Retinoid Receptor Expression and Its Correlation to Retinoid Sensitivity in Non-M3 Acute Myeloid Leukemia Blast Cells

Sören Lehmann, Crister Paul, and Hans Törnä
Department of Hematology, Karolinska Institute, Huddinge Hospital, 141 86 Stockholm, Sweden [S. L., C. P.], and Department of Medical Sciences, Section of Dermatology and Venereology, University of Uppsala, 75185 Uppsala, Sweden [H. T.]

ABSTRACT
All-trans-retinoic acid (ATRA) has significantly improved the treatment results in acute promyelocytic leukemia (M3). In non-M3 acute myeloid leukemia (AML), the effects are less clear, and there is a pronounced heterogeneity in the sensitivity to the growth-inhibitory effects of retinoids in leukemic cells from different non-M3 AML patients. Retinoids exert their effects through a number of nuclear receptors [retinoic acid receptors (RARs) and retinoid X receptors (RXRs)]. In this study, we determined the expression of RARα, RARβ, RARγ, and RXRα by real-time PCR in four cell lines and in blast cells from patients with non-M3 AML before and after ATRA incubation. All four receptors were expressed in cells from all 18 tested patient samples and in four myeloid cell lines. In the majority of the patient samples as well as in the cell lines, there was a pattern of high expression of RARα and RXRα and low expression of RARγ and RARβ. There was no correlation between the basal expression of any of the retinoid receptors and sensitivity to ATRA. A 24-h exposure to ATRA increased the expression of RARα, RARβ, RARγ, and RXRα in 46%, 77%, 30%, and 38% of the samples, respectively. The mean increase in receptor expression was most pronounced for RARβ and RXRα. There was a significant correlation between an increase in RARβ expression in response to ATRA and sensitivity to ATRA (P < 0.014). No such correlations were found for RARα, RARγ, and RXRα. The expression of the monocytoid marker CD14 was significantly correlated with increased expression of RARα (P = 0.03). We conclude that RARα, RARβ, RARγ, and RXRα are expressed in non-M3 AML blast cells and that ATRA-induced expression of RARβ may be a marker for retinoid sensitivity.

INTRODUCTION
Retinoids are important regulators of normal cell growth and differentiation (1, 2), but they also have profound effects on tumor cell growth in various malignant tumor species both in vitro and in vivo (3–9). The cellular effects of the retinoids are mediated through two types of intracellular receptors belonging to the steroid receptor superfamily, RARα3 and RXRs, each consisting of α, β, and γ isoforms. These receptors form RAR/RXR or RXR/RXR dimers that act directly on retinoid acid response elements within the promoter region of certain genes (10–12). The retinoid receptors are believed to mediate the physiological as well as the pharmacological effects of retinoids. The role of each subtype of the receptors may differ (11, 13), but overexpression of either RARα, RARβ, RARγ, or RXRα in transfected myeloid progenitor cells renders these cells sensitive to the growth-inhibitory effects of retinoids (14).

In APL (or M3 according to the FAB classification), ATRA is highly effective and has improved the complete remission rate as well as relapse-free and overall survival (15–19). These results have encouraged studies of treatment with retinoids in other malignancies. In non-M3 AML blast cells, the effect of retinoids is less pronounced compared to that in APL cells, but even so, leukemic cells from up to 70% of non-M3 AML patients are sensitive to ATRA or 9-cis-RRA (20, 21). Except for the heterogeneity in the response to retinoids between non-M3 AML cells from different patients, the sensitivity also varies between different tumor species. New and more receptor-specific ligands are continuously synthesized and studied to make treatment with retinoids more effective (22–25).

In several tumor species, attempts have been made to predict retinoid sensitivity in tumor cells by correlating the expression of the retinoid receptors to the growth-inhibitory effects of retinoids. In some studies, the up-regulation of RARβ in response to ATRA exposure has been shown to correlate with retinoid sensitivity both in vitro and in vivo in renal cell cancer (26–29). Fitzgerald et al. (30) also found a correlation between the basal expression of RARα and sensitivity to retinoids in breast cancer cells.

To investigate the relationship between receptor expression and retinoid sensitivity in non-M3 AML blast cells, we determined the mRNA expression of RARα, RARβ, RARγ, and RXRα by real-time PCR in non-M3 AML blast cells before and after exposure to ATRA. The results were then correlated with the antitumoral effects of ATRA in vitro and with several disease characteristics. RARα, RARβ, RARγ, and RXRα were expressed in all myeloid cell lines and in leukemic cells from all

3 The abbreviations used are: RAR, retinoic acid receptor; RXR, retinoid X receptor; ATRA, all-trans-retinoic acid; AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; FAB, French-American-British; RA, retinoic acid; AMR, ATP monitoring reagent.

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1 Supported by The Swedish Cancer Foundation, Stockholm, Sweden.
2 To whom requests for reprints should be addressed, at Department of Hematology, Huddinge Hospital, Karolinska Institute, 141 86 Stockholm, Sweden. Phone: 46-8-5858-0000; Fax: 46-8-5858-2525; E-mail: Soren.Lehmann@medhs.ki.se.
patients with non-M3 AML. There was no correlation between the basal receptor expression of any of the receptors and the growth-inhibitory effects of ATRA. However, ATRA-induced up-regulation of RARβ was significantly correlated with ATRA sensitivity. Moreover, we found a significant correlation between RARα expression and CD14 positivity.

MATERIALS AND METHODS

Cell Lines and Patient cells. The HL-60, K562, and KG1a cell lines were obtained from the American Type Culture Collection (Manassas, VA). A multidrug-resistant, P-glycoprotein-expressing, ATRA-resistant HL-60 cell variant, HL-60R, has been described by selection for resistance to doxorubicin as described previously by Jönsson et al. (31).

Leukemic cells from 18 non-M3 AML patients were collected from the bone marrow at the time of the diagnosis. Blast cells were isolated by the density gradient with Ficoll separation (Lymphoprep Nyregaard end Co AS, Oslo, Norway). The mean age of the patients was 66 years (age range, 37–83 years); 10 patients were male, and 8 patients were female. Three patients were classified as M1, three patients were classified as M2, one patient was classified as M3 (without t(15;17)), six patients were classified as M4, one patient was classified as M5b, one patient was classified as M6, and three patients were not classified. After cell separation, cells were washed twice in RPMI 1640 with 10% FCS (1.0 × 10⁶ cells/ml). The cells were then either analyzed immediately or cryopreserved in PBS with 20% FCS and 10% DMSO in vapor phase nitrogen. For determination of ATRA-induced change in receptor expression, a proportion of the blast cells were incubated in RPMI 1640 with 10% FCS and 1 μM ATRA for 24 h (5% CO₂, 37°C) before the receptor analysis.

Assessment of Viability. For assessment of retinoid sensitivity, patient cells were incubated in RPMI 1640 with 10% FCS with or without 1 μM ATRA or 9-cis-RA (Roche AB, Stockholm, Sweden). Cells were incubated in a humidified incubator at 5% CO₂ and 37°C for 96 h. All incubations were performed on fresh cells. The cell viability was assessed by a bioluminescence assay measuring ATP content as described previously (20, 32). The assay was performed automatically in a Bio Orbit (Turku, Finland) photometer. The AMR and the ATP standard were both supplied by Bio Orbita. The ATP standard was reconstituted in 1 ml of distilled water, giving a 10 μM solution. AMR was reconstituted with 5 ml of Tris-EDTA buffer [100 mM Tris and 2 mM EDTA (pH 7.75)]. Twenty μl of the cell sample were put in the cuvette. Automatically, 200 μl of AMR were dispensed in a cuvette placed in the photometer, and the resulting light emission was measured. ATP standard was automatically added (10 μl), and the emitted light was measured. The amount of ATP was calculated with correction for the blanks. The results were given as nanomoles of ATP/sample. The percentage of ATP in a sample compared to drug-free control was then calculated. The result of each exposure represents a mean of the two parallel exposures. To ensure the viability of controls, this was examined after 24 h of incubation and at the end of the incubation (after 96 h). All controls samples had >30% viable cells compared with day 1.

Immunophenotyping and Cytogenetic Analysis. Before immunophenotyping, erythrocytes were lysed in NH₄Cl for 8 min. Cells were then incubated with FITC-, phycoerythrin-, or PerCP-conjugated antibodies [Becton Dickinson (San Jose, CA) and Dakopatts (Glostrup, Denmark; RPEcy5 conjugate)] for 15 min using a routine panel of antibodies for fluorescence-activated cellsorting analysis of de novo AML. The samples were analyzed by three-color immunofluorescence in a FACScan flow cytometer (Becton Dickinson). A cutoff limit of 20% was set to define a sample as being negative or positive. In all samples defined as positive, 38–100% of the cells were positive, with the majority of the samples showing >50% positive cells. No negative sample showed more than 14% positive cells.

Cytogenetic analyses were performed at the time of the diagnosis. Either G banding or Q banding was made on the bone marrow material after a 24-h incubation. The chromosomes and the aberrations were classified according to the ISCN 1995.

Real-time PCR. Total RNA was isolated from the cells of AML patients using Trizol (Life Technologies Inc., Gaithersburg, MD) and transcribed into cDNA as described previously (33). The PCR reaction mixture included 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5.0 mM MgCl₂, 0.4 mM each deoxyribonucleotide triphosphate, and 1.25 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Sundbyberg, Sweden). Primers and fluorogenic probes were added to a final concentration of 0.4 and 0.2 μM, respectively (33). Total PCR volume was 25 μl, including 1 μl of the reverse transcription reaction that equals 50 ng of total RNA. Duplicate reaction tubes were set up for each sample and transcript under investigation. The tubes were placed in an ABI Prism 7700 System programmed for 40 sequential cycles, each comprising heating to 94°C for 15 s followed by 60°C for 30 s. A standard curve was generated by amplifying known amounts of a PCR product at the same time as the samples. PCR primers and TaqMan Fluorogenic DNA probes were as follows (sequences are given in 5’-3’ orientation): (a) β-actin, CTGGCTGCTGACCGAGG (forward primer), GAGAATCCACATGATCTGGGT (reverse primer), and CCGGCCTCCTGAGTGCTG (TaqMan probe); (b) RARα, CTCCATGCGCCTCTTCAT (forward primer), CGGCTTCGCCGCTCAAGTG (reverse primer), and CAGGCCTCTGAGTCTCCCTCAATTCCTTCAT (TaqMan probe); (c) RARβ, AGCTCACTGCTGACCGAGG (forward primer), TCTAGGTGGAGGGCAAAATGG (reverse primer), and AGAAGGGAAGCTACCCAACTCTCAATG (TaqMan probe); (d) RARγ, TGTGCGGAATATGGGCAACAC (forward primer), CTGATTCTGACTGCTCAG (reverse primer), and AAAAAATGGGAGCTACCCCACTCTCAATG (TaqMan probe); (e) RXRα, CCCTTGATCGAATCTGCTG (forward primer), AGAAGTGGGATCTCCGCTTG (reverse primer), and AGCAGGCGAACCACAGGCTTTCACCC (TaqMan probe).

Statistics. Mean values with the SE for each retinoid receptor were calculated from the molar ratios of each receptor (receptor mRNA:β-actin mRNA). The differences in the receptor expression between samples with respect to their CD status were evaluated with Student’s unpaired t test. The correlation between ATRA-induced effect on receptor expression and ATRA sensitivity was determined by Fisher’s exact test.
RESULTS

Expression of Retinoid Receptors in Four Myeloid Cell Lines. The expression of retinoid receptors and the housekeeping gene β-actin was investigated in the four myeloid cell lines (HL-60, HL-60R, KG1a, and K562), and the receptor expression is expressed as a molar ratio (receptor:β-actin). Receptor transcription was found in all four cell lines; however, the expression varied significantly (Fig. 1). RARα and RXRα expression was 100-1000-fold higher than that of RARβ and RARγ, with the exception of the K562 cells, in which RARα and RXRα expression was only 10-fold higher. RARα was most abundant in HL-60 cells, and expression of RARα was considerably lower in the multidrug-resistant HL-60R cells. Compared to the other cell lines, RARp and RXRα expression was high in the K562 cells, and RARγ expression was somewhat higher in HL-60R and K562 cells.

Expression of Retinoid Receptors in AML Patient Cells. Expression of all four receptors (RARα, RARβ, RARγ, and RXRα) could be detected in all of the 18 patient samples; however, as in the cell lines, the expression varied considerably (Fig. 2). The pattern of retinoid receptor expression was also similar to that seen in the cell lines, but with less pronounced differences in expression between the four receptors. As in the cell lines, the level of RARα and RXRα was higher than that of RARβ and RARγ. This pattern was seen in 15 of the 18 patients samples, but 3 of the patient samples exhibited another receptor profile with high expression of both RARβ and RARγ in 1 sample and low RXRα expression in 2 samples. RXRα showed the highest level of average expression of all receptors, and RARβ showed the lowest level of average expression of all receptors, somewhat lower than that for RARγ. The expression in cryopreserved cells did not differ from that in fresh cells.

Receptor Expression in Correlation to FAB Classification, CD Expression, and Chromosome Aberrations. Fifteen of the 18 patients were classified according to the FAB system. All subgroups exhibited a similar pattern of expression, except for the patient with M6 who presented with high RARβ and RARγ expression. There was also a higher expression of RARα in samples from patients with AML M4, but this difference was not statistically significant.

The correlation between the expression of CD markers and receptor expression was also analyzed. The expression was compared in cells that had been defined as either positive or negative for CD34, HLA-DR, CD14, CD15, CD13, CD33, and CD56. However, as only one and two of the samples were negative for CD13 and CD33, respectively, these CD markers were excluded from this analysis. For the remaining CD markers, no less than four cases were either negative or positive. The comparison between receptor expression and the expression of HLA-DR, CD15, and CD56 revealed no differences. For CD34-positive cells, the mean expression of RARα, RARγ, and RXRα tended to be lower than that seen in CD34-negative cells, but the difference was close to significance only for RARγ (P = 0.08; Table 1). The only statistically significant difference was the increased RARα expression in the CD14-positive cells (P = 0.03). Mean receptor expression and Ps for each receptor with regard to their CD34 and CD14 status are presented in Table 1.

Table 1 Retinoid receptor expression with respect to the CD3 and CD14 status

<table>
<thead>
<tr>
<th>CD34 status</th>
<th>CD14 status</th>
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<tr>
<td>RARα</td>
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<tr>
<td>RARβ</td>
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<tr>
<td>RARγ</td>
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<tr>
<td>RXRα</td>
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Retinoid Receptor Expression and Retinoid Sensitivity

The change in expression of each retinoid receptor after a 24-h ATRA incubation (decrease, $-; <$-5-fold increase, $+; >$-5-fold increase, $++; >$10-fold increase, $+++; >$15-fold increase, $++++$). The ATRA sensitivity was determined after 96-h incubation in 1.0 µM ATRA as cell viability compared to unexposed controls. ATRA resistance ($-$) was defined as >95% viability, and sensitivity was defined as 75–95% ($+$), 50–74% (+), or <50% (++) viability. $P$s represent the statistical significance for the correlations between ATRA sensitivity and change in receptor expression evaluated with Fisher’s exact test.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>ATRA sensitivity</th>
<th>RAR$\alpha$</th>
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<th>RAR$\gamma$</th>
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<tr>
<td>$P$</td>
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<td>0.014</td>
<td>0.59</td>
<td>0.27</td>
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Cytogenetic analyses could be assessed in 11 of the patients. In only one case did the chromosome aberration interfere with the locus of any of the genes coding for the four retinoid receptors. This case showed a monosomy of chromosomes 3, 9, and 12, corresponding to the gene locations of RAR$\beta$, RXR$\alpha$, and RAR$\gamma$, respectively. Although the basal expression of the three receptors was not decreased in this sample, the ability to up-regulate RAR$\beta$, RXR$\alpha$, and RAR$\gamma$ expression in response to ATRA was impaired compared with the other patient samples. Meanwhile, the ATRA-induced expression of RXRs expression was not impaired compared with the average expression. The changes in receptor expression after ATRA incubation in this single case were (expressed as the difference between basal and ATRA-induced expression) +35% for RAR$\beta$ (average, +330%), $-70\%$ for RAR$\gamma$ (average, +48%), $+150\%$ for RXR$\alpha$ (average, +360%), and $+210\%$ for RXR$\alpha$ (average, +12%).

**Correlation between Expression of Retinoid Receptors and Sensitivity to ATRA.** The average viability of the patient cells after 96 h of incubation in 1 µM ATRA was 78% (range, 10–119%) compared with that of unexposed cells. No correlation between the basal expression of the retinoid receptor and ATRA sensitivity was seen for any of the receptors, and correlation coefficients between ATRA sensitivity and receptor expression expressed as molar ratios were close to 0 for all four receptors. Because any of the investigated retinoid receptors is a potential mediator of the growth-inhibitory effects of ATRA, we also examined whether high expression of any of the receptors in patient cells or a high amount of total receptor mRNA in patient samples could be correlated with retinoid sensitivity. However, neither of these comparisons revealed any correlation. In one patient sample, ATRA increased the growth of leukemic cells, but the pattern of receptor expression was not different from that of the other cells.

In 13 of the patients, receptor expression was also examined after a 24-h exposure to 1 µM ATRA. Table 2 shows the effect of the ATRA incubation on the expression of each receptor in all patient samples. RAR$\beta$ was up-regulated by ATRA in the majority of the patient samples (77%). RAR$\alpha$, RAR$\gamma$, and RXR$\alpha$ were less commonly up-regulated (38%, 31%, and 46%, respectively). Although RAR$\beta$ up-regulation was the most common, the highest increment in expression was seen for RXR$\alpha$, showing a 25- and 15-fold increase in two patient samples.

Correlations were then performed between the growth-inhibitory effects of ATRA and the ATRA-induced change in receptor expression. We studied whether ATRA sensitivity, defined as a decrease in viability to <95% compared with unexposed controls, correlated with the change in receptor expression after ATRA exposure. These comparisons showed that an increase in RAR$\beta$ was significantly correlated with ATRA sensitivity ($P = 0.014$), a relationship that could not be found for any of the other receptors (Table 2). Comparing mean sensitivity to ATRA in samples that did up-regulate receptor expression with those that did not showed a significant difference for RAR$\beta$ [73% versus 103% viability for samples with and without RAR$\beta$ up-regulation ($P = 0.01$)]. All samples that did not up-regulate RAR$\beta$ were also ATRA resistant. Correlations between the retinoid sensitivity and ATRA-induced change in receptor expression were identical when receptor expression was compared with sensitivity to 9-cis-RA (significant correlation to RAR$\beta$ up-regulation but not to any change of RAR$\alpha$, RAR$\gamma$, or RXR$\alpha$; data not shown).

**DISCUSSION**

Since the introduction of ATRA in standard treatment of APL, there has been a constant search for the role of retinoids in other malignant diseases. New and more receptor-specific retinoids are continuously synthesized, and the knowledge about the mechanism of action of retinoids has grown considerably. A problem with retinoid-based therapies has been that the antitumor effects vary significantly between tumor species and between individual patients with the same diagnosis. Treatment with retinoids in non-M3 AML has attracted interest in recent years and new randomized trials have been initiated. A majority of the non-M3 AML blast cells are sensitive to either ATRA or 9-cis-RA *in vitro* but with a considerable proportion of the cells being resistant (20, 21). This stresses the need for more knowledge about the molecular mechanisms of retinoids and the demand for tools to predict retinoid sensitivity. In this study, we examined the expression of retinoid receptors before and after ATRA exposure and whether the expression correlated to retinoid sensitivity in non-M3 AML blast cells. We also correlated the expression to other characteristics of the cells.

Retinoid receptor mRNA for RAR$\alpha$, RAR$\beta$, RAR$\gamma$, and RXR$\alpha$ could be detected in all 18 patient samples and in four myeloid cell lines. In both the cell lines and the patient cells, expression of RAR$\alpha$ and RXR$\alpha$ was high compared to that of RAR$\beta$ and RAR$\gamma$, although the differences in the patient cells were less pronounced. This relationship between the expression of the different receptors is similar to what has been found in other malignant cells (28, 34–36). However, leukemic blast cells from AML patients are heterogeneous, and we did not
examine whether the receptor expression was altered in different leukemic subpopulations. The presence of subpopulations with different receptor expression may also explain why differences in the expression of the receptors (i.e., RARα and RXRα versus RARβ and RARγ) were less pronounced in patient cells compared with the cell lines. The different FAB classes exhibited mainly the same expression pattern, with exception of the single M6 case. However, no conclusion should be drawn from this single case.

In this study, we did not examine the expression at protein level. However, in previous studies on tumor tissues, there has been a good correlation between retinoid receptor mRNA and protein levels (34). This is also expected because the T1/2 for the proteins is short (37).

In contrast to the findings by Fitzgerald et al. (30), we found no correlation between retinoid sensitivity and basal receptor expression for any of the receptors on the mRNA level. This is also in accordance with most previous studies and reinforces the belief that the level of receptor expression itself does not determine the response to retinoids but that the cellular response is instead determined by other coregulating factors on transcriptional level (38–40).

Retinoids bind to nuclear receptors that form homodimers or heterodimers and then, together with coregulating factors, act on RA response elements and regulate the transcription of a variety of genes (11). One of the target genes is RARβ, which can be activated by the binding of a receptor complex to its promoter region (41, 42). A key role has been attributed to RARβ because it may act as a tumor suppressor and because the growth-inhibitory effects of retinoids correlate to the ability to up-regulate RARβ in response to ATRA exposure in some tumor species (1, 26–28, 43). Based on these previous findings, we also analyzed receptor expression in cells exposed to 1 μM ATRA for 24 h. In the patient cells, changes in receptor expression in response to ATRA were most prominent for RARβ and RXRα. For RARβ, a high proportion of the samples responded with up-regulation, whereas for RXRα, the amplitude of the up-regulation was considerable for some patient samples. Changes for RARα and RARγ were less prominent, and increased as well as decreased expression was seen. These results correspond to findings in renal cancer cell lines, melanoma cells, and myeloid progenitor cells (14, 27, 44).

By correlating the ATRA-induced change in receptor expression with ATRA sensitivity, we found a significant correlation between the sensitivity to ATRA and up-regulation of RARβ. This correlation was not seen for any of the other receptors. The finding is consistent with previous observations in other tumor species but has not been shown for AML. This clearly suggests a link between the growth-inhibitory effects of ATRA and RARβ up-regulation in non-M3 AML, and it could offer a possibility to predict the response to retinoids when RARβ expression is analyzed in vitro during ATRA treatment. This is currently being investigated in a clinical trial.

Retinoids usually induce growth inhibition and cause decreased cell survival but may also promote cell growth (20, 45, 46), and the predominant effect in hematological progenitor cells is dependent on the maturity of the cell (46, 47). It can be hypothesized that such diverse effects could be caused by changes in the expression of retinoid receptors during maturation and differentiation. There has also been one report about increased expression of RXRα on monocytoid differentiation (48). To study the relationship between differentiation of the leukemic blast cells and expression of retinoid receptors, we compared the receptor expression to the expression of some hematological differentiation markers. Although the number of samples was limited, we found an increase in RARα expression in samples expressing the monocytoid differentiation marker CD14. CD34-positive cells tended to have a lower expression of three of the receptors, but the difference was only close to significance for RARγ. For the other CD markers, either there were too few negative samples to be analyzed (CD13 and CD33) or the comparison failed to show any differences (HLA-DR, CD15, and CD56). The increased RARα expression in CD14-positive cells may also correspond to the tendency toward increased RARα expression that was found in AML samples with monocytoid differentiation (M4). However, because leukemic cells often express aberrant phenotypes, it is difficult to draw any conclusion regarding retinoid receptor expression during normal hematopoietic maturation and differentiation. We are currently investigating receptor expression in different subsets of normal hematopoietic cells.

Loss of heterozygosity at chromosome 3p24 has been found in lung cancer cells, and this has provoked hypotheses regarding the role of RARβ in the carcinogenesis of that disease (34). Only one patient sample carried chromosome aberrations that interfered with the locus of any of the retinoid receptor genes, and this patient presented with monosomy of three chromosomes (chromosomes 3, 9, and 12), representing the locus for the genes coding for RARβ, RARγ, and RXRα. Although the basal expression of the affected receptors was intact in these cells, the ability to up-regulate receptor expression in response to ATRA was impaired. However, because this represents the result in only one patient sample, it is difficult to draw further conclusions.

In summary, this is the first time, to our knowledge, that retinoid receptor expression has been analyzed with a real-time PCR technique in myeloid non-M3 leukemic cells. The results show that RARα, RARβ, RARγ, and RXRα are expressed to variable degree in these cells and that ATRA sensitivity correlates with ATRA-induced up-regulation of RARβ expression but not with the basal expression of any of the receptors. Retinoid protocols for non-M3 AML patients are currently under investigation, and the ability to up-regulate RARβ may be useful as a predictive test during treatment with retinoids.

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