All-Trans Retinoic Acid in Acute Promyelocytic Leukemias. II. In Vitro Studies: Structure-Function Relationship

By Christine Chomienne, Paola Ballerini, Nicole Balitrand, Marie-Thérèse Daniel, Pierre Fenaux, Sylvie Castaigne, and Laurent Degos

All-trans retinoic acid induces leukemic cells from patients with acute promyelocytic leukemia (M3) to differentiate in vitro to mature granulocytes which express the CD15 antigen and are capable of respiratory burst function. Of 35 M3 samples, only one failed to respond. In eight cases, we compared the efficacy of two naturally occurring isomers of retinoic acid, all-trans RA and 13-cis RA. Both isomers induce maximal differentiation at 10^{-6} mol/L. The maximal response was maintained at 10^{-7} mol/L for the all-trans but not for the 13-cis RA. We also observed that the metabolites 4-oxo-all-trans and 4-oxo-13-cis were effective at 10^{-6} mol/L. This order of magnitude difference in the in vitro differentiating potencies of all-trans RA and 13-cis RA in the blasts of promyelocytic leukemias predicts a difference in the clinical efficacy of the two drugs.

A Disequilibrium between proliferation and differentiation characterizes myeloid leukemic cells which appear blocked at a certain stage of their differentiation. This differentiation blockage is however sometimes reversible; leukemic cells in agar or in liquid suspension culture show spontaneous differentiation; they differentiate when incubated with various agents, and polymorphonuclear cells of some leukemic patients in complete remission have been shown by restriction fragment length polymorphism (RFLP) to arise from the leukemic clone. The study of leukemic cell differentiation has been greatly facilitated by establishment of leukemic cell lines that permits confirmation that the differentiated cells obtained originate from a leukemic progenitor.

Retinoic acid (RA) is one of the most potent and extensively studied differentiating agents. It inhibits proliferation and induces differentiation of malignant cells in vitro. Various natural and synthetic retinoids have been studied for their effects on hematopoietic cells, namely on the human myeloid leukemia HL-60 leukemia cell line. All-trans RA and 13-cis RA, two isomers of RA, equally stimulate the growth of normal CFU-GM cells. They have both been shown to inhibit the clonal cell growth of KG1 cells, and they both induce differentiation of U-937 cells with an EC_{50} of 10^{-7} mol/L. The proliferation of cells from patients with acute myeloid leukemia (AML) is either stimulated or inhibited in liquid or methylcellulose cultures. Leukemic cells from patients with acute promyelocytic leukemia (M3) differentiate in vitro in the presence of retinoids.

Because of their higher therapeutic indexes, the 13-cis-isomer and the ethyl ester of Vitamin A have been most studied in clinical trials. The ethyl ester, which we found ineffective in vitro in the U-937 cell line, was also reported to be ineffective in vivo. Several reports have been published concerning the effect of 13-cis-RA in hematopoietic disorders characterized by bone marrow (BM) maturation abnormalities (myelodysplastic syndromes, MDS) or bone marrow maturation arrest (AML). Until recently, the compound used for RA treatment of acute leukemias was the 13-cis isomer. In M3 patients refractory to conventional chemotherapy, at a daily dose of 100 mg/m^2, it was beneficial in isolated cases. The original experience of Huang et al with all-trans RA in 24 M3 patients treated with a daily dose of 45 to 100 mg/m^2 per day alone or in association with cytotoxic drug is even more striking: 23 patients obtained complete remission, and intravascular disseminated coagulation either disappeared rapidly or was not observed after onset of treatment. Our own experience with 22 M3 patients treated with all-trans RA alone corroborates the data of Huang et al.

We studied the in vitro effects of 13-cis RA and all-trans RA on leukemic cells from patients with M3. The effects of the two isomers on M3 cell differentiation are quantitatively different and suggest that in vitro data may correlate to in vivo efficacy.

**MATERIALS AND METHODS**

**Cell Samples**

BM and/or peripheral blood (PB) samples from patients with acute nonlymphocytic leukemia (ANLL) were collected in heparinized tubes. Diagnosis and French-American-British (FAB) classification were performed by Dr M.T. Daniel (Laboratoire Central d'Hématologie, Hôpital Saint Louis). From 1986 to 1988, 39 samples of ANLL were studied: 13 M1, 8 M2, 10 M3, 3 M4, and 5 M5 cases. Since 1989, 25 other patients with M3 were evaluated. Of these, 22 patients entered a clinical protocol for all-trans RA and were addressed by the different participating hematologic centers. Details of the clinical trial will be published elsewhere. All patients involved in this study were advised of the methods and possible risks of BM aspiration in accordance with institutional guidelines and gave informed consent.

After Ficoll-Hypaque (Eurobio, France) density gradient, T cells were removed by E rosetting with sheep erythrocytes and second Ficoll-Hypaque separation. Monocytes were eliminated after adherence by incubating the cell suspension in RPMI 1640 and 15% fetal calf serum (FCS) for 60 minutes at 37°C in a humidified CO_2 atmosphere in large Falcon plastic flasks. Nonadherent cells were

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then removed by gentle pipetting and resuspended in culture medium; 95% of the samples had a leukemic cell fraction with more than 90% blasts.

**Materials**

All-trans RA, 13-cis RA, and 4-oxo-all-trans RA, and 4-oxo-13-cis RA were provided by Dr. Bollag, Hoffman-LaRoche (Basel, Switzerland). They were suspended in absolute ethanol at an initial stock concentration of 10⁻² mol/L and further diluted in RPMI 1640 medium. The final concentrations of ethanol in culture medium had no effect on cell growth or differentiation. All dilutions and cultures were performed in subdued light, and stock solutions were stored at −70°C in foil-wrapped paper.

**Cell Culture and Differentiation Assay**

**Cell culture.** Leukemic cells were suspended in culture medium (RPMI 1640, 15% heat-inactivated FCS and antibiotics) at a concentration of 10⁶ cells/mL as recommended. All cultures were incubated at 37°C in a 5% CO₂ humidified atmosphere for a minimum of 5 days in the presence of the different retinoids. Cell aliquots were taken at days 3 and 5 or later for assessment of viability and differentiation status. For each cell sample, a control was cultured in the same conditions in the absence of retinoids.

**Survival and proliferation.** Survival and proliferation were assessed first by the trypan blue exclusion dye test: cell counts were assessed first by the trypan blue exclusion dye test: cell counts were confirmed after an exclusion test with acetic blue.

**Differentiation.** Differentiation was assessed on morphologic and functional criteria. Cytospin slide preparations were made from samples with or without RA. Slides were stained with May-Grumwald-Giemsa stain. Cells were categorized by standard morphologic criteria either as immature (blasts, promyelocytes, and promonocytes) or as mature (myelocyte stage and beyond). In most cases, α-naphthyl acetate esterase-stained preparations were examined in parallel. The nitroblue tetrazolium (NBT) reduction test was performed using standard methodology with phorbol myristate acetate (PMA) as previously described. Differential counts were performed under light microscopy on a minimum of 200 cells. The percentage of cells containing intracellular reduced black formazan deposits was thus determined. Modulation of the expression of specific differentiation cell surface antigens was studied by monoclonal antibody (MoAb) binding with CD33 (MY9) and CD34 (MY10) clusters from Coultronics, CD15 (lg-10) and CD14 (Leu M3) clusters from Becton Dickinson (Oxford, UK), and MO75 polymorphonuclear antibody from Biomakor by indirect immunofluorescence. Cells (10⁶) were incubated with the quantity of MoAb specified by the producer for 30 minutes at 4°C. After three washes, the cells were incubated 30 minutes more at 4°C in the dark in the presence of a second MoAb consisting of FITC-conjugated F(ab')₂ goat anti-mouse antiserum. The cells were washed three times. An aliquot was taken for preparation of slides and analysed on a fluorescence microscope (Zeiss, Oberkochen, FRG). The rest of the cells were resuspended in phosphate-buffered saline (PBS) with 1% paraformaldehyde and stored at 4°C in the dark until analysis on a cytofluorograph (Ortho 50H, Roissy, France, by R. Miglierina, Service Commun, Institut Hematologie). A logarithmic amplification allowed us to obtain reproducible percentages of fluorescein green fluorescence.

**Statistical Analysis**

Different measures of cell survival and differentiation status were analyzed: The effect of RA on cell concentration and cell viability was studied by comparing the cell concentration in the presence of RA at different times of incubation first to the initial cell concentration (R1 ratio) and second to the cell concentration in the control samples (R2 ratio). R1: viable cell concentration at day 3 or 5/viable cell concentration at day 0; R1-control day 3 or R1-control day 5 for the control samples and R1-RA day 3 or R1-RA day 5 for RA-treated samples. R2: viable cell concentration in RA-treated samples/viable cell concentration in the control (R2-day 3 at day 3 and R2-day 5 at day 5 of incubation).

We considered a differentiated leukemic cell as a cell that had reached a stage of granulocytic maturation corresponding, cytologically, to a metamyelocyte or band cell and possessing an intact respiratory burst with production of hydrogen peroxide (H₂O₂) and superoxide (O₂⁻). This late stage of differentiation of granulocytic differentiation can be visualized by the reduction of NBT. Differentiation status was represented by (a) the percentage of differentiated cells (NBT positive) or the absolute number of differentiated cells (percentage of NBT-positive cells times number of viable cells per milliliter) and (b) the difference of the percentages or absolute numbers of cells in inducer and control flask at day 3 or 5.

**RESULTS**

**Effect of All-Trans RA on AML3 Leukemic Cells**

The effects of continuous exposure of all-trans RA 10⁻⁶ mol/L on acute promyelocytic leukemic cells in suspension culture were studied in 35 samples of M3 patients. We wished to determine whether all-trans RA could stimulate terminal differentiation on fresh human M3 cells as in the human leukemic cell lines HL-60 and U-937. Of these 35 M3 samples, all cases but one responded (Fig 1). An
extremely low percentage of cells spontaneously acquired the
capacity to reduce NBT [1.3% ± 2.7% (median 0%) at day 0;
3.5% ± 5.6% (median 2%) at day 3; and 5% ± 6.3% (median
3%) at day 5]. At day 3, in the presence of all-trans RA, more
than 50% of the cells had the capacity to reduce NBT
(64% ± 24%, median 65%); this proportion reached 90% to
100% by day 5 (82.6% ± 18.9%, median 90%). These results
remained clearly significant when expressed as the total
number of NBT-positive cells per milliliter. In three samples,
a differentiation of the whole cell population was obtained
only after 9 or 11 days of incubation.
Morphologically, the modifications of the promyelocytic
leukemic cells during the differentiation induced by all-trans
RA are variable from one sample to another, as the promye-
locytic leukemic cells often are before treatment. After 5-day
incubation with RA, however, all leukemic cells have a

\[
\text{Table 1. Surface Antigen Analysis of M3 Cells Incubated With All-Trans RA} \ 10^{-6} \text{ mol/L for Five Days}
\]

<table>
<thead>
<tr>
<th>Patient</th>
<th>Source of Cells</th>
<th>MoAbs</th>
<th>1G10</th>
<th>Leu M3</th>
<th>M075</th>
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<tbody>
<tr>
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<td>7</td>
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<tr>
<td></td>
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<td>74*</td>
<td>29</td>
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<tr>
<td></td>
<td>-RA</td>
<td></td>
<td>38</td>
<td>23</td>
<td>30</td>
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</tr>
<tr>
<td>J</td>
<td>Do</td>
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<td>6</td>
<td>4</td>
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<tr>
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<td>+RA</td>
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<td></td>
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<td>F</td>
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DO, cells at day 0; +RA, cells incubated with all-trans RA; -RA, cells incubated without RA; ND, not done. Percentage of positive cells by indirect immunofluorescence evaluated on 10,000 cells analyzed on an Ortho cytofluorograph.

*Significant increases in antigen positivity for cells incubated with RA.
smaller cell volume, and a low nucleus/cytoplasm ratio and show alterations of the nucleus and cytoplasmic granulations. The cells are granulocytic at the stage of metamyelocytes and band cells. Segmented polymorphonuclear cells are rarely observed. The existence of a true asynchrony of maturation between nucleus and cytoplasm as the persistence of Auer rods confirms that the differentiated cell originates from the M3 cells. (Fig 2A and B). There was a substantial increase in the expression of the CD15 granulocytic differentiation antigen, recognized by the Ig10 MoAb (Table 1). As compared with the control samples, RA-treated cells showed a decrease or absence of self-renewal and reduction of \(^{3}\text{H}-\text{Td}\) incorporation in six samples tested (78% + 17%) (Fig 3).

Throughout the in vitro culture period, no modification of cell concentration occurred (R1-control day 3, 0.84 ± 0.3; R1-RA day 3, 0.98 ± 0.3, R1-control day 5, 0.92 ± 0.5, and R1-RA day 5, 1.0 ± 0.7). A trend in the increase of cell concentration in the RA-treated samples was noted, however. As compared with the initial cell concentration (R1-RA), 10 cases had a ratio greater than 1.1 (Table 2). No decrease in the percentage of differentiated cells was observed. Six of these cases had an increased ratio of the control (R1-C), which could reflect variation in the initial cell concentration at day 0. That the number of cells in the presence of RA was correlated to the control cell concentration of the same day \((r = 0.82, P < .001\) at day 3; and \(r = 0.80, P < .001\) at day 5) and remained superior to the

<table>
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<th>Cases with at least one index ratio &gt; 1.1 (*)</th>
<th>R1-C Day 5</th>
<th>R1-RA Day 5</th>
<th>R2-RA Day 5</th>
<th>Differentiated Cells Day 5 (%)</th>
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<td>32</td>
<td>0.92</td>
<td>1.24*</td>
<td>1.34*</td>
<td>85</td>
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</table>

Table 2. Index Ratio of Cell Concentration and Percentage of Differentiated Cells in M3 Samples After Five-Day Incubation With All-Trans RA 10^-8 mol/L

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control (R2-RA ratio >1.1) in 15 cases, however, may suggest that RA plays a part in the proliferation and survival of some M3 leukemic clones.

**Structure-Dose Effect of Retinoids in AML3 Leukemic Cells**

The effect of different concentrations of the two isomers, all-trans RA and 13-cis RA was tested in eight different M3 cases. The possibility of inducing terminal differentiation was dose dependent between $10^{-6}$ and $10^{-8}$ mol/L. At $10^{-6}$ mol/L, both isomers were equally effective in inducing NBT reduction. At $10^{-7}$ mol/L, however, a maximal effect was already achieved in all cases with all-trans RA and in only three cases with 13-cis RA ($P < .001$; sign test). At $10^{-8}$ mol/L, the effect of 13-cis RA was not detectable, whereas in certain cases a differentiation was already apparent with all-trans RA (Fig 4A). The number of viable cells after 3- and 5-day incubation with either isomer was variable and not significantly different whatever the concentration and differentiation effect obtained. There was no difference in cell viability between the two isomers at the concentrations used (Fig 4B).

**Effect of All Trans-RA on Acute Myeloid Leukemic Cells in Short-Term Culture**

To determine if the biologic effects of all-trans RA described above were specific to M3 leukemic cells, we studied 36 samples of randomly selected AML patients.
Fourteen cases acquired cytologic and functional features of terminally differentiated granulocytic cells: two of the 13 M1 cases, two of the 8 M2 cases, and 10 of the 10 M3 cases (Fig 5). The magnitude of the response in the M3 subtype was significantly higher than in the other subtypes, even when the nonresponders were excluded (P < .001, Mann-Whitney U test). In the absence or presence of RA, only seven cases (18%) showed a cell concentration (R1-C or R1-RA) ratio >1.1. Thirteen cases had a cell concentration in RA samples superior to the control of the same day (R2-day 5 >1.1): 6 of the 13 M1, 1 of the 8 M2, 3 of the 10 M3, 1 of the 3 M4, and 2 of the M5. Determination of the inhibition of ³H-TD incorporation after 1-hour incubation with Ara-C, allowed estimation of the proportion of multiplying cells. Of the different ANLL cell types, only the samples of the M3 group who had a R2-day 5 ratio >1.1 were highly correlated to the inhibition of ³H-TD incorporation with Ara-C P < .01 (all samples showing >70% inhibition). There was no correlation between all-trans RA effect on proliferation and differentiation on these different myeloid leukemic clones.

**DISCUSSION**

We compared the effects of two isomers of RA, all-trans and 13-cis, on the blast cells of patients with acute promyelocytic leukemia. First, this study confirms that the most studied isomer, all-trans RA, at a concentration of 10⁻⁴ mol/L induces a specific and consistent differentiating effect in M3 cells. In 36 randomly studied cases of ANLL, all 10 M3 samples responded to all-trans RA and only four of other AML subtypes (two M1 and two M2). On 35 other M3 sequential samples, all cases but one responded (a case having cytogenetic characteristics of M3 cells). These cells rapidly acquire the function of respiratory burst and cytologically reach the stage of metamyelocytes and band cells. Differentiation surface antigens specific of the polymorphonuclear stage were not expressed after 5 days in culture with RA, however, which could mean either that under such a short incubation time synthesis of certain differentiation antigens was not achieved or that this anomaly was characteristic of the differentiated leukemic cell. Only the CD 15 antigen was significantly increased, as already reported by Findley et al in three cases of childhood M3. In this model of suspension culture, there was an increase in cell number at day 5 in the presence of RA in half of the samples. In these cases, the differentiation response did not differ from the other cases. Because cell number in the presence of RA correlated to the cell number of the control, these data suggest that RA may play a role in the proliferation and survival of certain leukemic clones in vitro. Of the four patients who had a hyperleukocytosis with all-trans RA therapy, three are in the group of patients with at least one index cell concentration ratio greater than 1.1. These results are too preliminary, however, and the number of cases too small to allow a correlation between in vitro and in vivo proliferation. No leukemic cell culture on its own is a complete model in which to study in vitro leukemic cell proliferation. Studies on the effect of either isomer on clonogenicity and self-renewal of leukemic cells have been reported and show either inhibitory or stimulatory effects in ANLL samples. Our own data on M3 cases not included in this study are more in favor of an inhibitory effect on cell clonogenicity in the presence of RA.

This study also shows that the other isomer of RA, 13-cis RA, has an identical effect on M3 differentiation at 10⁻⁴ mol/L. The structure-dose studies performed in eight of the M3 cases of this study demonstrate, however, that a response of identical magnitude is obtained at 10⁻³ mol/L with all-trans RA, as reported by Breitman et al in three M3 patients, but the efficacy significantly decreases with 13-cis RA. Both drugs are ineffective at 10⁻⁴ mol/L. At 10⁻⁵ mol/L, differentiation is still observed. Cell viability was similar after 5-day incubation with both isomers, and cell concentration was not significantly different for concentrations greater than 10⁻⁵ mol/L. The difference in activity therefore was not related to a difference in cell toxicity. The two metabolites, 4-oxo-13-cis and 4-oxo-all-trans induced cell differentiation in M3 cells and had a structure-dose effect on M3 cells similar to that of the compound from which they originate.

This structure activity of the two isomers we observed in vitro was also confirmed by in vivo data. The efficacy of treatment of M3 cases with RA appears to be related to the dose and the compound given: Sampi et al reported no effect of the ethyl ester. We noted no effect of 13-cis at a dose of 45 mg/m². At 100 mg/m², 13-cis was observed and complete remission was documented. That a relapse of the disease was observed when the dose of 13-cis RA was
decreased was also in favor of a dose dependent effect of RA’s efficacy. At a dose of 45 mg/m² to 100 mg/m², Huang et al obtained CR with all-trans RA in 23 of 24 M3 patients and in our own experience 14 of 22 patients obtained CR. Clinical experience in MDS suggests that the response to 13-cis RA requires also a minimal dose. In M3 patients treated with all-trans RA (unpublished observations) the in vitro studies allowed us to suggest that the nonresponders (the only case that did not respond in vivo failed to differentiate in vitro) and the rare cases of poor prognosis attributed to partial response, early relapses, and increase of WBC counts are in the group with an increased cell concentration ratio. Thus, in this model differentiation therapy, in vitro and in vivo effects of the two isomers of RA appear to be correlated.

The mechanism(s) through which RA induces leukemic cell differentiation has not been elucidated although many effects subsequent to RA action have been observed at different levels of the cell. The structure-dose activity observed in leukemic cells with different retinoids suggest that their biologic actions are mediated by binding to specific receptors. To date, three RARs have been cloned and identified. The localization of the RA receptor α-gene close to the (15;17) translocation breakpoint specific to M3 cells has stimulated interest in its study. The published reports, however, show that the biologic effect of all-trans RA in M3 cells is related neither to the level of expression of RAR-α nor to its modulation by RA. Our results regarding the in vitro and in vivo structure activity of retinoids in M3 cells suggest that a receptor/binding protein mediates all-trans RA effect in M3 cells. Characteristics and function of these receptors and/or binding proteins are being studied in the laboratory.

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IN VITRO STUDIES OF RETINOIDS IN M3 LEUKEMIA


All-trans retinoic acid in acute promyelocytic leukemias. II. In vitro studies: structure-function relationship

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