A Retinoid-Resistant Acute Promyelocytic Leukemia Subclone Expresses a Dominant Negative PML-RAα Mutation

By Wenlin Shao, Laura Benedetti, William W. Lamph, Clara Nervi, and Wilson H. Miller Jr

The unique t(15;17) of acute promyelocytic leukemia (APL) fuses the PML gene with the retinoic acid receptor α (RAα) gene. Although retinoic acid (RA) inhibits cell growth and induces differentiation in human APL cells, resistance to RA develops both in vitro and in patients. We have developed RA-resistant subclones of the human APL cell line, NB4, whose nuclear extracts display altered RA binding. In the RA-resistant subclone, R4, we find an absence of ligand binding of PML-RAα associated with a point mutation changing a leucine to proline in the ligand-binding domain of the fusion PML-RAα protein. In contrast to mutations in RAα found in retinoid-resistant HL60 cells, in this NB4 subclone, the coexpressed RAβ remains wild-type. In vitro expression of a cloned PML-RAα with the observed mutation in R4 confirms that this amino acid change causes the loss of ligand binding, but the mutant PML-RAα protein retains the ability to heterodimerize with RXRα and thus to bind to retinoid response elements (RAREs). This leads to a dominant negative block of transcription from RAREs that is dose-dependent and not relieved by RA. An unrearranged RAα engineered with this mutation also lost ligand binding and inhibited transcription in a dominant negative manner. We then found that the mutant PML-RAα selectively alters regulation of gene expression in the R4 cell line. R4 cells have lost retinoid-regulation of RXRα and RAβ and the RA-induced loss of PML-RAα protein seen in NB4 cells, but retain retinoid-induction of CD18 and CD38. Thus, the R4 cell line provides data supporting the presence of an RAα-mediated pathway that is independent from gene expression induced or repressed by PML-RAα. The high level of retinoid resistance in vitro and in vivo of cells from some relapsed APL patients suggests similar molecular changes may occur clinically.

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analyzed both the unarranged RARα and fusion receptors of these clones for mutations that might explain their resistance to RA.

We report here a point mutation found in the ligand-binding domain of the fusion PML-RARα in the RA-resistant subclone, R4. R4 expresses PML-RARα protein detected by Western blot and the fusion protein binds RAR, but R4 nuclear extracts show ligand binding only by RARα. To determine that the mutation accounts for the observed loss of binding and transcriptional response to retinoids in R4 cells, we expressed in vitro PML-RARα or RARα proteins with this point mutation. The mutant PML-RARα and RARα proteins do not bind ligand, but retain their ability to bind RA and block the transcription of RA-responsive genes in a dominant-negative fashion.

MATERIALS AND METHODS

Cell culture. The parental NB4 promyelocytic leukemia cell line, three RA-resistant subclones, and the myeloid leukemia HL-60 cells were grown in RPMI medium (GIBCO BRL, Burlington, Ontario, Canada) plus 10% fetal bovine serum (10% FBS; Upstate Biotechnology Inc, Lake Placid, NY). Cos-1 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Wisent Inc, St-Bruno, Quebec, Canada) supplemented with 10% FBS. All cell cultures were incubated at 5% pCO2 at 37°C in humidified air.

DNA sequencing analysis. Total RNA was extracted from cells with guanidine isothiocyanate and prepared as previously described.27 Oligonucleotide primers were designed flanking the entire PML-RARα coding region and synthesized by BRI (Montreal, Quebec, Canada). Different domains of PML-RARα were synthesized by reverse transcription polymerase chain reaction (RT-PCR) and used as a template for dsDNA cycle sequencing. The components and the methods were provided by the reaction kit (GIBCO BRL). For each domain, sequencing was performed using 1 pmol of internal primer end-labeled with 1 μCi of [γ-32P] ATP (10 μCi/mL; Dupont-NEN, Boston, MA) by T4 polynucleotide kinase. PCR reactions were run on thermal cycler (GeneAmp PCR System 9600; Perkin-Elmer Cetus, Norwalk, CT) and analyzed by denaturing polyacrylamide gel electrophoresis.

Plasmid constructs. Two primers shown in Fig 1A were used for RT-PCR to amplify the RARα portion of the fusion PML-RARα (PRA ΔA) in R4. The amplified cDNA was subcloned to pBS/KS+ vector. Both pBS-PRA ΔA (R4) and pSG5-RARα or pSG5-PML-RARα were digested with BsoBI and BstEII restriction enzymes. The 800-bp fragment produced from pBS-PRA ΔA (R4) digestion containing the point mutation was ligated to the 5.1-kb fragment produced from pSG5-RARα or the 7.5-kb fragment from pSG5-PML-RARα digestion, generating a pSG5-RARα m4 or a pSG5-PML-RARα m4 construct. Both constructs were verified by sequencing analysis.

Assay for ligand binding activity. Cos-1 cells were transiently transfected by electroporation with pSG5 expression vectors containing either wild-type or mutant RARα and PML-RARα. Nuclear extracts were prepared from 1 to 5 × 106 cells and incubated for 18 hours at 4°C with 10 nmol/L [3H]-RA (50.7 Ci/mmol; DuPont-NEN), as previously described.27,28 The extracts were subsequently fractionated at 4°C by HPLC using a superose 6 HR 10/30 size exclusion column (Pharmacia, Uppsala, Sweden). The flow rate was 0.4 mL/min, fractions of 0.4 mL were collected, and radioactivity was determined using a liquid scintillation counter. The HPLC system was calibrated using a series of molecular weight (MW) markers, including the following: blue dextran, MW 2,000,000; thyroglobulin, MW 669,000; β-amylose, MW 200,000, bovine serum albumin, MW 66,000; and ovalbumin, MW 45,000.

Transcriptional activation assays. Cos-1 cells were grown in DMEM with 10% FBS and were seeded 1 day before transfection. Cells were rinsed with Opti-MEM (GIBCO BRL) and transfected by the lipofectamine method (GIBCO BRL) with 0.7 μg of receptor plasmid, 1 μg of reporter CAT plasmid, and 0.3 μg of pCMV-βGal as an internal control for transfection efficiency. Amounts of pSG5-RARα and pSG5-PML-RARα m4 were varied to have a total of 0.7 μg of plasmid. Cells were transfected for 4 hours and were replenished with 2 mL of DMEM with 10% FBS and were then incubated for 2 days with or without 10−8 mol/L RA (Sigma, St Louis, MO). The chloramphenicol acetyltransferase (CAT) activity was measured using a modified protocol of the organic diffusion method.29 Fifty microliters of cell extracts was incubated for 2 hours at 37°C with 200 μL of 1.25 mmol/L cold chloramphenicol (ICN, Costa Mesa, CA) dissolved in 100 mmol/L Tris, pH 7.8, and 0.25 μCi of 3H-labeled acetyl coenzyme A (NEN, Streetsville, Ontario, Canada). The reaction was extracted with Ready Organic Scintillation Cocktail (Beckman, Mississauga, Ontario, Canada), and 750 μL of the organic phase was counted on a scintillation counter. The CAT counts were normalized with β-Gal activity to obtain relative CAT activity.

Ribonuclease protection assay. Total cytoplasmic RNA was isolated and RNase protection analysis was performed as described previously.30,31 Hybridization of cRNA probes was performed at 45°C overnight, followed by the addition of 300 μL of RNase digestion buffer containing 40 μg/mL of RNase A and 700 U/mL of RNase T1. RNase digestion was performed at 25°C for 1 hour. The RNase-resistant fragments were resolved by electrophoresis on 6% urea-polyacrylamide sequencing gels. A GAPDH probe (Ambion Inc, Austin, TX) was included in all samples as a control for RNA loading. As approximate size markers, [32P]-labeledMsp I-digested fragments of pBR322 were run on all gels.

Northern analysis. Total RNA was electrophoresed on a 1% formaldehyde agarose gel and blotted onto Zeta probe (BioRad, Mississauga, Ontario, Canada) transfer membranes. The filters were hybridized to a DNA probe labeled by random priming (Pharmacia Biotech, Baie d’Urfe, Quebec, Canada). Hybridization and autoradiography were performed as previously described.27 The CD18 probe was isolated by RT-PCR. Two primers used for PCR correspond to CD18 sequences 931-950 and 1487-1506, respectively, and amplified a 576-bp CD18 cDNA fragment.

Western analysis. Nuclear extracts were diluted 1:1 with 2 × sodium dodecyl sulfate (SDS) sample buffer. Proteins were then fractionated by electrophoresis on a 8% SDS polyacrylamide gel and electroblotted onto a nitrocellulose membrane (Hybond C Super; Amersham, Milan, Italy). Proteins that reacted with the anti-RARα RPo(F) antibody32 (used at a 1:1,000 dilution) were detected using the ECL Western blotting detection kit (Amersham).

RESULTS

RA-resistant subclone R4 has a point mutation in the ligand-binding domain of PML-RARα. We have developed several RA-resistant subclones of NB4 by selection in RA-containing media without treatment with mutagens.25 Unlike RA-resistant subclones reported by Dermime et al,25 both Northern and Western analyses on these RA-resistant subclones showed expression of unarranged RARα and the fusion PML-RARα transcripts and proteins. Three resistant subclones (MR2, MR6, and R4) were subjected to DNA sequencing analysis using a dsDNA cycle sequencing system. Two primers depicted in Fig 1A were used in RT-PCR to specifically amplify the RARα part of PML-RARα (PRα
A point mutation at codon 890 in the ligand-binding domain of PML-RARα. (A) Schematic structure of PML-RARα, showing the approximate positions of two primers used in RT-PCR to amplify the RARα part of PML-RARα (PRαΔA). (B) Sequencing autoradiogram shows the T → C conversion at PML-RARα codon 890, which results in the exchange of leucine to proline.

The conformational change induced by the R4 mutation might also be expected to alter protein-protein interactions that are required for RARE binding and activation. Proline is a small cyclic amino acid that typically disrupts helical structures, causing an altered conformation of the protein.

Transcriptional inhibition by in vitro expressed mutant proteins. The conformational change induced by the R4 mutation might also be expected to alter protein-protein interactions that are required for RARE binding and activation. Proline is a small cyclic amino acid that typically disrupts helical structures, causing an altered conformation of the protein.

Corresponding to molecular weights of 50,000 and 16,000, probably representing the endogenous RARs and CRABPs present in Cos-1 cells. To determine whether the effects of this point mutation were dependent on the structure of the PML-RARα protein, we constructed a vector expressing RARα in which codon 890 was replaced by Pro (RARαm4). The binding of [3H]-tRA was analyzed as described above; no peaks at 50,000 corresponding to RARα-tRA specific binding were observed. Thus, this Leu → Pro mutation in the ligand-binding domain abolishes the ability of both RARα and PML-RARα to bind their ligand.
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Fig 2. (A) Wild-type RARα and PML-RARα as well as mutant PML-RARα and RARα were transiently transfected into Cos-1 cells. Nuclear extracts of transfected cells were subjected to Western analysis to examine the expression of exogenous proteins. Lane 1, Cos-1 cells transfected with mock; lane 2, transfected with RARα wt; lane 3, transfected with mutant RARα (RARα m4); lane 4, transfected with PML-RARα wt; lane 5, transfected with mutant PML-RARα (PML-RARα m4). (B) Specific nuclear tRA binding activity in Cos-1 cells transfected with (1) PML-RARα m4 or (2) RARα m4 in comparison to those transfected with wild-type receptors (3) and (4). Nuclear extracts were incubated with [3H]-tRA alone (●) or with [3H]-tRA in the presence of 200-fold excess of unlabeled tRA (○).
Table 1. Expression of Retinoid Receptor Isoforms and CD38 With or Without $10^{-6}$ mol/L tRA Treatment

<table>
<thead>
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<th>Receptor</th>
<th>NB4, Control</th>
<th>NB4, tRA (d3)</th>
<th>R4, Control</th>
<th>R4, tRA (d3)</th>
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<td>RARβ</td>
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</tr>
<tr>
<td>CD38</td>
<td>2.0</td>
<td>8.5</td>
<td>4.5</td>
<td>14.6</td>
</tr>
</tbody>
</table>

Expression of six retinoid receptor isoforms and CD38 in NB4 and R4 cells with or without $10^{-6}$ mol/L of tRA treatment. The bands appeared on RNase-protection blot were scanned by phosphoimager to quantify the data and are presented in the table as arbitrary units.

Expression of retinoid receptor isoforms and CD38 in NB4 and R4 cells. To determine whether the expression of this mutant PML-RARα in R4 directly affected retinoid receptor levels, we compared R4 with NB4 in their expression of the six retinoid receptor isoforms in a ribonuclease protection assay. The hybridizing bands specific for each receptor were scanned by phosphoimager and are presented in arbitrary units in Table 1. Neither RARγ nor RXRγ expression is observed in NB4 cells treated with tRA. Interestingly, a significant increase in RARβ expression is seen in NB4 cells. Both the induction of RARβ and decrease of RXRα expression upon RA are lost in R4 cells. However, we have found that one retinoid-regulated gene, myeloblastin, retains its response to RA in R4 cells.

Fig 3. Transcriptional activity of wild-type and mutant receptors. (A) TRE-tk-CAT reporter or (B) βRE-tk-CAT reporter was cotransfected with the indicated receptors. pSG5 is the vector alone. Relative CAT activity with ( ) or without ( ) $10^{-6}$ mol/L tRA treatment is shown with calculated fold induction below. Each data point represents results from three independent transfections. (C) The βRE-tk-CAT reporter was cotransfected with PML-RARα m4 and RARα wt expression vectors. The ratio of PML-RARα m4 to RARα was varied, and the total amount of receptor plasmids was kept at 0.7 μg for each transfection.
expression of two other RA-induced genes, CD38 and CD18 (leukocyte adherence β subunit), that have been shown to be transcriptionally upregulated during RA-induced differentiation in myeloid leukemia HL-60 cells. RA-dependent upregulation of both genes was observed in both parental NB4 cells and resistant subclone R4 cells. The RA-induced CD38 expression is presented in Table 1 by the RNase-protection method. Northern analysis was performed to examine the expression of CD18 (Fig 4). NB4,306, an RA-resistant NB4 subclone that does not express detected PML-RARα protein,25 was reported to have RA-dependent induction of CD18 expression in a manner similar to NB4 cells.41 In the two PML-RARα expressing cell lines we examined, CD18 expression is induced by RA. Thus, a significant subset of RA-regulated genes continue to be regulated in a variety of cells that are resistant to RA-induced differentiation.

DISCUSSION

Studies of retinoid-resistant subclones have led to insights into the molecular mechanisms of response to retinoids in RA-inducible embryonal carcinoma and leukemic cell lines.20,21 We applied the same strategy to study APL using the in vitro model provided by the NB4 cell line. We developed several RA-resistant NB4 subclones to investigate the molecular basis of RA-resistance in APL.20 We report here a point mutation found in the ligand-binding domain of the PML-RARα in an RA-resistant subclone, R4. The point mutation is localized at codon 890, changing leucine to proline. It is within the helix 11-helix 12 region of the RARα ligand-binding domain, 10 amino acids upstream to the AF-2 AD core domain. Renaud et al.35 compared the crystal structure of the retinoid receptor ligand-binding domain free of ligand with that of ligand-bound. They proposed a mouse trap mechanism: after the retinoid is attracted to the ligand-binding cavity by electrostatic forces, H11 is repositioned, causing H12 to swing to its final position. In this position, H12 serves as a lid for the ligand-binding cavity and stabilizes ligand binding to the pocket. This mechanism could be disrupted by the substitution of the cyclic amino acid proline, preventing interaction of the receptor with its ligand. By expressing in vitro PML-RARα engineered to contain the point mutation, we confirmed that this single mutation abrogates the ligand-binding ability of PML-RARα.

Two additional RA-resistant subclones, MR2 and MR6, were also subjected to DNA sequence analysis. No mutations or deletions were found in their PRα ΔA region. However, both cell lines show altered binding of high MW PML-RARα complex to ligand, suggesting that the protein-protein interaction mediated by PML domain of the fusion molecule may be altered. A recent report by Altabef et al10 showed that PML-RARα with two mutations in the PML domain transformed chicken hematopoietic progenitor cells and the mutant protein localization did not return to the normal pattern with RA treatment. We are currently sequencing the PML domain of these two resistant cell lines as well as exploring molecular mechanisms other than the alteration of the PML-RARα or the unrearranged RARα that may play a role in causing RA-resistance in these two cell lines.

RA-resistant HL60 subclones have been reported with mutations in the ligand-binding domain of RARα.14,16,20,21 Li et al10 and Robertson et al21 both found the same point mutation that leads to a truncated protein in their independently derived resistant subclones. The mutant RARα gene is either homozygous42 or coexpressed with the wild-type RARα and displays a dominant negative activity.21 Dore and Momparler22 reported a point mutation resulting in a protein that is unable to form RXR-RAR heterodimers, does not bind to retinoid response elements, and thus might not inhibit transcription as a dominant negative factor. However, in the R4 APL subclone, RA-resistance is caused by a point mutation found in the PML-RARα oncprotein, not in RARα. This mutation mediates a retinoid-independent dominant negative inhibition of the coexpressed wild-type RARα.

As shown by gel retardation assays, the mutant PML-RARα in R4 cells retains the ability to interact with RXRα and bind to the DNA βRE. In Cos-1 cells, both intact and mutant PML-RARα proteins inhibit transcriptional activity in the absence of ligand, again suggesting preservation of protein-protein or protein-DNA interaction. Although the intact PML-RARα becomes a transcriptional activator in response to RA, the mutant receptors continue to inhibit transcription in the presence of RA. PML-RARα plays a dual role in the APL phenotype: it mediates the block of differentiation but retains sensitivity to RA.56 Pharmacologic levels of RA release the differentiation block, perhaps by inducing proteolysis of PML-RARα protein.12,42 The RA-induced degradation of PML-RARα seen in NB4 is lost in R4, perhaps because PML-RARα–induced transcription is required to express or activate a protease, or, alternatively, the conformational change in the mutant PML-RARα confers resistance to proteolysis.

Studies suggested that the ratio of expression of PML-RARα to that of the unrearranged RARα is important in maintaining the dominant negative block of myelocytic dif-
fication.14 PML-RARα forms large multimeric complexes with itself, PML, RXR, and possibly a group of ligand-dependent transcription factors.29,36,45 Thus, PML-RARα and RARα may compete for common coactivators. PML-RARα has previously been shown to suppress transcription of RAREs in the absence of ligand,24 possibly by the sequestration of either PML, RXR, or other proteins. RA induces specific degradation of PML-RARα and so releases the block to transcription. However, because mutant PML-RARα is not degraded by RA, we were able to test its dominant negative function in the presence of ligand, and we find that it blocks the transcriptional activity of cotransfected wild-type RARα in a dose-dependent manner.

There is evidence that we can differentiate transcription mediated by PML-RARα from that by RARα. We compared the expression and retinoid regulation of six retinoid receptor isoforms in either PML-RARα or mutant PML-RARα expressing cell lines. The RA-dependent regulation of RXRα and RARβ observed in NB4 cells is lost in R4 cells, whereas myeloblastin, CD18, and CD38 continue to be regulated. The loss of RA-inducible regulation in some but not all genes in R4 cells indicates that the mutant PML-RARα selectively blocks RARα-regulated signaling. RARα may mediate induction of certain genes even in the presence of dominant negative PML-RARα.

APL is a unique example in oncology of a molecular translocation that can be treated by therapy targeted directly towards the defect, the chimeric PML-RARα gene. However, an aberrant form of PML-RARα found in R4 cells prevents it from responding to RA normally and results in the resistance of cells to RA treatment. The high level of retinoid resistance in vitro and in vivo of cells from some relapsed APL patients suggests that similar molecular changes may occur clinically.

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