Apoptosis Induction by Retinoids in Eosinophilic Leukemia Cells: Implication of Retinoic Acid Receptor-α Signaling in All-Trans-Retinoic Acid Hypersensitivity

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Abstract

Hypereosinophilic syndrome (HES) has recently been recognized as a clonal leukemic lesion, which is due to a specific oncogenic event that generates hyperactive platelet-derived growth factor receptor-α-derived tyrosine kinase fusion proteins. In the present work, the effect of retinoids on the leukemic hypereosinophilia-derived EoL-1 cell line and on primary HES-derived cells has been investigated. We show that all-trans-retinoic acid (ATRA) inhibits eosinophil colony formation of HES-derived bone marrow cells and is a powerful inducer of apoptosis of the EoL-1 cell line. Apoptosis was shown in the nanomolar concentration range by phosphatidylserine externalization, proapoptotic shift of the Bcl-2/Bak ratio, drop in mitochondrial membrane potential, activation of caspases, and cellular morphology. Unlike in other ATRA-sensitive myeloid leukemia models, apoptosis was rapid and was not preceded by terminal cell differentiation. Use of isoform-selective synthetic retinoids indicated that retinoic acid receptor-α-dependent signaling is sufficient to induce apoptosis of EoL-1 cells. Our work shows that the scope of ATRA-induced apoptosis of malignancies may be wider within the myeloid lineage than thought previously, that the EoL-1 cell line constitutes a new and unique model for the study of ATRA-induced cell death, and that ATRA may have potential for the management of clonal HES. (Cancer Res 2006; 66(12): 6336–44)

Introduction

Molecularly targeted therapy of malignancies is a promising new concept based on the selective pharmacologic modulation of key signaling/transcription mechanisms specific to a given tumor/leukemia type. All-trans-retinoic acid (ATRA), the first clinically relevant example of such treatment, targets the promyelocytic leukemia (PML)/retinoic acid receptor (RAR)-α fusion oncoprotein in acute promyelocytic leukemia (APL). APL blasts undergo terminal granulocytic differentiation on treatment with ATRA and are thereafter eliminated from the body by apoptosis, leading to complete remission (1). ATRA treatment in combination with chemotherapy has dramatically improved the prognosis of APL (1). Despite the marked differentiation-inducing effect of the molecule observed in various other in vitro solid tumor and leukemia models, ATRA treatment of malignancies is, however, currently limited to PML/RAR-α-positive APL in the clinic.

Recently, the identification of an interstitial deletion, del(4) (q12q12), on chromosome 4 that generates the FIP1L1-like-1/platelet-derived growth factor receptor-α (FIP1L1/PDGFRα)-transforming tyrosine kinase fusion protein in a significant subset of cases of hypereosinophilic syndrome (HES) permitted to unequivocally establish the clonal, leukemic origin of these lesions (2). In addition, as the tyrosine kinase activity of the FIP1L1/PDGFR-α fusion protein can be inhibited by molecules such as imatinib mesylate (STI571 or Gleevec), HES that expresses this fusion protein can be successfully treated with imatinib mesylate (2–6). However, clinical experience has shown that treatment of sensitive types of leukemia with targeted therapy, such as imatinib mesylate or ATRA as a single agent, often leads to relapse and emergence of resistant clones (7, 8), and, indeed, imatinib mesylate resistance in HES has already been described (2, 9).

Although certain sublines of the HL-60 and AML14 myeloid leukemia cell lines present some eosinophilic features (10, 11), the FIP1L1/PDGFR-α-positive (PML/RAR-α and BCR/ABL negative) EoL-1 cell line (12), established from an acute eosinophilic leukemia that followed a chronic HES (13, 14), has recently emerged as a key model system for the investigation of the molecular and cellular biology of FIP1L1/PDGFR-α-positive malignancies of the eosinophilic lineage (15). The EoL-1 cell line constitutes the only in vitro tool currently available for the study of this type of malignancy and has recently been used by many laboratories for the investigation of eosinophil-granulocytic differentiation (13, 16, 17) and the function of the FIP1L1/PDGFR-α fusion protein (5, 15, 18).

ATRA can exert marked biological responses, including cell differentiation and apoptosis, in several experimental models of malignancy, including myeloid leukemias (19). In addition, ATRA has also a suppressive effect on normal eosinopoiesis in vivo (20). However, the effect of ATRA on the behavior of neoplastic eosinophil cells has not been studied thus far. Therefore, in the present study, we investigated the effects of retinoids on EoL-1 cells and on eosinopoiesis of HES-derived bone marrow in vivo. We show that EoL-1 cells are exquisitely sensitive to retinoic acid and undergo extensive programmed cell death at very low doses of ATRA and, importantly, that ATRA also inhibits eosinophil colony formation of bone marrow cells obtained from FIP1L1/PDGFR-α-positive HES.

Materials and Methods

Cell culture and treatments. The EoL-1 and HL-60 human cell lines were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and American Type Culture
Collection (Manassas, VA), respectively, and NB4 cells were kindly provided by Dr. Michel Lanotte (U685 Institut National de la Santé et de la Recherche Médicale, Paris, France). Cells were grown in RPMI 1640 supplemented with glutamax-1, 2 mmol/L glutamine plus 15% (for HL-60) or 10% (for NB4 and EoL-1 cells) FCS (Life Technologies, Inc., Grand Island, NY) that supported growth of EoL-1 cells as suspension of single cells without significant cell clumping.

At the beginning of experiments, exponentially growing cells were resuspended in complete medium at a density of 2 x 10^6/mL, and drugs were added from concentrated stock solutions made in DMSO. Final concentration of DMSO did not exceed 0.1% and did not interfere with the assays. ATRA and AM580 (+5,6,7,8-tetrahydro-5,5,8,9-tetramethyl-2-naphthamido)benzoic acid (naphthalaminobenzoic acid) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France), Ro-41 5253 (p-[E]-2'-3',4'-dihydro-2'-4'-dimethyl-7'-(hep-tolyoxy)-2'H-1-benzo[b]imidazopyran-6'-yl)propenyl]benzoic acid 1',1'diiodide) was a gift from Drs. Eva-Maria Guttknecht and Pierre Weber (Hoffmann-La Roche SA, Basel, Switzerland), and imatinib mesylate was provided by Novartis, Inc. (Basel, Switzerland).

Cell counts and viability were determined by trypan blue exclusion using a hemocytometer. Morphologic assessment of cells was made on cytopsin preparations stained with May-Grunwald-Giemsa (MGG).

Early apoptosis was detected on a FACScalibur flow cytometer (BD Biosciences, Le Pont de Claix, France) and by Annexin V and propidium iodide staining according to the instructions of the manufacturer (Annexin V-FITC kit, Beckman Coulter, Marseille, France; ref. 21).

Mitochondrial membrane potential (ΔΨm) was measured on a FACScalibur flow cytometer by staining cells with 20 nmol/L 3,3'-dihexyloxacarbocyanine iodide DiOC6(3) (Uptima Interchim, Montlucon, France) for 30 minutes at 37°C in serum-free RPMI 1640 in the dark (22).

Surface marker expression was determined on a FACScalibur flow cytometer by incubating 1 x 10^6 cells for 30 minutes at 4°C with monoclonal antibodies directed against CD11b, CD11c, CD14, CD49d, CDw125, or isotype-match control antibodies (BD Biosciences). Detection of intracellular interleukin (IL)-5Rα was done after fixation and permeabilization with 0.1% Triton X-100 in PBS.

**Transfections.** Thirty million EoL-1 cells in 250 μL Opti-MEM (Life Technologies) were electroporated at 280 V, 950 μF with the hRAR-β2 luciferase reporter gene (8 μg; ref. 23), and, as control, the β-galactosidase vector (pCH110; 1 μg) using an IBI Gene Zapper 450/2500 apparatus (Eastman-Kodak, New Haven, CT). Cells were then resuspended in complete medium, and, 3 hours later, drugs were added. Assays for luciferase (Luciferase Assay kit, Promega, Charbonnieres, France) and β-galactosidase (β-Gal Reporter Gene Assay kit, Roche, Fontenay-sous-bois, France) were done on harvested cells after an overnight treatment with drugs according to the protocols of the manufacturers. Results are expressed as fold induction based on the relative luciferase activity (arbitrarily set at 1) displayed in the absence of any drug. Experiments were normalized to β-galactosidase (24).

**Immunoblotting and antibodies.** Cells were quickly washed twice with ice-cold NaCl (150 mmol/L), precipitated with 5% trichloroacetic acid overnight at 4°C, and then centrifuged (25). Protein pellet was dissolved in sample buffer (25), and equal amounts of lysates (50 μg protein/well) were run on SDS-PAGE and electroblotted onto nitrocellulose (Hybond enhanced chemiluminescence (ECL), Amersham, Freiburg, Germany).

Blocking of nonspecific sites was done by incubating the membranes for 1 hour with TBS supplemented with 0.1% Tween 20 (TBST) and 5% nonfat dry milk (TBST-milk). Immunostaining was done by incubating the membranes under agitation overnight at 4°C with primary antibodies diluted in TBST-milk for caspase-3 (2,000-fold), caspase-8, caspase-9, and McI-1 (1,000-fold; Cell Signalling, Beverly, MA); Bcl-2 (2,000-fold; DAKO, Trappes, France); Bak (500-fold) and Bcl-x (2,000-fold; PharMingen, San Diego, CA); Bak (2,000-fold), PDGFR-α (C-20), and P-V754-PDGFR-α (1,000-fold; Santa Cruz Biotechnology, Santa Cruz, CA); β-actin (4,000-fold) and poly(ADP-ribose) polymerase (PARP, 2,000-fold; Sigma-Aldrich); and plasma membrane calcium ATPase (PMCA; 1,000-fold; Ozyme, Saint-Quentin en Yvelines, France). The 9c9a6 anti-RAR-α antibody, kindly provided by Prof. Pierre Chambon and Dr. Cécile Rochette-Egly (Institut de Genetique et de Biologie Moléculaire et Cellulaire, Illkirch, Illkirch, France) was diluted 500-fold in TBST. After three washes in TBST-milk, membranes were incubated with appropriate peroxidase-conjugated secondary antibodies for 30 minutes in TBST-milk and washed. Signal was detected using the ECL system and quantified by scanning nonsaturated luminograms using the ScionImage software (version 4.02, Scion Corp.).

**Collagen gel culture of human bone marrow.** Bone marrow samples were obtained from residual biopsy material from healthy donors and from a HES patient after informed consent. The patient, a 29-year-old man, was treated during 4 months with 500 to 1500 mg/d hydroxyurea, and the symptoms persisted. After 2 weeks of hydroxyurea withdrawal, a bone marrow biopsy was taken for the detection of the FIP1L1/PDGFR-α transcript by reverse transcription-PCR and for cell culture. At this point, he presented marked hepatosplenomegaly by ultrasonography, WBC:117 G/L, neutrophils:74.76 G/L, basophils:0.02 G/L, monocytes:1.37 G/L, lymphocytes:1.63 G/L, and eosinophils:3.88 G/L. After detection of the FIP1L1/PDGFR-α transcript, treatment with 100 mg/d imatinib mesylate was implemented, resulting in disappearance of hepatosplenomegaly and other symptoms within 1 week (WBC:22 G/L, Hgb:126 g/L, PLT:156 G/L, and eosinophils:0.00 G/L).

Mononuclear cells of normal and patient bone marrow were isolated by density gradient centrifugation on lymphocyte separation medium (density, 1.077; Eurobio, Courtaboeuf, France). Cells were grown in collagen gel cultures using a modified protocol from the Megacult-C kit (Stem Cell Technologies, Inc., Vancouver, British Columbia, Canada). Cells were plated for the patient and normal donors at 1 x 10^6 and 2.5 x 10^6 per chamber of double chamber slides, respectively, in RPMI 1640 supplemented with Megacult-C medium, agar human leukocyte-conditioned medium with 10% fetal bovine serum, and collagen in the presence or absence of ATRA as shown in the figures. Human recombinant IL-3, recombinant granulocyte macrophage colony-stimulating factor (GM-CSF), and recombinant IL-5 (R&D Systems, Lille, France) were added at a final concentration of 10 ng/mL. Bone marrow cultures were made in duplicate, and colony-forming units were enumerated on day 14 of culture after MGG staining or immuno-histochemical staining (DAKO LSAB+ System, DAKO) with an antibody directed against major basic protein (MBP; BMK13, Chemicon, Hampshire, United Kingdom) according to the instructions of the manufacturer.

The results presented in this work are representative of three independent experiments.

**Results**

**ATRA induces the death of EoL-1 cells.** EoL-1 cells were cultured in the presence or absence of various concentrations of ATRA in the 1 nmol/L to 10 μmol/L range, and cell viability was determined by trypan blue exclusion daily for 15 days. ATRA induced complete cell death in the low nanomolar concentration range. Although untreated cells, as well as cells treated with 1 nmol/L ATRA, continued to proliferate and maintain ~100% viability, cell death could be detected >10 nmol/L ATRA (Fig. 1A). The time course of cell death was dependent on the concentration of ATRA, with faster kinetics at higher doses of the drug. ATRA-induced cell death proceeded somewhat more slowly than that induced by imatinib at maximally efficient concentrations (1 mmol/L).

To investigate further the exquisite sensitivity of EoL-1 cells to ATRA, the dose-dependence of ATRA-induced EoL-1 cell death was explored in greater detail in the 1 to 10 nmol/L concentration range at day 12. Reduction of cell viability was clearly detectable already at a concentration of 2 to 4 nmol/L of exogenously added ATRA, with only 20% viable cells remaining at day 12 in the presence of 4 nmol/L of the drug (Fig. 1B).

ATRA treatment of EoL-1 cells induced cell clumping. Untreated EoL-1 cells grew in suspension, predominantly as single cells, with...
occasional small cell aggregates consisting of only a few (<10) cells. Treatment with various concentrations of ATRA induced the formation of large cell aggregates from day 1, which could reach the size of several hundred cells (Fig. 5A, 1) and which could be dissociated by gentle pipetting. Aggregation was accompanied by an almost complete loss of viable single cells in the culture, and death of cells proceeded within the aggregates and could be detected by trypan blue staining (Fig. 5A, 3).

On ATRA treatment, the promyelocytic NB4 and myeloblastic HL-60 leukemia cell lines, which constitute the most widely used and best characterized in vitro models of ATRA-induced cell differentiation within the myeloid lineage, undergo terminal neutrophil granulocytic differentiation in 4 and 6 days, respectively (26, 27). Moreover, induction of neutrophil-granulocytic differentiation followed by apoptosis of promyelocytic blasts constitutes the basis of ATRA treatment of APL. To compare ATRA sensitivity of EoL-1 cells to that of HL-60 and NB4, cells were incubated with 100 nmol/L ATRA for 4 days, and viability was determined by trypan blue exclusion. A faster and more marked death response of EoL-1 cells could be observed when compared with HL-60 or NB4 cells (Fig. 1C). The viability of HL-60 and NB4 cells remained essentially unaltered, whereas the viability of EoL-1 cells decreased to ~50%, indicating that ATRA-induced cell death proceeds faster than that of HL-60 or NB4 cells.

To study morphologic changes that take place during ATRA-treatment of EoL-1 cells, cytospin preparations were made from cells treated with 100 nmol/L drug for up to 7 days and stained with MGG. Untreated cells displayed a blastic morphology with large, round nuclei and high nuclear/cytoplasmic ratio (Fig. 5B, 1). During treatment, some cells showed a discrete modification in cell size, diminution of nuclear/cytoplasmic ratio, and occasional appearance of nuclear invaginations and vacuoles (Fig. 5B, 2-6). It is worth mentioning that remaining viable cells that persisted in culture in late stages of treatments (such as day 7; Fig. 5B, 6) did not display signs of more advanced myeloid differentiation, such as indented or multilobed nuclei or specific cytosolic granules or eosinophilic staining patterns, and nitroblue tetrazolium cytochemical staining remained negative as well (data not shown).

To document more precisely the expression of proteins involved in eosinophil differentiation, analysis of the modulation of the immunophenotype of EoL-1 cells during ATRA treatment was done by flow cytometry using CD11b (integrin αM subunit), CD11c (integrin αC subunit), CD49d (integrin αδ chain), CD14 (lipopolysaccharide receptor), and CDw125 (IL-5Rα) specific antibodies and appropriate isotype-matched negative controls. ATRA treatment for 3 days led to a marked induction of CD11b and CD11c expression in the plasma membrane, and this was accompanied by a parallel decrease of the CD49d level (Fig. 1D). Our results agree with previous studies, showing that EoL-1 cells under normal culture conditions express a high level of CD49d and very little CD11b and that the expression of CD49d decreases and CD11b increases under differentiating stimuli (16, 28). On the other hand, ATRA had no effect on CD14 and intracellular or extracellular (l) staining was determined using a FACScalibur flow cytometer. Columns, mean of three independent experiments; bars, SE.

Figure 1. ATRA induces death of EoL-1 cells. The percentage of viable EoL-1 cells treated or not treated with the indicated concentrations of ATRA (continuous lines) or 1 nmol/L imatinib (dotted line) was assessed by trypan blue staining every day during 15 days (A) and after 12 days of treatment (B). C, the percentage of viable HL-60, NB4, and EoL-1 cells treated or not treated by ATRA (10⁻⁷ mol/L) was assessed by trypan blue staining after 4 days of treatment. D, EoL-1 cells were treated (white bars) or not treated (black bars) with ATRA (10⁻⁷ mol/L). Cells were harvested 3 days after treatment, and the specific expression of CD11b, CD11c, CD14, CD49d, and CDw125 (IL-5Rα) was assessed by direct staining using specific antibodies. Cell surface (e) or intracellular (l) staining was determined using a FACScalibur flow cytometer. Columns, mean of three independent experiments; bars, SE.
treatment, morphologic changes characteristic for apoptosis could be observed, including cell shrinkage with maintained plasma membrane integrity, condensation, and fragmentation of nuclei. Such apoptotic bodies became preponderant by day 7 of ATRA treatment, and this was followed on longer incubations by a complete loss of the viability of the culture (Fig. 5B, 4-6).

Taken together, our results show that ATRA regulates the expression of integrins specifically expressed during eosinophilic differentiation, has no effect on IL-5Rα expression (Fig. 1D), and does not induce mature eosinophil morphology (Fig. 5B), indicating that the cell death effect of ATRA is not linked to the induction of terminal eosinophilic differentiation.

Characterization of ATRA-induced EoL-1 cell death. To characterize the molecular events taking place during ATRA-induced death of EoL-1 cells, general apoptosis markers corresponding to various stages of the apoptotic process were investigated. Early apoptosis was detected by phosphatidylserine externalization as measured by FITC-labeled Annexin V binding and propidium iodide staining. Treatment of cells with ATRA induced, in a time-dependent manner, the externalization of phosphatidylserine in EoL-1 cells (Fig. 2A and B). Although untreated cells were predominantly unlabeled by Annexin V or propidium iodide, treatment with 100 nmol/L ATRA for 3 days resulted in a marked increase of the number of Annexin V-positive, propidium iodide-negative cells, indicative of apoptosis. On longer treatment, this was followed by the appearance of Annexin V and propidium iodide double-positive cells, indicative of more advanced stages of apoptosis and cell destruction.

Two major pathways lead to the induction of apoptosis. Although the extrinsic apoptotic pathway, triggered by the activation of the death receptors and leading to caspase-8 activation, is a well-established mechanism of the apoptosis of mature eosinophils (29, 30), data are currently not available about the intrinsic pathway in this lineage. The intrinsic pathway involves proteins of the Bcl-2 family and the mitochondria to which signals converge (31). This process results in the loss of mitochondrial membrane potential and release of proapoptotic proteins, such as cytochrome c, leading to the formation of the apoptosome, which in turn cleaves and activates caspase-9 (31).

As ATRA-induced apoptosis in leukemic neutrophil-granulocytic differentiation implicates mitochondria and proteins of the Bcl-2 family, the mitochondrial apoptosis pathway was further investigated by measuring ΔΨm in control and ATRA-treated EoL-1 cells by flow cytometry using DiOC6(3) (22), a fluorescent carbocyanine dye. This molecule distributes into mitochondria depending on the membrane potential of the organelle and therefore a decrease of DiOC6(3) fluorescence indicates diminished ΔΨm. Treatment of EoL-1 cells with 100 nmol/L ATRA induced a significant drop in DiOC6(3) fluorescence when compared with untreated cells, indicating that ATRA treatment at days 1 to 4 leads to a gradual decrease of ΔΨm (Fig. 2C).

We investigated also the expression of proteins of the Bcl-2 family by Western immunoblotting in EoL-1 cells treated with ATRA for 3 days. ATRA treatment decreased Bcl-2 and increased Bak expression, whereas the expression levels of other Bcl-2 family members, such as Bax, Bcl-xL, and Mcl-1, did not change significantly (Fig. 3A). Taken together, these data indicate that ATRA treatment induced an ~3.5-fold proapoptotic shift in the Bcl-2/Bak ratio (Fig. 3B).

Extrinsic and intrinsic pathways lead to activation of initiator caspases, such as caspase-8 and caspase-9, which can activate the effector caspases, such as caspase-3 and caspase-6, which cleave, degrade, or activate other cellular proteins (32). Activation of procaspase-8, procaspase-9, and procaspase-3 was monitored daily in EoL-1 cells treated with 100 nmol/L ATRA for 4 days by detection of active caspase fragments by Western blot.

Figure 2. ATRA induces phosphatidylserine externalization and ΔΨm decrease. EoL-1 cells were treated or not treated with ATRA (10−7 mol/L). A, apoptotic cells were detected by Annexin V binding and propidium iodide labeling after the indicated times of treatment. Apoptotic cells in the lower right quadrant (LR), necrotic cells in the upper right quadrant (UR), and viable cells in the lower left quadrant (LL) were detected. B, percentage of apoptotic and necrotic cells. C, ΔΨm was studied by flow cytometry in untreated (dotted lines) or treated (continuous lines) EoL-1 cells with 10−7 mol/L ATRA after the indicated time of treatment. Decreased ΔΨm was detected as a leftward shift in the curve shape of the DiOC6(3) signal.
of proteolytically active caspase-3 (17 kDa), as well as caspase-8 (36 and 40 kDa) and caspase-9 (34 kDa) could be detected in ATRA-treated EoL-1 cells from day 2 (Fig. 3C). Cleavage of caspase substrates was investigated by the detection of proteolytic fragments of PARP, a well-established caspase substrate (33) and of PMCA, a recently identified target of caspase action (34). Treatment of EoL-1 cells with 100 nmol/L ATRA induced PARP cleavage with the appearance of an 85-kDa proteolytic PARP fragment (Fig. 3D). Cleavage followed a time course compatible with that of caspase-3 activation and could be completely inhibited by the pan-caspase inhibitor z-VAD-(O-Me)-fmk (data not shown). Proteolytic fragmentation of PMCA was also observed in ATRA-treated EoL-1 cells, and this led to the formation of a 125-kDa PMCA fragment, in accordance with the literature (Fig. 3D; ref. 34). Taken together, these data indicate that ATRA-induced apoptosis in EoL-1 cells is caspase dependent.

**Analysis of ATRA-activated signal transduction in EoL-1 cells.** The biological effect of retinoids is mediated by ligand-inducible transcription factors that belong to the RAR and RXR gene families. Both gene families are composed of 3 members (RAR-α, RAR-β, and RAR-γ and RXR-α, RXR-β, and RXR-γ, respectively), which may perform distinct functions involved in the induction of differentiation, growth inhibition, or apoptosis (35).

To identify the retinoid receptor pathway involved in apoptosis induction in EoL-1 cells, experiments with retinoid receptor isoform-specific ligands were carried out. AM580, a selective RAR-α-specific agonist induced cell death of EoL-1 cells (Figs. 4A and 5C, 1-3). AM580 (10 nmol/L) was ~10-fold more potent in apoptosis induction when compared with ATRA (100 nmol/L). This agrees with the relative potencies of the two molecules observed in other experimental settings (36). Similar to other cell types (36), ATRA could transactivate the RAR-α-sensitive RAR-β2 promoter also in EoL-1 cells (Fig. 4B). ATRA-induced death of EoL-1 cells could be inhibited by the RAR-α-specific antagonist Ro-41 5253 (Figs. 4C and 5C, 4-6; ref. 37). Endogenous RAR-α protein could be detected in EoL-1 cells, and ATRA treatment induced RAR-α protein down-regulation (Fig. 4D). This is in accordance with the ATRA-induced proteolytic degradation of RAR-α observed in other myeloid cell types (38). Altogether, these observations indicate that RAR-α is expressed and functional in EoL-1 cells and is sufficient to initiate ATRA-induced apoptosis. On the other hand, different concentrations of ATRA had no discernible effect on the expression level of the state of tyrosine phosphorylation (P-Y754-PDGFR-α) of the FIP1L1/PDGFR-α fusion protein, whereas imatinib totally abrogated the autophosphorylation of the tyrosine kinase fusion protein at Y754 of the PDGFR-α moiety (Fig. 4E).

**ATRA inhibits eosinophil colony formation.** To extend our results and to explore the clinical relevance of our observations, we investigated eosinophil colony formation on normal and HES-derived bone marrow mononuclear cells in the presence and absence of ATRA.

Bone marrow–derived mononuclear cells of healthy donors and of a FIP1L1/PDGFR-α-positive HES patient were grown in collagen containing semisolid cultures as described in Materials and Methods. The number of eosinophil and noneosinophil colonies was determined by cellular morphology on MGG-stained slides.
preparations and by immunocytochemical staining for MBP, an eosinophil granule protein that constitutes a specific marker of this lineage (39). Figure 5D, 1 and 2 shows morphologic features of eosinophilic cells (bilobed nuclei and crystalloid granules) and cells of noneosinophilic colonies (predominantly of neutrophil-granulocytic morphology), respectively. Figure 5D, 3 and 4 shows positive MBP immunostaining (red immunocytochemical staining) in eosinophilic and lack of staining in noneosinophilic colonies, respectively. Under ATRA treatment, a specific decrease of the absolute number of eosinophil colonies (Fig, 6C) as well as a decrease of the eosinophil/noneosinophil colony ratio (Fig. 6d and B) could be observed by morphologic as well as immunocytochemical criteria on MGG- and MBP-stained samples of HES myeloid progenitors, respectively. As shown in Fig. 6D, 5 and 6, when HES mononuclear cells were grown in collagen cultures in the presence of 1 µmol/L ATRA, residual eosinophilic colonies displayed a marked apoptotic morphology with nuclear condensation and numerous apoptotic bodies.

These observations show for the first time that ATRA exerts at clinically attainable concentrations a selective antiproliferative effect on eosinopoiesis of HES-derived bone marrow mononuclear cells.

Discussion

Retinoids are natural and synthetic vitamin A derivatives and analogues that are involved in many important biological processes, including vision, morphogenesis, differentiation, growth, metabolism, and cellular homeostasis. ATRA regulates biological processes by binding to specific nuclear receptors, RARs and RXRs (35). The antiproliferative activity observed for ATRA is thought to be mostly due to direct growth inhibition and induction of differentiation. ATRA can induce the differentiation of many tumor cell lines in vitro (40–42), but, currently, its efficacy in vivo is restricted to APL, where ATRA targets the PML/RAR-α-chimeric oncoprotein, inducing the progressive differentiation of the leukemic cells and their elimination from the body (1).

Whereas some synthetic retinoids are potent inducers of apoptosis (43), although apoptosis induction has been reported for ATRA in some cell lines, this is usually weak and is induced at relatively high concentrations (26, 43–45). The mechanisms by which ATRA induces apoptosis are not well understood. The molecule was shown to down-regulate the expression of the antiapoptotic protein Bcl-2 (45–47) and to induce the decrease of mitochondrial respiration (48) and calcium sequestration (49) causing cell death by apoptosis (50). In addition to the activation of the intrinsic apoptotic pathway, ATRA has also been reported to induce apoptosis in APL blasts through the induction of the expression of the tumor necrosis factor–related apoptosis-inducing ligand (51).

Recently, imatinib mesylate that is in clinical use for the treatment of chronic myeloid leukemia, has been shown to induce remission in some cases of HES (4). This led to the identification of the imatinib mesylate–inhibitable FIP1L1/PDGFR-α-oncogenic fusion protein (12). The discovery of FIP1L1/PDGFR-α, observed in a majority of cases of HES, permitted to establish the clonal, leukemic nature of this disorder. HES can be life threatening due to infiltration of vital organs by excessive amounts of eosinophils, which can release toxic substances normally involved in antiparasitic host defense. In addition, HES can transform to acute leukemia (18). Thus far, the only currently available in vitro cellular model for the study of FIP1L1/PDGFR-α-positive HES is the EoL-1 cell line, established from an acute eosinophilic leukemia that arose following chronic HES (13, 14). EoL-1 cells express the FIP1L1/PDGFR-α fusion oncprotein (15). In vivo, imatinib mesylate
induces the apoptosis of EoL-1 cells (12). However, the exact molecular mechanisms of this effect remain to be established.

To our knowledge, no published work has addressed thus far the effects of retinoids on apoptosis of malignant cells belonging to the eosinophilic lineage (52), although ATRA has been reported to exert a suppressive effect on normal eosinopoiesis ex vivo (20).

In the present work, we show that ATRA induces the apoptosis of EoL-1 cells and inhibits eosinopoiesis of FIP1L1/PDGFR-α-positive HES-derived bone marrow mononuclear cells. Apoptosis proceeds somewhat more slowly than that induced by imatinib and could be shown by phosphatidylserine externalization, activation of initiating and executor caspases, proteolytic cleavage of caspase substrates, proapoptotic shift of the ratio of proapoptotic versus antiapoptotic Bcl-2 family proteins, decreased ΔΨm, and cell morphology. Interestingly, apoptosis could be induced in EoL-1 cells at extremely low ATRA concentrations, with clearly detectable cell death in the slightly supraphysiologic range (4 to 10 nmol/L) of ATRA. Unlike in the myeloblastic HL-60 and promyelocytic NB4 cells, in EoL-1 cells, although integrin expression varied, death was not preceded by terminal differentiation as shown by cell morphology or IL-5Rα expression. These characteristics taken together suggest that EoL-1 cells constitute a new experimental model with unique features for the study of the apoptosis-inducing effect of retinoids.

The understanding of the exact molecular mechanisms set in motion by ATRA in EoL-1 cells requires further research. However, the capacity of an RAR-α-specific agonist to induce and of an...
RAR-α-specific antagonist to prevent ATRA-induced cell death, in combination with the demonstration of functional RAR-α in EoL-1 cells, strongly suggests that RAR-α-dependent signaling is sufficient to initiate apoptosis in these cells. In addition, the observation that ATRA-induced cell death was independent from initial cell density in the 1 × 10^3 to 2 × 10^5/mL range and the inability of exogenously added IL-5 to rescue ATRA-treated cells suggest that cell death is not initiated by autocrine/paracrine mechanisms or by the acquisition by the cells of an IL-5-dependent phenotype.

In this work, activation of initiating (caspase-8 and caspase-9) and effector caspases (caspase-3) has been observed in ATRA-treated EoL-1 cells. Activation of caspase-9, which is induced in the mitochondrial apoptotic pathway, has been observed in parallel with a decrease of ΔΨm. In addition, caspase-8 activation was also observed as well as cleavage of caspase substrates (PARP and PMCA). Because of the considerable complexity of apoptotic pathways and, in particular, due to the existence of various positive feedback mechanisms (53, 54), the precise identification of the primary initiating event of ATRA-induced apoptosis in EoL-1 cells requires further studies. Our observations indicate, however, that ATRA-induced killing of EoL-1 cells proceeds via RAR-α-induced caspase-dependent apoptosis, without influencing the activity of the fusion protein as measured by the state of its autophosphorylation or its level of expression. This suggests that ATRA-induced apoptosis is independent from the expression or activity of FIP1L1/PDGFR-α tyrosine kinase fusion protein.

Rather than being uniform, the mechanisms of the control and the induction and execution of programmed cell death display cell-type and stimulus-dependent characteristics. Apoptosis of normal mature eosinophils can be induced in the presence of prosurvival cytokines, such as IL-3, IL-5, or GM-CSF, by glucocorticoids (55), transforming growth factor-β (56), or by activation of the Fas receptor in vitro, and Fas-dependent signal transduction (30) and reactive oxygen species (56) are involved in this process. The signaling pathways that control apoptosis constitute an intricate network, in which cross-talk between various proapoptotic and antiapoptotic mechanisms operates in a highly dynamic and interconnected manner, and this has not been investigated in eosinophils in great detail thus far. The marked sensitivity and rapidity of ATRA-induced apoptosis of EoL-1 cells clearly distinguish these cells from other known models of ATRA-induced cell death (26, 43–45). This model therefore may prove useful for the in-depth analysis of the molecular mechanisms of ATRA-induced cell death.

Our results may have some relevance in the context of the clinical management of HES. We show that the formation of eosinophil colonies from the bone marrow mononuclear cell fraction of a FIP1L1/PDGFR-α-positive HES patient can be inhibited in vitro by clinically attainable concentrations of ATRA (1 μmol/L; refs. 1, 57), as detected by early (MBP expression) as well as late eosinophil differentiation markers (i.e., formation of colonies that display mature eosinophil-granulocytic morphology). This effect was specific in that ATRA treatment had no significant effect on eosinopoiesis from normal bone marrow samples under the conditions used in our experiments. Morphologic analysis of residual eosinophilic colonies in ATRA-treated HES collagen cultures revealed the presence of nuclear condensation and numerous apoptotic bodies compatible with apoptosis. Due to the sensitivity of the tyrosine kinase activity of the FIP1L1/PDGFR-α fusion protein to inhibition by imatinib mesylate, neoplastic HES is currently treated by imatinib mesylate (2–6). However, spontaneous as well as acquired resistance to this drug has been observed in a significant subset of patients (2, 7, 9). Therefore, our observations support the idea that ATRA, which is used in clinical practice for APL, may be of some use also in non-APL-type malignancies, such as HES.

Figure 6. ATRA inhibits eosinophil colony formation in HES. Bone marrow mononuclear cells obtained from normal donors (Normal) and from a case of FIP1L1/PDGFR-α-positive HES (Patient) were grown as described in Materials and Methods in the presence or absence of 10^-6 mol/L ATRA. Eosinophil (E) and noneosinophil (NE) colonies were identified morphologically on MGG-stained preparations (A) and independently by MBP immunostaining (B). Results are percentage of eosinophil (white bars) versus noneosinophil colonies (black bars). C, absolute number of eosinophil and noneosinophil colonies. Columns, mean of three independent experiments; bars, SE.

<table>
<thead>
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<th>Patient</th>
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<td>13 ± 1</td>
<td>9 ± 3</td>
<td>14 ± 4</td>
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<td>26 ± 2</td>
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