Granulocytic Differentiation of Human NB4 Promyelocytic Leukemia Cells Induced by All-trans Retinoic Acid Metabolites

Nadia Idres, Gérard Benoît, Maria A. Flexor, Michel Lanotte, and Guy G. Chabot

Institut National de la Santé et de la Recherche Médicale (INSERM U496), Institut Universitaire d'HématoLOGie (Université Paris 7), Hôpital Saint-Louis (AP-HP), 75475 Paris (Cedex 10), France

ABSTRACT

The metabolism of all-trans retinoic acid (ATRA) has been reported to be partly responsible for the in vivo resistance to ATRA seen in the treatment of human acute promyelocytic leukemia (APL). However, ATRA metabolism appears to be involved in the growth inhibition of several cancer cell lines in vitro. The purpose of this study was to evaluate the in vitro activity of the principal metabolites of ATRA [4-hydroxysterinoic acid (4-OH-RA), 18-hydroxy-retinoic acid (18-OH-RA), 4-oxo-retinoic acid (4-oxo-RA), and 5,6-epoxy-retinoic acid (5,6-epoxy-RA)] in NB4, a human promyelocytic leukemia cell line that exhibits the APL diagnostic t(15;17) chromosomal translocation and expresses the PML-RARα fusion protein. We established that the four ATRA metabolites were indeed formed by the NB4 cells in vitro. NB4 cell growth was inhibited (69–78% at 120 h) and cell cycle progression in the G2 phase (82–85% at 120 h) was blocked by ATRA and all of the metabolites at 1 μM concentration. ATRA and its metabolites could induce NB4 cells differentiation with similar activity, as evaluated by cell morphology, by the nitroblue tetrazolium reduction test (82–88% at 120 h) or by the expression of the maturation specific cell surface marker CD11c. In addition, nuclear body reorganization to macropunctated structures, as well as the degradation of PML-RARα, was found to be similar for ATRA and all of its metabolites. Comparison of the relative potency of the retinoids using the nitroblue tetrazolium reduction test showed effective concentrations required to differentiate 50% of cells in 72 h as follows: ATRA, 15.8 ± 1.7 nM; 4-oxo-RA, 38.3 ± 1.3 nM; 18-OH-RA, 55.5 ± 1.8 nM; 4-OH-RA, 79.8 ± 1.8 nM; and 5,6-epoxy-RA, 99.5 ± 1.5 nM. The ATRA metabolites were found to exert their differentiation effects via the RARα nuclear receptors, because the RARα-specific antagonist BMS614 blocked metabolite-induced CD11c expression in NB4 cells. These data demonstrate that the principal ATRA Phase 1 metabolites can elicit leukemia cell growth inhibition and differentiation in vitro through the RARα signaling pathway, and they suggest that these metabolites may play a role in ATRA antileukemic activity in vivo.

INTRODUCTION

Retinoids (vitamin A or retinol, and its derivatives) are a family of molecules involved in many physiological processes such as embryogenesis, cell growth, vertebrate development, differentiation, and homeostasis (reviewed in Ref. 1). The retinoids effects are known to be mediated by two classes of nuclear receptors, the RARs and the RXRs, each comprising three subtypes (α, β, γ), with various isoforms of each subtype (2). These receptors belong to the superfamily of nuclear hormone receptors and act as ligand-activated transcription factors of numerous genes (reviewed in Refs. 2 and 3). Retinoid receptors interact as either homo- or heterodimers on specific hormone response elements (RA responsive elements or RX responsive elements) of target gene promoters (4–6). The transcriptional activity of these receptors is further positively (holo-receptor) or negatively (apo-receptor) modulated by ligand binding (reviewed in Refs. 7 and 8). In addition, intracellular CRABPs play a regulatory function in RA signaling (9). Biological responses to ATRA may, therefore, be modulated by the type of nuclear receptors present in cells, by nuclear cofactors, by the CRABPs, and by the availability of specific retinoid ligands (reviewed in Ref. 10).

The differentiating properties of ATRA have led to the development of therapies for the prevention and treatment of various human cancers (11) including APL, in which retinoids are particularly active (12). APL is a subtype of myeloid leukemia (M3), characterized by the accumulation of cells blocked at the promyelocytic stage. This leukemia exhibits a specific chromosomal translocation t(15;17) involving the PML locus on chromosome 15 and the RARα locus on chromosome 17, thus generating a chimeric gene PML-RARα, translated into a chimeric nuclear receptor PML-RARα (13–17). PML-RARα functions as an aberrant receptor and is considered to be the cause of APL (18).

Although several clinical studies have established that ATRA can induce leukemia cell differentiation and remission in almost all patients (12, 19), resistance to this therapy develops rapidly (20). This acquired resistance appears to be attributable in part to the decline in ATRA plasma levels below therapeutic concentrations after repeated administration, presumably caused by the induction of hepatic cytochrome P450s that may increase its clearance (21–23). It has also been proposed that increased CRABPs contribute to resistance by decreasing nuclear availability of ATRA (24). More recently, mutations in the E-ligand-binding region of the RARα chimeric protein PML-RARα have been linked to ATRA resistance (25–29).

ATRA metabolism is complex and only partially understood at the present time (reviewed in Ref. 30). It can be oxidized by cytochrome P450s to various metabolites including 4-OH-RA, 4-oxo-RA, 18-OH-RA, and 5,6-epoxy-RA, which may further metabolized to water-soluble glucuronides. Although ATRA metabolites are often considered to be catabolic products possessing low biological activity, some metabolites have been reported to be active in embryogenesis and to inhibit the growth of certain cancer cell lines (31–36). However, metabolism also seems to be involved in the response of certain cancer cells to ATRA, as shown by a study of breast cancer cell lines in which cell lines sensitive to ATRA growth inhibition (T47D and MCF7) could metabolize ATRA to polar metabolites (e.g., 4-oxo-RA and 4-OH-RA), whereas resistant cell lines (MDA-MB231 and MDA-MB418) metabolized ATRA poorly (35). Similar results involving ATRA metabolism to polar compounds by other sensitive cancer cells have also been reported (37).

The abbreviations used are: RAR, retinoic acid receptor; RA, retinoic acid; APL, acute promyelocytic leukemia; ATRA, all-trans RA; 4-OH-RA, 4-hydroxy-RA; 18-OH-RA, 18-hydroxy-RA; RXR, retinoids X receptor; CRABP, cellular RA binding protein; HPLC, high-performance liquid chromatography; NBT, nitroblue tetrazolium; NB, nuclear body; PMA, phorbol 12-myristate 13-acetate; PE, phycoerythrin.

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2 To whom requests for reprints should be addressed, at Institut Universitaire d’Hématologie (Université Paris 7), Hôpital Saint-Louis (AP-HP), 75475 Paris (Cedex 10), France. Phone: 33-1-53-72-21-30; Fax: 33-1-42-40-95-57; E-mail: gchabot@chu-stlouis.fr.

3 The abbreviations used are: RAR, retinoic acid receptor; RA, retinoic acid; APL, acute promyelocytic leukemia; ATRA, all-trans RA; 4-OH-RA, 4-hydroxy-RA; 18-OH-RA, 18-hydroxy-RA; RXR, retinoids X receptor; CRABP, cellular RA binding protein; HPLC, high-performance liquid chromatography; NBT, nitroblue tetrazolium; NB, nuclear body; PMA, phorbol 12-myristate 13-acetate; PE, phycoerythrin.
As the metabolism of ATRA appears to be involved in its mechanism of action in certain cancer cell lines, the purpose of this study was to evaluate the in vitro effects of the principal oxidized ATRA metabolites using the human NB4 promyelocytic leukemia cell line that expresses the characteristic APL t(15;17) chromosomal translocation (38). Our results show that NB4 cells can metabolize ATRA to polar metabolites (4-OH-RA, 4-oxo-RA, 18-OH-RA, and 5,6-epoxy-RA), and that these metabolites are active retinoids in vitro with regard to cell growth inhibition, cell cycle arrest in G1 phase, cell differentiation, NB reorganization, and degradation of the chimeric protein PML-RARα. In addition, when using a specific RARα antagonist, ATRA metabolites are shown to act via the RARα nuclear receptor signaling pathway, as does the parent compound.

MATERIALS AND METHODS

Chemicals. ATRA, its isomers 13-cis-RA and 9-cis-RA, NBT, PMA, and propidium iodide were purchased from Sigma-Aldrich (St-Quentin-Fallavier, France). 4-OH-RA, 4-oxo-RA, 18-OH-RA, and 5,6-epoxy-RA were kindly donated by Dr. Michael Klaus (Hoffmann-La Roche Ltd., Basel, Switzerland) and Dr. Louise H. Foley (Hoffmann-La Roche Inc., Nutley, NJ). The RARα antagonist BMS614 was synthesized by Bristol-Myers Squibb and kindly donated by Dr. H. Gronemeyer (Institut de Génétique et de Biologie Moleculaire et Cellulaire, Illkirch, France). Solution of retinoids were prepared in ethanol at 10⁻² M and stored protected from light at −20°C.

Cell Culture. The NB4 human APL cell line, developed in our laboratory (38), was maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FCS (Bayer Diagnostics, Puteaux, France), 2 mM l-glutamine, 50 units/ml penicillin G, and 50 µg/ml streptomycin (Life Technologies, Inc.). The cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂. Cell density was assessed with an electronic particle counter and size analyzer ( Coulter Electronics, Hialeah, FL). Solution of retinoids at 10⁻² M in ethanol) were further diluted in culture medium to the final concentration indicated in each experiment. Final concentrations in ethanol in culture medium did not exceed 0.01%. Stock solutions of retinoids were regularly checked for purity using reversed-phase HPLC analysis and did not show any contamination by ATRA. Cell cultures were diluted with fresh medium every 48 h, and drug concentrations were adjusted accordingly. All of the experimental procedures were light protected.

ATRA Metabolism in NB4 Cells and HPLC. Cells (10⁶) were incubated at 37°C in 2 ml of the above medium with 1 µM ATRA for 24 h. After acidification (HCl, 1%), cells and medium were extracted with ethyl acetate (39), the organic phase recuperated by centrifugation, and evaporated under a gentle nitrogen stream. The dry residue was reconstituted in 100 µl of methanol, and 50 µl was injected onto a HPLC system composed of a Varian 5000 (Amersham; Ref. 42).

RESULTS

ATRA Metabolism in NB4 Cells. We first evaluated the ability of human NB4 promyelocytic leukemia cells to metabolize ATRA. Fig. 1 shows the ATRA metabolites formed after 24 h in the presence of 1 µM ATRA. The metabolites formed were 4-oxo-RA, 4-OH-RA, 18-OH-RA, and 5,6-epoxy-RA, which presented concentrations of 0.06, 0.03, 0.08, and 0.39 µM, respectively. The stereoisomers 13-cis-RA and 9-cis-RA presented concentrations of 0.07 and 0.16 µM, respectively, and ATRA of 0.19 µM.

NB4 Cell Growth Inhibition and Cell Cycle Arrest by ATRA Metabolites. NB4 cell growth in the presence of exogenously added ATRA metabolites was assessed at a concentration of 1 µM for 120 h (Fig. 2A). Compared with untreated cells, the growth inhibition was found to be similar for ATRA and the 4 metabolites tested, yielding 50% growth inhibition between 72 and 96 h. At 120 h, cell growth was still similar for all compounds, and ranged from 22 to 31% of
untreated cells. Cell growth declined to 11–13% of controls for ATRA and its metabolites at 168 h (data not shown). In parallel experiments, the influence of ATRA or its metabolites on the cell cycle distribution was assessed for 5 days (Fig. 2). A time-dependent cell cycle accumulation in the G1 phase was observed for all compounds tested, yielding 82–84% G1 accumulation at day 5, at the expense of the S and G2-M phases. These data are in agreement with the observed growth arrest.

**NB4 Cell Differentiation by ATRA Metabolites.** Because the ATRA metabolites appeared to be as effective as the parent compound with regard to cell growth inhibition and cell cycle arrest, we assessed the differentiating potential of these retinoids in NB4 cells using cell morphology, NBT reduction, and CD11c expression. Morphological differentiation using May-Grünewald Giemsa staining of NB4 cells exposed to either ATRA or its metabolites was carried out every day for 5 days. The first signs of morphological differentiation were observed at 72 h for all compounds, and the differentiation was more pronounced for 18-OH-RA and 5,6-epoxy-RA than for ATRA, 4-oxo-RA or 4-OH-RA (data not shown). At 96 h, the granulocytic maturation pattern was similar for all retinoids, with polyplobular nuclei, decreased nuclear:cytoplasmic ratio, and decreased cytoplasm staining (Fig. 3A). ATRA and its metabolites induced similar kinetics of differentiation with comparable efficacy at 1 μM reaching 82–88%
Because differentiation of NB4 cells by ATRA is mediated through the nuclear receptor pathways involved in this response, it was of interest to investigate the nuclear receptor pathways involved in this response. Because the ATRA metabolites could induce differentiation of NB4 cells by ATRA Metabolites, it was of interest to determine the relative potency of ATRA and its metabolites in the induction of NB4 cell differentiation, with effective concentrations to elicit 50% (EC50) of NBT positive cells ranging from 7 nM to 1 μM for 72 h (Fig. 5). ATRA was more efficient than its four metabolites in the induction of differentiation, with effective concentrations to elicit 50% (EC50) of NBT positive cells of 38.3 ± 6 nM for 4-oxo-RA, 55.5 ± 1.8 nM for 18-OH-RA, 79.8 ± 1.8 nM for 4-OH-RA, and 99.5 ± 1.5 nM for 5,6-epoxy-RA. However, at higher concentrations (from 0.25 to 1 μM), ATRA and its metabolites showed similar differentiation efficiency of about 80% NBT positive cells.

Effect of RARα Antagonist on the Induction of Differentiation by ATRA Metabolites. Because the ATRA metabolites could induce NB4 promyelocytic leukemia cell differentiation, it was of interest to investigate the nuclear receptor pathways involved in this response. Because differentiation of NB4 cells by ATRA is mediated through the RARα receptor, we therefore blocked this pathway using the RARα-specific antagonist BMS614. NB4 cells were simultaneously treated with ATRA or its metabolites at 0.1 μM, in the absence or presence of the RARα-specific antagonist BMS614 (1 μM) for 48 h (Fig. 6). As expected, BMS614 abolished ATRA-induced CD11c expression as shown by the decrease in fluorescence intensity to near control values. Likewise, induction of CD11c expression by the ATRA metabolites was blocked by BMS614, which indicated that the metabolites induced maturation through the RARα signaling pathway, as does the parent compound.

DISCUSSION

Although ATRA is considered to be the best single agent available for APL therapy, it is limited in that clinical resistance can develop

Table 1  CD11c integrin cell surface expression of NB4 cells exposed to ATRA or its metabolite

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% CD11c positive cells 24 h</th>
<th>% CD11c positive cells 48 h</th>
<th>% CD11c positive cells 72 h</th>
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<tbody>
<tr>
<td>ATRA</td>
<td>67</td>
<td>79</td>
<td>70</td>
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<tr>
<td>4-oxo-RA</td>
<td>53</td>
<td>61</td>
<td>51</td>
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<tr>
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<tr>
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<td>62</td>
<td>67</td>
<td>65</td>
</tr>
<tr>
<td>5,6-epoxy-RA</td>
<td>67</td>
<td>81</td>
<td>76</td>
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* a The CD11c-labeled controls and the isotype (IgG2a) control cells showed 1% positive cells.
* b Cells were exposed to retinoid concentrations of 1 μM for the indicated times.

Fig. 4. A, NB reorganization by ATRA and its metabolites. Intracellular distribution of PML and PML-RARα proteins in NB4 cells exposed to ATRA or its four metabolites at 1 μM for 5 days was analyzed by immunocytochemistry. The nuclei from control cells presented fine and numerous labeled dots containing PML (Fig. 4A), corresponding to the micropunctuated NBS typically found in APL cells. When the cells were exposed to ATRA, the morphological reorganization was characterized by an increase in NB size corresponding to macropunctated NBS, and a decrease in their number. A similar pattern of NB reorganization to that seen with ATRA was observed for all of the metabolites tested (Fig. 4A). As early as 24 h, NB reorganization could be seen, which reached a maximum at 72 h. No major difference in NB size was observed between 72 h and 120 h for ATRA or its metabolites (data not shown). Concomitant to NB reorganization, degradation of the PML-RARα chimeric protein by ATRA metabolites was also assessed. ATRA metabolites treatment induced PML-RARα degradation as did ATRA (Fig. 4B). The protein became barely detectable at 48 h, with the emergence of a lower molecular weight band corresponding to degradation of the chimeric protein (Fig. 4B).

Fig. 5. Determination of the relative potency of ATRA and its metabolites. NB4 cells were exposed to ATRA or its metabolites at concentrations ranging from 7 nM to 1 μM; the NBT reduction test was performed after 72 h, and positive cells were counted. Values are the mean of three separate experiments; error bars have not been shown for legibility.
rapidly (20). One cause of ATRA resistance is attributable to its increased metabolism with rapid clearance often observed in relapsing patients (43). ATRA has, therefore, been considered to be the unique effective compound, and its metabolites have been considered as catabolic products. However, several authors have proposed that ATRA metabolism may be required for cell growth inhibition of various cancer cell lines in vitro, although no causal relationship has been shown (35, 37, 44). Because very little information is presently available concerning the biological activity of the principal Phase I ATRA metabolites, the purpose of this work was to evaluate the in vitro effects of the main oxidized metabolites of ATRA using the NB4 human promyelocytic leukemia cell line (38). The NB4 cell line is a relevant model for this study in that it exhibits the chromosomal translocation t(15;17) specific for APL, with the resultant production of the chimeric protein PML-RARα. It can be differentiated by ATRA treatment and may be used to elucidate mechanisms of differentiation in APL, a paradigm of a disease sensitive to differentiation therapy.

Our results show that NB4 cells metabolize ATRA to the more polar metabolites 4-oxo-RA, 4-OH-RA, 18-OH-RA, and 5,6-epoxy-RA. ATRA metabolism is cytochrome P450-dependent and appears to involve several isofoms including CYP2C8 (45) and CYP26 (46, 47; reviewed in Ref. 48). More recently, Marill et al. (49) have identified several other human cytochrome P450s significantly involved in the metabolism of ATRA.

ATRA and its metabolites were found to be equally active in terms of growth inhibition of NB4 leukemia cells at 1 μM concentration. These data are in agreement with those of van der Lee et al. (35) and van Heusden et al. (33), who also observed growth inhibition of breast cancer cell lines with ATRA metabolites (4-oxo-RA, 4-OH-RA, and 5,6-epoxy-RA). Rat rhabdomyosarcoma cells have been reported to be sensitive to growth inhibition by 4-oxo-RA, 4-OH-RA, and 5,6-epoxy-RA (34). However, the NB4 leukemia cell line used in this report appears to be more sensitive than solid tumor cell lines, because ~90% growth inhibition was obtained in these cells compared with 40–50% reported in MCF-7 cells or rhabdomyosarcoma cells (33–35). To our knowledge, this is the first report on the growth inhibitory activity of the principal Phase 1 metabolites of ATRA in a promyelocytic leukemia cell line and, also, the first account of the activity of the 18-OH-RA.

The ATRA metabolites were also shown to be active in regard to differentiation induction in the NB4 cells as evaluated by several criteria including cell morphology, NBT reduction test, and CD11c integrin expression. These metabolites were as active as the parent compound at concentrations between 0.2 and 1 μM. At lower concentrations, differences were seen between ATRA and its metabolites in their relative potency (EC50) to induce maturation of NB4 cells in the NBT reduction test. Although ATRA was found to be the most potent retinoid tested (EC50, 15.8 nM), 4-oxo-RA (EC50, 38.3 nM) is also very active, inasmuch as only a 2.4-fold difference was observed between this metabolite and the parent compound. It is of interest to note that the EC50 found for 4-oxo-RA is achievable in vivo in humans after ATRA administration, because concentrations in the range of 19–30 ng/ml (61–96 nM) can be reached (50). In some studies, 4-oxo-RA has also been reported to be more active than ATRA in the modulation of positional specification in Xenopus laevis embryos (31). With regard to the EC50 found for 4-oxo-RA, 4-OH-RA, and 5,6-epoxy-RA, similar relative potency data have been reported in the differentiation of rat rhabdomyosarcoma cells (34). The relative potency of these natural retinoids may, therefore, be dependent on the cell type and on the physiological phenomenon considered. Comparison of the relative potency of ATRA metabolites may also be biased in the sense that the metabolites were applied exogenously to the cells, as opposed to the physiological situation in which the metabolites are formed intracellularly, via the cytochrome P450s.

Concomitant to the induction of differentiation in the NB4 cells by the ATRA metabolites, we observed a reorganization of the nuclear PML-containing bodies, and a degradation of the PML-RARα chimeric protein, as has been previously observed with ATRA (51–53). These data suggest that the metabolites share the same signaling pathway as ATRA. To verify this, we used a RARα-specific antagonist (BMS614) in combination with the metabolites. CD11c expression analysis showed a complete inhibition of CD11c expression induction when NB4 cells were cotreated with BMS614, which indicated that ATRA metabolites act through the RARα pathway for differentiation induction.

In summary, these data demonstrate new biological properties of the ATRA metabolites and indicate that they are not ineffective catabolic products. These metabolites may participate in the ATRA mechanism of action on cell growth inhibition and differentiation via the RARα receptor signaling pathway. In conclusion, the principal ATRA Phase I metabolites are active retinoids which, provided that manageable toxicity and in vivo pharmacological concentrations were achievable, could be of interest for differentiation therapy of sensitive human cancers.

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