC23 cells (44) were transfected with retroviral constructs. Marrow was transduced by spinoculation with fresh retroviral supernatants (filtered through 0.45- μ m [pore-size] filters) at $1,100 \times g$ in the presence of 2 μ g of Polybrene/ml for 1.5 h on two consecutive days. Unsorted cells after transduction were introduced into lethally irradiated (9 Gy, in two equal doses 3 to 6 h apart) or sublethally irradiated (4.5 Gy, as a single dose) mice.

Peripheral blood counts and bone marrow differential counts. Blood was obtained from the retro-orbital sinus. White blood cell (WBC) count, hemoglobin count, and platelet count were measured with the Hemavet 850 cell counter (CDC Technologies). Blood smears and marrow smears were stained with Wright's Giemsa stain. Peripheral blood differential WBC counts (total of 200 cells each) and bone marrow differential counts (total of 400 cells each) were determined as described previously (32). Some of the data included in Fig. 1 and 4 for control, *PML-RARA* preleukemic, and *PML-RARA* leukemic mice were previously published (29, 30).

Histopathology. Tissues were initially fixed in either Bouin's fixative or a buffered formalin solution. Sternum fixed in formalin were decalcified for 2 to 3 h prior to embedding (formic acid 11%, formaldehyde 8%). Paraffin embedded sections were stained with hematoxylin and eosin (H&E).

Immunophenotyping. After depletion of red blood cells, 300,000 bone marrow and spleen cells were resuspended in 100 to 200 μ l of fluorescence-activated cell-sorting buffer (buffered saline with 2% heat-inactivated fetal bovine serum and 2.5% cell dissociation buffer [Gibco-BRL]). Cells were incubated with unlabeled anti-CD16/CD32 antibodies (Fc block) for 15 min prior to the addition of conjugated antibodies. The antibodies used included fluorescein isothiocyanate-conjugated antibodies to Ly-6G(Gr-1), CD59, CD86, CD34, CD45R(B220), CD90.1(Thy1.1), CD41, and CD71; phycoerythrin (PE)-conjugated antibodies to CD11b(Mac-1), CD31, CD117(c-kit), Ly-71(F4/80), CD19, CD3, CD61, and Ly-76(Ter119); Tricolor-conjugated antibodies to CD45; and biotin-conjugated antibody to h β_c , followed by treatment with PE-conjugated streptavidin. Antibodies were incubated with the cells for 15 to 30 min in the dark on ice. Stained cells were washed and analyzed on a FACScan or FACSCalibur apparatus (Becton Dickinson), and at least 10,000 events were collected for each sample. Fluorescence-activated cell sorting data were analyzed with CellQuest (Becton Dickinson).

Transplantation of leukemias. Spleen cells of animals with leukemia were washed and resuspended in buffered saline. A total of 10^6 cells were injected into sublethally irradiated recipients by intravenous injection through the lateral tail vein.

Additional studies. Treatment with ATRA by implanting 5-mg 21-day release pellets was performed as described previously (1). A total of 10^6 cells from spleens of ill mice were transplanted into sublethally irradiated recipients by intravenous injection through the lateral tail vein. Cytogenetic and spectral karyotyping analysis of spleen cells was performed as described previously (37). For Southern blotting of PML-RAR α / β_c V449E leukemias, the enzymes utilized were *Kpn*I (to assess presence of provirus) or *Bam*HI (to assess the number of integration sites) and the probe was the 1.1-kb *neo* cassette derived from pRuf-Neo by digestion with *Bgl*II and *Cla*I.

Statistical analysis. Statistical analyses were performed with Excel 2000 by using the Student *t* test or, for comparisons of survival curves, with GraphPad Prism 3.0 by using the log-rank test.

RESULTS

PML-RAR α has a modest impact on myelopoiesis in vivo. Although PML-RAR α has been observed to inhibit the differentiation of cell lines and of primary cells in vitro (12–14, 48,

FIG. 1. PML-RAR α has a modest effect on bone marrow myelopoiesis. (A) Bone marrow differential cell counts are shown. Control or healthy preleukemic PML-RAR α bone marrows were stained with Wright's Giemsa. Percentages of nucleated cells were derived from 400 cell differential counts. Imm/Bl, immature forms/blasts; Inter, intermediate forms (including neutrophilic and monocytic cells); Neu, mature forms, neutrophilic; Lym, lymphocytes; Eos, eosinophilic cells; Mon, mature monocytes. Bars: controls ($\square$), $n = 16$; preleukemic PML-RAR α ($\blacksquare$), $n = 4$. *, preleukemic PML-RAR α marrow shows, compared to control bone marrow, a modest increase in the number of immature forms/blasts ($P = 0.01$). The graphs depict arithmetic means $\pm$ the standard deviation. (B) Flow cytometric immunophenotyping of controls or healthy preleukemic PML-RAR α transgenic bone marrows. Histograms reflect gate = R1. Compared to the control bone marrow, preleukemic PML-RAR α transgenic bone marrow shows a decrease in Ly-6G(Gr-1) and an increase in CD31 expression. (C) Cytology and histopathology of control or preleukemic PML-RAR α transgenic mice. Subpanels: a, b, c, and d, control FVB/N; e, f, g, and h, preleukemic PML-RAR α ; a and e, bone marrow smears stained with Wright's Giemsa (magnification, $\times 400$); b to d and f to h, H&E-stained histopathology sections of bone marrow (magnification, $\times 400$) (b and f), spleen (magnification, $\times 100$; insets, magnification, $\times 400$) (c and g), and liver (magnification, $\times 100$) (d and h).

54), we had observed that the expression of an *MRP8 PML-RAR α* transgene in mice had only a modest effect on hematopoiesis. Careful analysis of myelopoiesis in these mice revealed subtle abnormalities (1, 29) (Fig. 1). Although peripheral blood counts are indistinguishable from control animals, in the bone marrow there is an increase in immature myeloid cells (Fig. 1A). Immunophenotypic analysis revealed that, in addition to the decrease in Ly-6G(Gr-1) expression we previously

observed, the bone marrow of *MRP8 PML-RAR α* transgenic mice showed a modest increase in CD31 compared to controls (Fig. 1B). This immunophenotypic profile reflects an increase in immature cells because Ly-6G(Gr-1) expression increases when neutrophilic cells mature and CD31 expression decreases with myeloid maturation. Cytologic and histopathologic exam-

FIG. 2. PML-RAR α blocks differentiation that accompanies IL-3 receptor activation. (A) Bone marrow differential cell counts are shown. Control or healthy preleukemic *PML-RAR α* transgenic mice were injected with IL-3 for 4 days. Bars: littermate controls ($\square$), $n = 4$; *PML-RAR α* ($\blacksquare$), $n = 4$. *, IL-3-injected *PML-RAR α* transgenic animals had, compared to IL-3-injected controls, significantly more immature forms/blasts ($P = 0.003$) and significantly fewer lymphocytes ($P = 0.02$). Trends toward fewer mature neutrophilic cells and erythroid cells were also evident. (B) Differential cell counts of in vitro assays. Control or preleukemic *PML-RAR α* transgenic bone marrows were harvested, depleted of erythroid cells, and cultured in IL-3 with or without ATRA for 24 h. Bars: littermate controls ($\square$), $n = 3$; *PML-RAR α* ($\blacksquare$), $n = 4$. Abbreviations are as described in Fig. 1A with the following additions. In these in vitro assays, intermediate-stage cells that were clearly neutrophilic (Inter/Neu) were enumerated separately from those that appeared monocytic (Inter/Mo); mature mono-

cytes and macrophages were enumerated together (Mo/Mac). ination revealed only subtle changes in the bone marrow; the splenic and hepatic histopathology were not evidently different from those of control animals (Fig. 1C).

PML-RAR α blocks the differentiation that normally accompanies cytokine receptor activation. We initially selected IL-3 to test the hypothesis that growth factor stimulation would cooperate with PML-RAR α to cause leukemia. There were a number of reasons for this choice. First, IL-3 expression had been observed in 3 of 16 APLs studied (9). Second, the finding that IL-3 cooperated with *Hoxb8* to rapidly induce leukemia in mice provided a precedent for our work (46). Third, because the granulocyte-macrophage colony-stimulating factor (GM-CSF)/IL-3/IL-5 receptors have been extensively studied, this system is amenable to delineating critical downstream signals (5, 7, 16).

We injected control and *PML-RAR α* transgenic mice with IL-3 for 4 days and assessed the effects of the cytokine. As has been observed previously, short-term treatment with IL-3 had little effect on the peripheral blood (42). In the bone marrow, however, the increase in myelopoiesis and the shift toward less-mature cells normally caused by IL-3 were enhanced by the presence of PML-RAR α (Fig. 2A). In *PML-RAR α* transgenic mice, IL-3 caused more than 30% of the bone marrow cells to accumulate at the immature form/blast stage of differentiation. In addition, there was a trend toward decreased red blood cell precursors. An increased number of blasts and a loss of normal hematopoiesis are features of AML. These findings indicated that, although PML-RAR α had minimal effects on differentiation under steady-state conditions in vivo, this chimeric fusion protein markedly interfered with differentiation when a cytokine stimulus was present.

The ability of PML-RAR α to block IL-3 induced differentiation was corroborated by in vitro culture of control and transgenic bone marrow cells (Fig. 2B). Short-term culture of *PML-RAR α* transgenic bone marrow in the presence of IL-3 was characterized by a marked deficit in mature neutrophils, an abnormality that was substantially abrogated by the addition of ATRA.

The combination of PML-RAR α and IL-3 rapidly causes a leukemia-like syndrome. In order to further examine the effects of combining PML-RAR α with cytokine activation, we utilized retroviral transduction of mouse bone marrow. We harvested bone marrow from 5-fluorouracil-treated control and *PML-RAR α* transgenic mice. The bone marrow cells were transduced with an IL-3-expressing retrovirus, and 300,000 to

cytes and macrophages were enumerated together (Mo/Mac). (I) Control or *PML-RAR α* transgenic bone marrow in IL-3 after 24 h; (II) control or *PML-RAR α* transgenic bone marrow in IL-3 plus ATRA at 10^{-7} M; (III) control or *PML-RAR α* transgenic bone marrow in IL-3 plus ATRA at 10^{-5} M. *, for *PML-RAR α* cells, immature forms/blasts were markedly increased in IL-3 in culture after 24 h (compared to FVB/N, $P < 0.0001$), and mature neutrophils were markedly decreased ($P = 0.03$). In the presence of ATRA, *PML-RAR α* marrow yielded significantly fewer immature forms/blasts and significantly more intermediate and mature neutrophilic cells (compared to *PML-RAR α* without ATRA, $P \leq 0.01$ at 10^{-7} M and 10^{-5} M ATRA). Of note, at 10^{-5} M ATRA, the numbers of immature forms/blasts and mature neutrophilic cells were not significantly different between the FVB/N and *PML-RAR α* cultures.

FIG. 3. IL-3 transduction of *PML-RARA* transgenic bone marrow yields a rapidly fatal disease. Survival curves are shown. Control or *MRP8 PML-RARA* transgenic bone marrow was transduced with a retrovirus that expressed IL-3. Lethally irradiated recipients received 3×10^5 to 5×10^5 cells. Sublethally irradiated mice received 5×10^4 cells. The presence of *PML-RARA* was associated with more rapidly progressing disease than that observed in the controls. Significance values (*PML-RARA*/IL-3 versus control/IL-3): $P = 0.003$ for lethally irradiated recipients and $P < 0.0001$ for sublethally irradiated recipients. Lethally irradiated recipients: *PML-RARA*/IL-3, $n = 4$; control/IL-3, $n = 6$. Sublethally irradiated recipients: *PML-RARA*/IL-3, $n = 19$; control/IL-3, $n = 15$.

500,000 cells were injected into lethally irradiated nontransgenic histocompatible recipients. Recipients of *PML-RARA* bone marrow transduced with IL-3 became ill with a lethal myeloid disease within 14 to 16 days, whereas mice receiving IL-3 transduced normal marrow became ill within 39 days to 11 weeks (Fig. 3).

If cytokine stimulation is sufficient to induce disease, then injection of even relatively small numbers of transduced cells into sublethally irradiated mice should also be rapidly fatal. We therefore injected 50,000 cells after transduction into sublethally irradiated recipients. Again, the combination of *PML-RARα* and IL-3 caused lethal myeloid disease rapidly, whereas IL-3 alone caused illness more slowly and not all mice became sick (Fig. 3).

We compared the character of the disease that arose in recipients of control bone marrow transduced with IL-3 (control/IL-3-transduced mice) with that seen in recipients of *PML-RARA* transgenic bone marrow transduced with IL-3. The peripheral blood of control/IL-3-transduced mice showed a marked elevation in WBC counts, normal platelet numbers, and moderate anemia in some animals (Fig. 4A). Numerous mature neutrophilic forms were present, accompanied by a substantial number of intermediate forms, but few immature forms/blasts (Fig. 4B). Monocytosis and eosinophilia were also evident. The bone marrow of these mice showed a marked increase in myeloid cells in which a normal pattern of differentiation was maintained (mature > intermediate > immature) (Fig. 4C). These results are similar to those previously described (2, 57). Although the peripheral blood of *PML-RARα*/IL-3 mice also showed increased WBC counts, moderate anemia and marked thrombocytopenia were present (Fig. 4A). The leukocytosis was different from that seen in the control/IL-3 animals: differentiation was impaired with a shift toward less-mature forms (Fig. 4B). In the bone marrow, the

combination of *PML-RARα* and IL-3 was characterized by expansion of myeloid cells with numerous immature forms/blasts but few mature neutrophils (Fig. 4C). Flow immunophenotyping (Fig. 4D) corroborated our morphological analysis: compared to control/IL-3-transduced bone marrow, the myeloid cells of *PML-RARα*/IL-3-transduced bone marrow expressed increased markers of immaturity [CD34, CD90.1(Thy1), CD31] and metabolic activity (CD71, the transferrin receptor), as well as somewhat higher levels of CD11b(Mac-1) and Ly-71(F4/80), a marker expressed on mature monocytes and eosinophils that can also be expressed at low levels on immature myeloid cells. Interestingly, the level of Ly-6G(Gr-1) expression, which is lower than that of controls for preleukemic *PML-RARA* transgenic mice, was induced to normal levels by IL-3.

The ability of *PML-RARα* to block differentiation that normally accompanies IL-3 stimulus was also evident in smears and sections of bone marrow and in the spleen and liver (Fig. 4E). Of note, the livers of both control/IL-3 and *PML-RARα*/IL-3 mice were heavily infiltrated with myeloid cells. Whereas spleens of control/IL-3 mice showed red pulp expansion with a mix of myeloid cells, erythroid cells, and megakaryocytes, the red pulp of *PML-RARα*/IL-3 mice was expanded predominantly with immature myeloid cells. The blood counts, marrow morphology, histopathology, and rapid clinical course of disease observed in *PML-RARα*/IL-3 recipients are characteristic of AML in mice.

To assess the response of the *PML-RARα*/IL-3 disease to ATRA, sublethally irradiated recipients of *PML-RARα*/IL-3 bone marrow were treated with ATRA 10 days after injection. The median survival of ATRA-treated mice was 45% longer than the survival of untreated or placebo-treated mice (untreated/placebo treated, $n = 20$, 16 to 19 days; ATRA treated, $n = 4$, 25 to 26 days; $P = 0.001$).

Karyotypic analysis of six *PML-RARα*/IL-3 diseased recipients was performed (Table 1). Five cases lacked clonal abnormalities, whereas in one case, 2 of 11 metaphase cells exhibited a Robertsonian translocation (sample 740), which would not be expected to result in altered gene expression. These results contrast with the high frequency of clonal cytogenetic changes and the distinct pattern of recurring numerical abnormalities that we observed in *PML-RARα* (91%) and *PML-RARα*/BCL-2 (100%) leukemias (29, 37). These cytogenetic findings indicate that the combination of *PML-RARα* and IL-3 may be fully sufficient to generate the leukemia-like syndrome observed.

An activated IL-3 receptor cooperates with *PML-RARα* to induce a transplantable AML with features of APL. To further

TABLE 1. Karyotypic analysis of *PML-RARα*/IL-3 mice

Sample no.	Karyotype ^a
730.....	40,XX[13]/NCA:40,XX,t(2;4)(F3;D1)[1]
739.....	40,XX[9]/NCA:39,X,ins(13;X)(B;A2D),-18[1]
740.....	39,XX,der(8;17)(A1;A1)[2]/40,XX[9]*
741.....	40,XX[8]/NCA:41,XX,+7[1]/40,XX-9,+15[1]
742.....	40,XX[10]
743.....	40,XX[10]

^a NCA, nonclonal abnormality; *, Robertsonian translocation (centric fusion) noted in two cells.

investigate the ability of cytokine stimulation to cooperate with PML-RAR α in leukemogenesis, as well as to establish a system in which to analyze the mechanisms underlying this cooperation, we utilized activated versions of the IL-3 receptor. The IL-3 receptor is part of a family of cytokine receptors that includes GM-CSF and IL-5. In humans, these three receptors have distinct alpha chains but share a beta chain (β_c). In mice there is an alternative β chain (β_{IL-3}) that combines with the IL-3 receptor alpha chain to generate a second class of IL-3 receptor. We have utilized activated versions of human β_c that have been previously demonstrated to confer cytokine independence to mouse cells and have been used to enhance our understanding of GM-CSF/IL-3/IL-5 signal transduction (Fig. 5) (21, 22, 39). Although activating mutations in β_c have not

FIG. 4. PML-RAR α and IL-3 disease has features of AML. (A to C) Blood and bone marrow analyses of diseased recipients of IL-3-transduced control and PML-RAR α transgenic bone marrow are shown. The results are compared to normal FVB/N mice and leukemias that arose spontaneously in MRP8 PML-RAR α transgenic animals. (A) Peripheral blood: WBC, 10,000/ μ l; hemoglobin (HGB), grams/deciliter, platelet count (PLT), 100,000/ μ l. *, control/IL-3 mice exhibited leukocytosis ($P = 0.01$), displayed a trend toward anemia, and were not thrombocytopenic. PML-RAR α /IL-3 mice exhibited leukocytosis, anemia, and thrombocytopenia ($P \leq 0.004$). Bars: control FVB/N ($\square$), $n = 14$; leukemias from PML-RAR α mice (hatched), $n = 10$; control/IL-3 ($\blacksquare$), $n = 6$; PML-RAR α /IL-3 ($\blacksquare$), $n = 10$. (B) Peripheral blood: percentage of nucleated WBCs. *, compared to control/IL-3 mice, PML-RAR α /IL-3 mice had increased immature forms/blasts ($P = 0.01$), increased intermediate forms ($P = 0.01$), and decreased mature neutrophils ($P = 0.0001$). Bars: control FVB/N ($\square$), $n = 8$; leukemias from PML-RAR α mice (hatched), $n = 6$; control/IL-3 ($\blacksquare$), $n = 5$; PML-RAR α /IL-3 ($\blacksquare$), $n = 5$. (C) Bone marrow: percentage of nucleated cells. *, compared to control/IL-3 mice, PML-RAR α /IL-3 mice had increased immature forms/blasts ($P = 0.01$) and decreased mature neutrophils ($P = 0.0003$). Not shown are mast cells that comprised 4% of cells in control/IL-3 marrow, 0.7% of cells in PML-RAR α /IL-3 marrow, and <0.25% of cells in control and PML-RAR α leukemic marrows. Bars: control FVB/N ($\square$), $n = 16$; leukemias from PML-RAR α mice (hatched), $n = 6$; control/IL-3 ($\blacksquare$), $n = 5$; PML-RAR α /IL-3 ($\blacksquare$), $n = 5$. (D) Flow cytometric immunophenotyping of control/IL-3 or PML-RAR α /IL-3 bone marrow. Subpanels: a to d, control/IL-3; e, f, g, and h, PML-RAR α /IL-3; a and e, bone marrow smears stained with Wright's Giemsa (magnification, $\times 400$); b to d and f to h, histopathology sections stained with H&E; b and f, bone marrow (magnification, $\times 400$); c and g, spleen (magnification, $\times 400$); d and h, liver (magnification, $\times 100$).

FIG. 4—Continued.

been reported in human AML (11), we used this system to investigate mechanisms of cooperative leukemogenesis. We transduced control and *PML-RARA* transgenic bone marrow with wild-type (β_c wt) and activated (β_c V449E; β_c V433E if the signal sequence is excluded) receptors and reconstituted lethally irradiated recipients. Mice were monitored for the development of disease, and ill animals were analyzed. β_c V449E cooperated with PML-RAR α to induce leukemias (Fig. 6A). These leukemias were characterized by leukocytosis, anemia, and thrombocytopenia. Immature forms/blasts were evident in the peripheral blood, and bone marrows were marked by the accumulation of numerous immature myeloid cells (Fig. 6B). Flow immunophenotyping corroborated the observed morphology since the leukemias were composed of immature myeloid cells that expressed both Ly-6G(Gr-1) and CD11b(Mac-1), as well as CD117(c-kit) and CD34. In addition, there was strong expression of CD61, CD59, and CD31. The cells lacked the lymphocyte markers CD3, CD19, and CD45R, as well as the erythroid marker Ly-76(Ter119) (Fig. 6C). Cytology of bone marrow and histopathology of tissues showed the illness to be a disseminated disease of immature myeloid cells (Fig. 6D). In contrast to our observation that we could not consistently transmit PML-RAR α /IL-3 disease to secondary recipients, the PML-RAR α / β_c V449E leukemias were readily transplantable (Fig. 6E).

PML-RAR α / β_c V449E leukemias were responsive to retinoic acid. Sublethally irradiated recipients of 10^6 cells from a PML-RAR α / β_c V449E animal were treated with placebo or 5-mg ATRA pellets on day 14 after transplantation of the leukemia. The median survival of ATRA-treated mice was 33% longer than the median survival of placebo-treated mice (placebo treated, $n = 6$, 19 to 22 days; ATRA treated, $n = 6$, 27 to 32 days; $P = 0.001$). Interestingly, this effect on survival was similar to the 45% prolongation observed in PML-RAR α /IL-3 mice treated with ATRA (see above) but appeared to be less than the twofold median prolongation of survival caused by ATRA when PML-RAR α leukemias without activation of the IL-3 receptor were treated (29, 36). We also examined the karyotypes of the PML-RAR α / β_c V449E leukemias. As shown in Table 2, seven of nine (78%) leukemias had clonal abnormalities, which included the gain of chromosome 15 in two cases and gain of chromosome 8 in seven cases. Four cases showed loss of an X chromosome.

	β_c wt	β_c V449E	β_c I374N	β_c V449E-Y593F	β_c V449E-Y766F	β_c V449E-Y6xF
1						
2						
3						
374	I	N				
4						
449	V	E		E	E	E
Box 1						
593	Y			F		F
628	Y					F
711	Y					F
766	Y				F	F
822	Y					F
882	Y					F

FIG. 5. Diagram of h β_c retroviral constructs that gives a schematic representation of activating β_c constructs. The extracellular domains are numbered 1 to 4 from the amino terminus. Six intracellular tyrosines residues are indicated. Amino acid I374 is located in cytokine receptor domain 4 (CRD4) of the extracellular region; activated receptor β_c I374N has an isoleucine at amino acid 374 mutated to asparagine. Amino acid V449 is located in the transmembrane region; activated receptor β_c V449E has a valine at amino acid 449 mutated to glutamic acid. β_c V449E/Y593F, in addition to V449E, also has a tyrosine mutated to a phenylalanine at amino acid 593. Similarly, β_c V449E/Y766F has a V449E mutation and a tyrosine at position 766 mutated to a phenylalanine. β_c V449E/Y6xF has six cytoplasmic tyrosine residues mutated to phenylalanine in addition to the V449E transmembrane mutation.

FIG. 6. PML-RAR α cooperates with activated h β_c receptor to induce AML. (A) Survival curves. Control or MRP8 PML-RAR α transgenic bone marrow was transduced with pRufNeo-h β_c V449E or pRufNeo-h β_c (wild-type) retroviruses. (I) Lethally irradiated recipients received 5×10^5 cells. Curves: control/ β_c V449E, $n = 6$; control/ β_c (wild type), $n = 6$; PML-RAR α / β_c V449E, $n = 8$; PML-RAR α / β_c (wild type), $n = 4$. (II) Sublethally irradiated mice received 5×10^4 cells. Curves: control/ β_c V449E, $n = 9$; control/ β_c (wild type), $n = 5$; PML-RAR α / β_c V449E, $n = 9$; PML-RAR α / β_c (wild type), $n = 5$. Lethally irradiated PML-RAR α / β_c V449E recipients were sick within 60 days with a median of 49 days and sublethally irradiated recipients PML-RAR α / β_c V449E mice were sick within 80 days with a median of 60 days. Compared to PML-RAR α / β_c (wild type) mice (some of which developed leukemia after 160 days), the decreased latency of leukemia in PML-RAR α / β_c V449E mice was statistically significant ($P = 0.02$ for lethally irradiated recipients and $P < 0.01$ for sublethally irradiated recipients). (B) Blood and bone marrow analyses of diseased recipients of β_c V449E transduced PML-RAR α transgenic bone marrow. Bars (peripheral blood counts [top graph]): PML-RAR α / β_c V449E, $n = 12$; WBC, 10,000/ μ l; hemoglobin (HGB), grams/deciliter; platelet count (PLT), 100,000/ μ l. PML-RAR α / β_c V449E leukemic mice showed high blood cell counts and are anemic and thrombocytopenic. Peripheral blood differential cell counts ($n = 5$) are shown in the middle panel as a percentage of nucleated WBCs. PML-RAR α / β_c V449E mice showed increased immature forms/blasts, increased intermediate forms, and decreased mature neutrophilic forms compared to control mice (Fig. 4B). Bone marrow differential cell counts ($n = 5$) are shown in the bottom panel as a percentage of nucleated cells. Counts are markedly different from those of control mice. (C) Flow cytometric immunophenotyping of PML-RAR α / β_c V449E bone marrow. Bone marrow samples of mice transduced with PMLRAR α / β_c V449E were stained with FITC-, PE- and Tricolor-conjugated antibodies. Histograms reflect gate = R1 and R2. *, antibody recognizes CD90.2. Because FVB/N mice express CD90.1, the few cells stained represent cross-reactivity or nonspecific staining. (D) Cytology and histopathology of leukemic recipients of β_c V449E transduced PML-RAR α transgenic bone marrow. Subpanels: a, bone marrow smear stained with Wright's Giemsa (magnification, $\times 400$); b to d, histopathology sections stained with H&E; b, bone marrow (magnification, $\times 400$); c, liver (magnification, $\times 100$); d, spleen (magnification, $\times 100$) and higher-magnification insets of the spleen (magnification, $\times 400$). (E) Leukemias were readily transplantable. A survival curve of sublethally irradiated recipients of PML-RAR α / β_c V449E leukemias ($n = 9$) is shown.

In light of these clonal karyotypic abnormalities, we examined whether splenic DNA of PML-RAR α / β_c V449E leukemias also demonstrated a monoclonal (or oligoclonal) pattern of retroviral insertion sites. Interestingly, Southern blot analyses of four leukemias, including two leukemias for which we

had cytogenetic data (samples 108 and 363), demonstrated that proviral DNA was present in the leukemias (Fig. 7A) and that there was more than one site of integration in each sample, with various band intensities indicative of multiple clones (Fig. 7B). A number of explanations for the contrasting clonality

C

E

FIG. 6—Continued.

results obtained by karyotypic and Southern blot analyses are considered below (see Discussion).

Tyrosine phosphorylation of activated beta common is essential for cooperation with PML-RAR α . Mutations in the juxtamembrane region (extracellular) and transmembrane region of β_c can activate the receptor and confer cytokine independence onto factor-dependent cell lines and primary hematopoietic cells (22, 39). Whereas β_c V449E exemplifies the transmembrane mutations, β_c I374N (β_c I358N, signal sequence excluded) exemplifies the juxtamembrane class of mutations.

D

Because these classes of mutations differ in their biological and biochemical effects (21, 40), we compared the combination of β_c I374N and PML-RAR α with what we had observed with PML-RAR α plus β_c V449E. In contrast to the latter, β_c I374N did not cooperate with PML-RAR α to induce leukemia (Fig. 8).

Previous studies had demonstrated that β_c V449E exhibited ligand-independent phosphorylation of the intracellular tyrosine residues of β_c , whereas β_c I374N did not exhibit detectable ligand-independent tyrosine phosphorylation (21). We therefore hypothesized that signals derived from the phosphorylated tyrosines of β_c were responsible for the ability of this activated cytokine receptor to cooperate with PML-RAR α in leukemogenesis. We tested this hypothesis by utilizing β_c V449E/Y6xF in which tyrosines 593, 628, 711, 766, 822, and 882 have all been mutated to phenylalanines. Although β_c V449E/Y6xF retains two tyrosines located near the transmembrane-intracellular junction, β_c V449E/Y6xF did not cooperate to induce acute leukemia (Fig. 8).

Particular tyrosines of β_c have been implicated in specific biochemical functions. Y593F (Y577F, signal sequence excluded) is the binding site for Src homology 2 domain-containing transforming protein (SHC) and may also contribute to phosphorylation of protein tyrosine phosphatase, non-receptor type 11 (SHP-2) (10, 20, 43). Y766F (Y750F, signal sequence excluded) can contribute to STAT activation (although it is not necessary for such activation) and may enhance tyrosine phosphorylation on β_c (10, 19, 55). Interestingly, both β_c V449E/Y593F and β_c V449E/Y766F were able to cooperate with PML-RAR α to induce leukemia (Fig. 8), indicating that these individual tyrosines were not essential for cooperative leukemogenesis. These leukemias were phenotypically similar to those arising in PML-RAR α / β_c V449E recipients (data not shown). There was a trend toward prolonged survival in the presence of these point mutations ($P < 0.05$). Nevertheless, we

TABLE 2. Karyotypic analysis of PML-RARα/β_c V449E leukemias

Sample no.	Karyotype ^a
108.....	40,XY[14]/NCA:39,-X,-Y,+14[1]
109.....	42,XY,+8,+15[10]
112.....	41,XY,+8[2]/40,XY[8]
362.....	40,X,-X,+8[2]/NCA:41,XX,+15[1]
363.....	40,X,-X,+15[7]/40,X,-X,+8[2]/40,XX[3]
365.....	40,XX[14]/NCA:41,XX,+8[1]
366.....	40,X,-X,+8[2]/40,XX[10]
396.....	39,X,-X[3]/40,idem,+8[6]/NCA:40,idem,+15[1]
398.....	41,XX,+8[8]/NCA:41,idem,-9,+16[1]/40,XX[1]

^a NCA, nonclonal abnormality.

cannot definitively conclude that these point mutations influenced latency because (i) there are small numbers of mice per group, (ii) at least one animal in each group became ill in <50 days, and (iii) it is possible that differences in viral titers influenced survival.

DISCUSSION

We have utilized *MRP8 PML-RARA* mice to investigate how genetic changes cooperate in myeloid leukemogenesis. We observed that, whereas PML-RARα had only modest effects on myelopoiesis and cytokine stimulation alone resulted in

FIG. 7. PML-RARα/β_c V449E leukemias have multiple proviral insertion sites. Genomic DNAs derived from spleen samples of PML-RARα/β_c V449E leukemic mice were used to assess retroviral integration. Filters were probed with a 1.1-kb Neo probe derived from pRufNeo. The sizes of molecular weight markers are shown in kilobases. (A and B) Genomic DNAs were digested with *Kpn*I (which cuts in each viral long terminal repeat) to assess the presence of provirus (A) or digested with *Bam*HI to assess the number of integration sites (B). *Bam*HI cleaves once within the retroviral sequence; therefore, each band represents a site of retroviral integration.

FIG. 8. Tyrosine phosphorylation of hβ_c is essential for cooperation with PML-RARα. Compared to PML-RARα/β_c V449E leukemic recipients, mice that received *PML-RARA* bone marrow transduced with β_c I374N (*n* = 17) and β_c V449E-Y6xN (*n* = 8) did not become ill in the first 200 days. Both β_c V449E/Y593F (*n* = 7) and β_c V449E/Y766F (*n* = 3) mice developed leukemia. The phenotype of these leukemias was similar to that observed in PML-RARα/β_c V449E mice (data not shown).

expanded myelopoiesis with retained differentiation, PML-RARα blocked the differentiation that normally accompanies growth factor stimulus. IL-3 combined with PML-RARα to rapidly generate a lethal disease with pathological features of AML. An activated β_c chain similarly cooperated with PML-RARα to generate a transplantable AML. Analysis of various activated β_c chains showed that (i) the ability to confer cytokine independence, per se, is not sufficient for cooperative leukemogenesis; (ii) signals induced by phosphotyrosines are critical for transformation in this system; and (iii) a tyrosine residue critical for SHC activation is not required for cooperation with PML-RARα.

The combination of PML-RARα and IL-3 resulted in a disease with features of murine AML: accumulation of numerous immature forms/blasts, anemia and thrombocytopenia, dissemination of immature cells outside of the bone marrow and spleen, and rapid lethality. Injections of small numbers of cells immediately after transduction into sublethally irradiated mice was fatal in <21 days. In addition, in contrast to previous studies of leukemias in *MRP8 PML-RARA* transgenic mice, clonal cytogenetic changes were observed in only one of six leukemias studied, and in the one case, only a minority of cells showed a nonpathogenic chromosomal abnormality. These results strongly suggest that PML-RARα plus IL-3 are sufficient to cause a leukemia-like syndrome.

We wanted to demonstrate that cytokine receptor activation would cooperate with PML-RARα in a cell autonomous manner in order to develop a system in which we could delineate the mechanisms contributing to leukemic transformation. For this reason, we made use of an activated β_c chain of the GM-CSF/IL-3/IL-5 receptor family. Like IL-3, β_c V449E cooperated with PML-RARα and in fact gave rise to a transplantable AML with features of APL.

Differences between our results with IL-3 and β_c V449E retroviruses are likely due in part to cell nonautonomous effects of the IL-3 retrovirus. Mice reconstituted with bone marrow transduced with the IL-3 retrovirus have markedly elevated IL-3 levels in serum (46). IL-3 is therefore able to influence not

only the transduced cells but also nontransduced cells in the transplanted marrow and in host cells as well. The shorter latency of disease in PML-RAR α /IL-3 mice compared to PML-RAR α / β_c V449E recipients is therefore probably due, at least partially, to the impact of IL-3 on nontransduced cells. Supporting this possibility is the fact that, whereas injection of 50,000 PML-RAR α cells transduced with IL-3 was fatal to sublethally irradiated recipients in <20 days (Fig. 3), injection of 1,000,000 cells from PML-RAR α / β_c V449E leukemic mice took >20 days to cause lethality (Fig. 6E, $P < 0.001$) and the latency of leukemia after injection of 50,000 cells from PML-RAR α / β_c V449E leukemic mice into sublethally irradiated recipients was 67 days (data not shown). Cell nonautonomous effects of IL-3 might also contribute to the lack of transplantability of PML-RAR α /IL-3 disease to secondary recipients. In addition, the fact that five of six karyotypes of PML-RAR α /IL-3 disease were normal, whereas seven of nine PML-RAR α / β_c V449E leukemias were abnormal could similarly reflect the ability of secreted IL-3 to expand both transduced and untransduced PML-RAR α -expressing cells.

Our findings that the PML-RAR α / β_c V449E leukemias exhibited clonal karyotypic abnormalities, whereas the patterns of proviral insertion suggested oligo- or polyclonality, raise interesting questions regarding the sufficiency of the combination of PML-RAR α and β_c V449E to fully transform normal blood cells into acute leukemia. One possibility is that PML-RAR α plus β_c V449E is sufficient for transformation, that this is reflected in the multiple insertion sites present in the spleens of leukemic mice, and that the clonal karyotypic abnormalities reflect the rapid acquisition of cytogenetic changes, with clonal selection, taking place within already-transformed cells. Alternatively, PML-RAR α plus β_c V449E may induce a preleukemic state characterized by expanded myelopoiesis that is not initially acute leukemia. The proviral insertion patterns could reflect persistence of multiple preleukemic clones in the spleens of leukemic mice. If this is the case, then additional genetic changes, reflected as clonal karyotypic abnormalities, are necessary and, in this system, inevitable. Inducible systems represent a powerful approach for assessing sufficiency in malignant transformation (45). Studies of mice carrying a PML-RAR α transgene in combination with an inducible activated cytokine receptor could clarify the number of steps required for leukemogenesis.

An important finding from this work was that growth factor independence does not necessarily permit cooperation with PML-RAR α in leukemogenesis, that is, differences among events able to confer factor independence can influence the potency of cooperation. We observed that although β_c I374N, like β_c V449E, is able to confer growth factor independence and activate mitogen-activated protein kinase, JAK2, and STAT5 (21), β_c I374N did not readily cooperate with PML-RAR α to generate AML. What then underlies this difference between these two versions of an activated receptor? At the cellular level, β_c I374N and β_c V449E have different effects on the murine factor-dependent FDB cell lines (40). Both confer factor independence, but FDB cells that grow and survive due to the presence of β_c I374N undergo differentiation, whereas FDB cells dependent on β_c V449E remain undifferentiated. Therefore, when these activated receptors are expressed in vivo, the signals generated by β_c I374N might foster greater

maturation than those caused by β_c V449E. Of note, activated FLT3, which like β_c V449E has been reported to favor continued proliferation while impairing maturation (60), does cooperate with PML-RAR α to cause leukemia (27, 53). At the molecular level, known qualitative differences between the β_c I374N and β_c V449E mutants that might underlie their different effects include the following: (i) β_c I374N results in ligand-independent association with the α chain of mouse GM-CSF receptor; (ii) β_c V449E but not β_c I374N results in phosphorylation of the tyrosines in the intracytoplasmic region of β_c ; and (iii) such phosphorylation results in SHC activation in cells that express β_c V449E but not β_c I374N (21, 23; T. Blake and T. Gonda, unpublished observations). A lack of SHC activation in PML-RAR α cells transduced with β_c I374N was a possible explanation for the lack of cooperative leukemogenesis we observed with β_c I374N, but the β_c V449E/Y593F construct, which lacks the SHC binding site, still cooperated with PML-RAR α to cause leukemia. The fact that mutations of six tyrosines of β_c in the β_c V449E/Y6xY construct blocked cooperation indicates that signals generated by phosphorylation of these tyrosines are critical, and the fact that β_c V449E/Y766F did cooperate highlights the redundancy of the signals generated by interactions with these tyrosines (10, 19, 43, 55). The use of additional β_c mutations, as well as complementary approaches to assessing the importance of downstream effectors, should permit our model system to be further utilized in identifying critical mediators through which activated cytokine receptors contribute to AML.

Given the subtle impact of PML-RAR α on myelopoiesis in vivo, what is the role of this protein in APL? The transcriptional effects of PML-RAR α , including the ability of PML-RAR α to repress transcription through recruitment of histone deacetylases (41) and DNA methyltransferases (8), likely underlies the ability of this protein to cause a subtle increase in immature myeloid cells. In addition, by inhibiting PML's ability to induce apoptosis, PML-RAR α may foster the acquisition of additional pathogenic mutations in this expanded immature cellular compartment (47). Finally, as we have demonstrated, PML-RAR α contributes to a profound block in differentiation in the presence of additional genetic lesions. PML-RAR α exemplifies a principle that seems to apply to transcription factor mutations that contribute to human AML: it has multiple effects that each individually contribute to transformation.

One type of cooperating event, activation of cytokine receptors on its own fosters proliferation and survival and maintained differentiation. An appropriate balance in the cellular response to growth factors permits expansion of the immature or mitotic compartment while ensuring the generation of large numbers of mature effector cells. Recent data suggest that growth factor-induced activation of RARs may in fact be the basis for the differentiation response. For example, both GM-CSF and IL-3 enhance the activity of RARs (24, 52). Although under steady-state conditions loss of RAR activity has, like the expression of PML-RAR α , mild effects on myelopoiesis (26, 35), it is plausible that PML-RAR α blocks the increased RAR activity induced by growth factor stimulation. This block would thus abrogate a differentiation signal required to maintain neutrophil production in the presence of increased cytokine receptor activation.

It is notable that specific expression of a number of translo-

cation proteins in myeloid cells, including PML-RAR α , AML1-ETO, and CBF β -SMMHC, can have modest in vivo effects (1, 15, 17, 18, 31). It therefore appears that the genetic changes of AML central to impaired differentiation are able to block maturation only when accompanied by changes that promote autonomous growth. This idea is supported by the observations that activated FLT3 cooperates with PML-RAR α (27, 53) and that BCR-ABL cooperates with AML1-MDS1-EVI1 or NUP98-HOXA9 (4, 6) to cause acute leukemia. By understanding the critical events that underlie cooperative effects, we should be able to therapeutically tip the cells of human AMLs back toward normal behavior, as has been done successfully for APL. Our findings explore one pathway for leukemic transformation, i.e., cooperation of abnormal transcription factors with activated cytokine receptors, and provide a system for identifying the particular signals that are critical for this process. Other pathways for leukemic transformation almost certainly exist, including overexpression of complementing transcription factors (e.g., *Hoxa9* plus *Meis1a* [34]), as well as unidentified events in the karyotypically complex leukemias seen after myelodysplasia, cytotoxic chemotherapy, and in the elderly. Further work is needed to reveal whether these different types of AML share fundamental molecular abnormalities.

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