Identification of B94 (TNFAIP2) as a Potential Retinoic Acid Target Gene in Acute Promyelocytic Leukemia

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Abstract

Acute promyelocytic leukemia (APL) is characterized by a block to myeloid differentiation caused by expression of the fusion oncoprotein promyelocytic leukemia-retinoic acid receptor α (PML-RARα). The purpose of this study was to identify genes that are regulated in a PML-RARα-dependent fashion by retinoic acid (RA), because such genes may be integrally involved in APL pathogenesis and/or myeloid differentiation. A cDNA microarray approach was used to identify genes induced in response to RA in TF1 myeloid leukemia cells expressing PML-RARα (TF1-PR cells). The B94 gene (TNFAIP2; Unigene Hs.101382), originally identified as a tumor necrosis factor α-inducible gene in endothelial cells, was one of several genes found to be induced by RA specifically in TF1-PR cells, but not in TF1-neo (control) cells. The induction of B94 was most pronounced in cells expressing the PML-RARα short isoform and was negligible in cells that expressed a mutant PML-RARα protein containing a deletion of the PML-coiled-coil domain. B94 induction by RA occurred within 1 h, did not require new protein synthesis, and was inhibited by actinomycin D, suggesting rapid transcriptional activation. B94 was also induced by RA in NB4, U1, and HL-60 cells, but not in other hematopoietic cell lines tested, suggesting that its up-regulation by RA may be specific to cells that express PML-RARα or are at the late myeloblast or promyelocyte stage of myeloid development. A screen of bone marrow cells from normal donors or patients with acute myelogenous leukemia showed that B94 was highly expressed in normal marrow and in marrow from patients with acute myelogenous leukemia French-American-British subtypes M2-M5, but was repressed in marrow cells from APL patients. Treatment of APL blasts in vitro with all-trans-RA resulted in up-regulation of B94 mRNA. These results suggest that B94 plays a role in myeloid development and support the hypothesis that B94 is a target gene of PML-RARα in APL.

Introduction

APL3 is a malignancy of the myeloid lineage characterized by arrested differentiation at the promyelocytic stage of myeloid development (1, 2). It is one of the most treatable of human malignancies, and most patients are cured with ATRA in combination with anthracycline chemotherapy (3). The fusion oncogene implicated in APL, PML-RARα, plays both causative and curative roles in this disease: in the absence of ATRA, it blocks transcription of genes required for myeloid differentiation, whereas in the presence of ATRA, it appears to facilitate expression of such genes, leading to differentiation and perhaps to apoptosis of leukemic cells (1, 2). One approach to understanding the role of PML-RARα in APL is to identify genes that are transcriptionally repressed by this molecule and induced by ATRA specifically (or preferentially) in PML-RARα-positive cells.

Several lines of evidence suggest that PML-RARα inhibits myeloid differentiation by interfering with transcriptional activation of RA target genes (4, 5). RAR/retinoid X receptor heterodimers bind RA target gene promoters in association with nuclear receptor corepressors (Silencing mediator for retinoid and thyroid hormone receptors or nuclear receptor corepressor and mSin3A) and HDACs. Histone deacetylation yields a chromatin conformation unfavorable for transcription; however, the binding of RA to its cognate receptor results in dissociation of the N-CoR complex and restoration of a transcriptionally active chromatin structure. Compared with native RARα, the PML-RARα-corepressor interaction is far less sensitive to RA, resulting in histone deacetylation and transcriptional repression even in the presence of physiological concentrations of RA. The ability of pharmacological doses of RA to dissociate the PML-RARα-corepressor complex underlies the current approach to APL treatment and is felt to restore expression of genes involved in myeloid differentiation (4, 6, 7). Identification of these RA target genes is an important avenue of future research in APL, and several candidate genes have been identified (8, 9).

B94 is a cytokine-inducible immediate early gene that was originally identified as a TNF-α-inducible transcript in human endothelial cells (10). Developmental studies in the mouse indicate that B94 mRNA is expressed in hematopoietic and lymphoid tissues (11), suggesting that B94 plays a role in blood cell development. Two recent genome-wide screens have identified B94 transcripts in microglial cells (12), postulated to arise from the monocytic lineage, and in peripheral blood neutrophils (13), further supporting a role for B94 in hematopoietic development. The growth factor-dependent TF1 leukemia cell line (14) is permissive for expression of PML-RARα (15), and this cell line was used to identify genes that are repressed by PML-RARα and induced by RA in a PML-RARα-dependent manner. In the current report, we show that B94 is one such gene and provide preliminary evidence that B94 is a potential target for transcriptional repression by unliganded PML-RARα in APL cells.

Materials and Methods

Chemicals and Supplies. Except as noted, chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO), Penicillin-streptomycin, fungizone, and qualified, heat-inactivated FBS were from Life Technologies. Inc. (Grand Island, NY). M-Plasmocin was obtained from InVivoGen (San Diego, CA). Recombinant human TNF-α was from Genzyme (Cambridge, MA), and antihuman TNF-α-neutralizing antibody (purified goat IgG) was purchased from R&D Systems (Minneapolis, MN). Rabbit polyclonal anti-RARα was from Santa Cruz Biotechnology (Santa Cruz, CA), and STAT-60 RNA isolation reagent was purchased from Tel-Test “B” (Friendswood, TX). The Fast Track Kit for mRNA isolation was obtained from Invitrogen (Carlsbad, CA). Radioligand [α-32P]dCTP was purchased from Dupont-New England Nuclear (Wilmington, DE). The human B94 cDNA expressed sequence tag...
lysates from control TF1 cells (Neo) followed by quantitation using a densitometer. The data shown are the averages of two independent experiments. The presence or absence of actinomycin D (added 30 min before ATRA) was determined by Northern blotting as described above. Band intensity was quantitated by PhosphorImager analysis, Northern blotting and hybridization as described in Materials and Methods. CHX and ATRA for 4 h. Northern blot analysis was performed using B94 and β-actin probes, followed by quantitation using a densitometer. D. NB4 cells were treated with ATRA for 4 h in the presence or absence of actinomycin D (added 30 min before ATRA). B94 RNA was detected by Northern blotting as described above. Band intensity was quantitated by PhosphorImager analysis, and B94 mRNA level is plotted in arbitrary units. The data shown are the averages of two independent experiments.

Clinical Samples. Samples of blood and fresh and/or cryopreserved bone marrow were obtained from donors after informed consent under protocols approved by the Roswell Park Cancer Institute Institutional Review Board. Before RNA extraction or cryopreservation, mononuclear cells were prepared by Ficoll-Hypaque density gradient centrifugation. AML cases were categorized using conventional French-American-British criteria, and all cases of M1 (APL) were confirmed by cytogenetic and/or molecular analysis.

RNA Isolation, Northern Blotting, and Microarray Analysis. Total RNA was isolated using STAT-60 reagent, and polyA mRNA was isolated using the Fast Track Kit from Invitrogen. Northern blots were hybridized using ExpressHyb solution (Clontech) according to the manufacturer’s instructions. A 2.0-kb fragment of chicken β-actin was used as a probe to control for the amount of RNA loaded. A 525-bp B94 cDNA fragment was isolated from 5’-EST clone 487045 after digestion with EcoRI and NotI and agarose gel purification using the NucleoTrap Kit (Clontech). The fragment was sequenced in the Roswell Park Cancer Institute Biopolymer Facility to confirm its identity with the previously published (10) human cDNA. Autoradiography was carried out using Kodak Bio-Max MS Film. Phosphorimaging analysis was performed with a Storm 860 PhosphorImager (Molecular Dynamics), and densitometry was carried out with a Molecular Dynamics Computing Densitometer. To prepare cDNA probes for hybridization to cDNA microarrays, polyA mRNA from treated cells was isolated and reverse-transcribed in the presence of Cy-5- or Cy-3-labeled dUTP essentially as described previously (16). Hybridization to microarrays and data acquisition were performed as reported previously (16).
Regulation of B94 in APL

Western blot hybridization was performed as described previously (15) using rabbit polyclonal anti-RARα (Santa Cruz Biotechnology).

Construction of PML-RARα Coiled-Coil (ΔC) Mutant. Briefly, the PML-RARα L form cDNA was digested with BssHII, religated, and cloned into the expression vector pCINeo (Promega) as described previously (15). The BssHII fragment removed encompasses amino acids 216–330, all within PML exon 3, including the distal part of the third zinc finger, and the first three of four clusters in the coiled-coil domain.

Results

Induction of B94 by ATRA in PML-RARα-positive TF1 cells. TF1 myeloid leukemia cells (14) were engineered to express either the L or S isoform of PML-RARα (TF1-PRα L or TF1-PRα S cells), as well as a mutant L form PML-RARα that contains a deletion of most of the PML coiled-coil domain (TF1-PRα C; Fig. 1C). The lack of induction of B94 in TF1-PRα C cells compared with clones expressing the L isoform, as shown previously (Ref. 15; Fig. 1B). The lack of induction of B94 in TF1-PRα S and TF1-PRα D cells was not due to lack of expression of this mutant protein, as shown in Fig. 1B. All TF1 clones, as well as parental TF1 cells, expressed endogenous RARα (Fig. 1B; Ref. 15) and retinoid X receptorα (data not shown). These data indicate that ATRA induction of B94 is mediated by PML-RARα and is critically dependent on the presence of the coiled-coil (dimerization) domain of PML. B94 was not induced in TF1-PRα cells by TPA, arsenic trioxide, or the histone deacetylase inhibitor trichostatin A (data not shown).

Mechanism of B94 Induction by ATRA and Induction in Other Hematopoietic Cell Lines. B94 was also induced by ATRA in the PML-RARα-positive cell lines NB4 and U9118 (Ref. 17 and 18; 2B; data not shown) as well as in the PML-RARα-negative cell line HL-60 (data not shown). To study the time course and determine the mechanism of induction of B94 by ATRA, TF1-PRα S cells were treated with ATRA for various times in the presence or absence of the protein synthesis inhibitor cycloheximide (Fig. 2C). Maximum induction of B94 occurred after 8 h of ATRA treatment, but up-regulation of B94 mRNA was evident within 1 h of ATRA addition; B94 induction by ATRA was not blocked by cycloheximide. To determine whether B94 was up-regulated at the level of transcription, NB4 cells were treated with ATRA in the presence of the RNA polymerase inhibitor actinomycin D. Actinomycin D completely abrogated B94 induction (Fig. 2D), suggesting that up-regulation of B94 by ATRA is due to a direct effect on transcription rather than stabilization of its mRNA. B94 regulation was also assessed during monocytic differentiation in KG-1, TF1, and HL-60 cells, which respond to TPA by differentiation into macrophages. Treatment of KG-1 and TF1 cells with TPA over a 24-h period failed to up-regulate B94 (data not shown); however, TPA did induce B94 in HL-60 cells (Ref. 11; data not shown). Thus, HL-60 cells, which have the capacity to develop into either neutrophils (with ATRA) or macrophages (with TPA), expressed increased levels of B94 with either agent. No increase of B94 was observed in K562 cells induced to undergo erythroid differentiation with heme or Adriamycin, or in DAMI cells induced to undergo megakaryocytic differentiation with TPA (data not shown).

B94 Is Induced Independently by ATRA and TNF-α in TF1-PR Cells. Because B94 was originally identified as a TNF-α-inducible gene, we considered the possibility that ATRA induction of B94 was indirect and mediated by intervening production or secretion of TNF-α, particularly because ATRA has been shown to induce cytokine expression (including TNF-α expression) in hematopoietic cell lines (19, 20). Although the cycloheximide data discussed above suggested that intervening protein synthesis was not required for B94 induction, it remained possible that posttranslational mechanisms could have resulted in increased TNF-α secretion. TNF-α alone induced B94 gene expression in TF1-PRα S and TF1-PRα D cells (Fig. 3) and the PML-RARα-positive NB4 cells (data not shown). This approximately 3-fold induction was totally abrogated in TF1-neo cells by pretreatment with a neutralizing anti-TNF-α antibody (Fig. 3). The 5-fold induction of B94 by ATRA in TF1-PRα S cells was unaffected by addition of the anti-TNF-α antibody (Fig. 3), proving that intermediary synthesis/secrection of TNF-α was not responsible for induction of the B94 gene by...
ATRA in these cells. It is also apparent from Fig. 3 that the combination of ATRA plus TNF-α was at least additive, if not synergistic, in the induction of B94 in TF1-PR S cells.

Tissue-Specific Expression of B94. To assess B94 tissue-specific expression, a blot containing total RNA from an array of human tissues (RNA Master Blot; Clontech) was probed with the B94 cDNA fragment used in all Northern blot experiments. Total RNA for each tissue on this dot blot has been normalized with respect to expression levels of housekeeping genes. Based on dot intensity, the relative amount of B94 transcript was quantitated, and a plot of tissue-specific expression is presented in Fig. 4. B94 was expressed in essentially all tissues, but the highest levels were observed in peripheral blood leukocytes, spleen (fetal and adult), and lymph node. Substantial expression was also observed fetal kidney, fetal and adult lung, and placenta. The lowest level of B94 expression was in the pituitary gland and whole brain tissue (adult and fetal).

B94 Expression in Bone Marrow from Patients with APL and Other AML Subtypes. B94 expression was assessed in normal and leukemic bone marrow samples (Fig. 5A). B94 was highly expressed in normal marrow, chronic myeloid leukemia marrow, and in marrow from patients with AML subtypes M0–M2. In contrast, B94 was expressed at much lower levels in leukemic cells from four patients with APL (Fig. 5A). This was not due to degradation of RNA in the APL samples, as shown by the actin control hybridization. Despite its low level in APL marrow, B94 was induced by ATRA in APL blasts cultured in vitro (Fig. 5B).

Discussion

PML-RARα, the oncogene implicated in APL pathogenesis, has been shown to inhibit myeloid differentiation in the absence (or at low concentrations) of RA but to facilitate differentiation in the presence of high concentrations of retinoids (reviewed in Ref. 1). The mechanism is presumed to involve repressive and/or inductive effects on expression of genes involved in myeloid development. At low or physiological levels of RA, PML-RARα functions as a transcriptional repressor by recruiting nuclear corepressors and HDACs to the promoter regions of RA target genes (2, 4, 6, 7). The resultant deacetylation of histones results in a closed chromatin conformation that is repressive for gene transcription (21). High levels of RA (achieved therapeutically in treatment of APL patients) result in the release of corepressors from the PML-RARα molecule, recruitment of transcriptional coactivators, and changes in chromatin conformation that facilitate gene transcription. Several potential PML-RARα target genes have been identified, including C/EBP-ε (8), p21WAF1/CIP1 (9), and type II transglutaminase (22). In this report, we provide evidence that B94 is also a target gene of PML-RARα. In the absence of RA, this gene is repressed in APL cells (Fig. 5A) or in PML-RARα-positive cells (Fig. 3), whereas it is strongly induced by ATRA in APL blasts, in cell lines derived from APL patients, and in a leukemic cell line engineered to express PML-RARα (Figs. 2, 3, and 5).

Although the function of the B94 protein is unknown, the tissue distribution of B94 transcripts supports a role for this molecule in hematopoiesis. In mice, B94 mRNA is present in many tissues during embryonic development, including developing vascular structures,
Although our data and previously published reports (10, 11) suggest that B94 is involved in hematopoietic development, it is likely to have a broader role in cellular physiology. Over 100 B94-specific expressed sequence tags from diverse organs and tissues have been identified in the Unigene dataset, suggesting that B94 is a widely expressed gene that may play a common role in many different tissues. B94 SAGE tags have also been found in a number of publicly available SAGE libraries, particularly in a library constructed from pooled RNA from ovarian carcinoma cell lines, where the frequency of B94 tags was 1010 tags/million. B94 SAGE tags were also found at a high level in several libraries from normal human mammary epithelial cells. The 654-amino acid human B94 protein is an intracellular protein that is homologous (23% amino acid identity; 46% similarity) to rat Sec6 (GenBank accession number U32575), a homologue of a yeast protein (SEC6) that is involved in the Golgi-to-plasma membrane stage of the yeast secretory pathway. A human sec6 homologue (GenBank accession number AF055006) has also been identified. Both mature neutrophils and macrophages contain numerous granules that contain proteases and other microbicidal substances, and these granules eventually fuse with either the plasma membrane or with phagosomes to effect killing of pathogenic organisms. Given the homology of B94 with rat and human sec6, it is possible that B94 plays a role in granule or vesicle trafficking within myeloid (and other) cells.

Compared with TF1-PR cells containing intact PML-RARα (S or L isoform), B94 induction by ATRA was reduced to negligible levels in TF1-PR cells expressing a mutant PML-RARα construct harboring a deletion of most of the PML coiled-coil domain (Figs. 1 and 2). This region of the PML protein mediates homo- and heterodimerization of PML and PML-RARα (23) and is involved in numerous functions of PML-RARα, including differentiation inhibition (24), transcriptional cooperativity with AP-1 (25), and binding to the retinoblastoma protein (26). By interaction with native PML, PML-RARα is presumed to titrate out and effectively inactivate PML in APL cells, resulting in aberrant transcriptional responses in these cells. PML may itself be a transcriptional repressor (27) or may modulate transcription indirectly by interacting with proteins such as cAMP-responsive element binding protein (28) and fos (29) that control critical transcriptional responses in cells. We hypothesize that lack of interaction of the mutant PML-RARα with a critical transcriptional coregulator (PML itself, cAMP-responsive element binding protein, or fos) explains the loss of B94 induction by ATRA in the TF1-PRAC cells, because a similar PML-RARα coiled-coil mutant retained DNA binding and transactivation potential in vitro (24).

The induction of B94 by multiple cytokines in addition to ATRA suggests that it may be involved in a general response of the organism to inflammation or tissue damage, a speculation supported by the observation of B94 mRNA induction during angiogenesis (10, 11). ATRA itself induces a number of proinflammatory cytokines, including IL-8, TNF-α, and IL-1β, in APL cells (30), which suggested that up-regulation of B94 by ATRA in TF1-PR cells could have been an indirect event, particularly because TNF-α also induces B94 in these cells (Fig. 3). However, our results indicate that the induction of B94 by ATRA and the induction of B94 by TNF-α are independent events, thus suggesting distinct mechanisms of transcriptional activation of the B94 promoter by these two agents. ATRA and TNF-α have been shown to cooperate in activation of the IL-8 promoter via a mechanism that involves nuclear factor κB (31), and such a mechanism may also be operative in the case of B94. Ultimately, the role that B94 plays in hematopoiesis will only become clear with detailed functional

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5 ncbi.nih.gov/SAGE.
studies, including gene knockout experiments in mice, which is in progress. The currently available data suggest that the regulation and function of B94 will be complex, but a compelling case can be made that a full understanding of its function may provide important insights into both normal and malignant myelopoiesis.

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References


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