Mechanisms of Retinoid Resistance in Leukemic Cells: Possible Role of Cytochrome P450 and P-Glycoprotein

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Retinoic acid (RA) regulates the differentiation and proliferation of a wide variety of different cell types and all-trans RA induces complete remission in a high proportion of patients with acute promyelocytic leukemia (APL). However, clinical resistance to retinoids may develop and poses a serious problem for differentiation-inducing therapy. We studied the effects of RA in combination with a cytochrome P450 inhibitor (clotrimazole) and a P-glycoprotein antagonist (verapamil) on cell growth and differentiation of RA-resistant HL-60 cells and fresh RA-resistant leukemic cells from two APL patients. RA-resistant HL-60 cells and APL cells differentiated to mature granulocytes when cultured with all-trans RA and either clotrimazole and verapamil but not with either of the agents alone. These findings were confirmed in these cells by their increased expression of CD11b antigen and migration-inhibitory factor-related protein-8/14 mRNAs and MDR1 with the clinical resistance to chemotherapy. Taken together, either of these pathways might be important in development of retinoid resistance in leukemic cells. Therefore, to address the possible role of cytochrome P450 and P-glycoprotein in RA resistance of leukemic cells, we studied the effects of either a cytochrome P450 inhibitor or a P-glycoprotein antagonist in combination with ATRA on cell growth and differentiation of RA-resistant HL-60 cells and fresh retinoid-resistant leukemic cells from two patients with APL.

MATERIALS AND METHODS

Cells. Wild-type and RA-resistant HL-60 cells (generous gift of Dr R.E. Gallagher, Montefiore Medical Center, Bronx, NY) were maintained in serum-free culture medium (Cosmned 001; Cosmo Bio Co, Tokyo, Japan) with 100 U/mL penicillin and 100 mg/mL streptomycin in a humidified atmosphere with 5% CO2; no serum was used to eliminate the influence of endogenous RA in fetal bovine serum concentrations of RA progressively decreased with continuous treatment with ATRA, one mechanism for RA resistance is an alteration in metabolism of the drug. Review of retinoid metabolism suggest that several key points exist in which retinoid resistance might occur. RA is hydroxylated at the cyclohexenyl ring to form a 4-hydroxy-RA metabolite by a cytochrome P450-dependent mono-oxygenase system. Thus, intracellular RA levels in myeloid leukemic cells may decrease as the cells increase their expression of cytochrome P450 enzymes. Another possibility is the induction of expression of P-glycoprotein, which is an energy-dependent drug efflux pump that decreases intracellular drug accumulation. P-glycoprotein, which is encoded by the multidrug-resistance-1 (MDR1) gene, is overexpressed in a variety of tumor cells, and studies have shown a correlation of expression of P-glycoprotein with clinical resistance to chemotherapy. Taken together, either of these pathways might be important in development of retinoid resistance in leukemic cells. Therefore, to address the possible role of cytochrome P450 and P-glycoprotein in RA resistance of leukemic cells, we studied the effects of either a cytochrome P450 inhibitor or a P-glycoprotein antagonist in combination with ATRA on cell growth and differentiation of RA-resistant HL-60 cells and fresh retinoid-resistant leukemic cells from two patients with APL.

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serum. After obtaining written informed consent following institutional guidelines, bone marrow samples were obtained from five patients with APL including two RA-resistant patients. Mononuclear cells were separated over Ficoll-Hypaque (density, 1.077; Sigma Chemical Co, St Louis, MO) and washed twice with phosphate-buffered saline (GIBCO BRL, Gaithersberg, MD). Cells were maintained in RPMI-1640 medium (GIBCO BRL) with 10% fetal bovine serum (Cytosystems, New South Wales, Australia). ATRA (10−8 mol/L; Sigma) was always added to the culture medium for the maintenance of RA-resistant HL-60 cells.

At least 4 days before each experiment, the cells were washed with phosphate-buffered saline; then retinoid and chemicals were added to the medium. Cells (2 × 10^5/mL) were suspended with test materials at appropriate concentrations. After incubation for 4 days, cells were harvested and examined for induction of differentiation by morphology. The morphology of the cells was evaluated from cytospin slide preparations stained with Wright-Giemsa.

HepG2 (human hepatoblastoma) cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in minimum essential medium with nonessential amino acids and sodium pyruvate (GIBCO BRL). A 200-fold trimetrexate-resistant subline of human leukemia cell lines MOLT-3, MOLT-3/TMQ200, was a gift from Dr T. Ohnuma (Mount Sinai School of Medicine, New York, NY) and was maintained in RPMI 1640 medium with 10% fetal bovine serum.

Chemicals. ATRA was dissolved in 100% ethanol to a stock concentration of 1 mmol/L, was stored at −20°C, and was protected from light. The cytochrome P450 inhibitor (clotrimazole; gift from Bayel Yakuhin, Ltd, Tokyo, Japan), and 3-methylcholanthrene (Sigma) were dissolved in 100% ethanol. In each experiment, controls were run using the same concentration of ethanol as was present in the experimental plates, and this concentration of diluent had no effect on proliferation and differentiation of cells.

Assays for cellular proliferation. Cellular proliferation was measured by cell viability and nonradioactive cell proliferation assay system (MTT assay; Boehringer Mannheim, Tokyo, Japan). Cells (2 × 10^5) were incubated with ATRA with or without the cytochrome P450 inhibitor or P-glycoprotein antagonist for 4 days in 96-well microtiter plates (Flow Laboritories, Irvine, CA). Viability was assayed by trypan-blue dye exclusion. MTT assay was performed according to the manufacturer's specifications (Boehringer Mannheim).

Flow cytometric analysis. For analysis of cellular differentiation, expression of cell surface antigens was studied by two-color direct immunofluorescence staining technique. Cells were incubated for 60 minutes with human AB serum (Sigma) to block Fc receptors and then were stained by using fluorescein isothiocyanate (FITC)-conjugated mouse antihuman CD14 and phycoerythrin-conjugated mouse antihuman CD11b (both from Becton Dickinson, Mountain View, CA). For P-glycoprotein detection, indirect immunostaining was performed after blocking the Fc receptors by human AB serum; cells were stained with monoclonal antibody MRK16 (Kyowa Medex Co, Ltd, Tokyo, Japan), followed by staining with goat antimouse second antibody conjugated to FITC. Control studies were performed with a nonbinding control murine IgG, and IgG1 isotype antibodies (Becton Dickinson). Analysis of fluorescence was performed on a FACScan flow cytometer (Becton Dickinson).

RNA isolation and Northern blotting. Total RNA was extracted by acid guanidinium thiocyanate-phenol-chloroform method. Total RNA (20 pg/lane) was electrophoresed on formaldehyde-agarose gels (GIBCO-BRL) and was transferred to nyloncellulose membranes (Hybond N*; Amersham Japan, Tokyo, Japan). Briefly, the labeled probes were hybridized for 16 to 24 hours in 50% formamide, 2× SSC (1× SSC = 150 mmol/L NaCl and 15 mmol/L sodium citrate [pH 7.0]), 5× Denhardt's, 0.1% sodium dodecyl sulfate, and 10% dextran sulfate (Sigma). Filters were washed to a stringency of 0.1× SSC at 65°C and were exposed to Kodak XAR film (Eastman Kodak, Rochester, NY). Autoradiograms were exposed for 3 days.

Molecular probes. A human c-myc cDNA probe (EcoRI-EcoRI; 2.2 kb) was purified from plasmid pSR18/19 (a gift from Japanese Cancer Research Resourcers Bank, Tokyo, Japan). Migration-inhibitory factor-related protein-8 (MIF-8) and MRP-14 cDNA probes were constructed by polymerase chain reaction (PCR) of first-strand cDNA from 1.25-dihydroxyvitamin D3-treated HL-60 cells, and details have been described previously.² The β-actin probe was EcoRI-BamHI (0.7 kb) fragment from plasmid pHFaB-A-3. All probes were labeled with [α-32P]dCTP (3,000 Ci/mmol; Amersham) by random priming kits (Takara Shuzo Co, Ltd, Tokyo).

Reverse-transcription PCR (RT-PCR) assay for CYP1A1 and MDR-1 expression. Expression of CYP1A1 and MDR-1 gene was investigated by the RT-PCR method. The RT reaction was performed using 1 μg of total RNA, 100 pmol of random hexamer (Boehringer Mannheim, Indianapolis, IN), 10 U RNase inhibitor (Promega, Madison, WI), 200 U Moloney murine leukemia virus-RT (GIBCO BRL), and deoxyribonucleotides (final concentration, 0.5 mmol/L each; Pharmacia, Tokyo, Japan) in a total volume of 20 μL and under conditions recommended by the suppliers. After cDNA synthesis, PCR was sequentially performed using CYP1A1, MDR-1, and β-actin primers to generate 423-bp, 243-bp, and 838-bp fragments, respectively. The primers used in this study were as follows: for CYP1A1, sense 5'-TCA CAG ACA GCC TTG AG-3' (residues 928-947) and antisense 5'-GAT GGG TTG ACC CAT AGC TT-3' (residues 1341-1360)²³; for MDR-1, sense 5'-AAC CCT AGT ACC AAA GAG GCT CTG-3' (residues 2041-2064) and antisense 5'-GCC TAG AAA CAA AAA TGG TGA AAG GAA A-3' (residues 2260-2282)²; and for β-actin, sense 5'-ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG-3' (residues 294-325)²⁴ and antisense 5'-CGT CAT ACT CCT GTC TGA TCC ACA TCT GC-3' (residues 1100-1131)²² were PCR was performed using denaturing steps at 95°C for 1 minute, annealing steps at 55°C for 1 minute, and extending steps at 72°C for 2 minutes, respectively. At the end of 30 cycles, further extension for 7 minutes at 72°C was included. All reactions were performed in a total volume of 50 μL containing 2 μL of cDNA (of 20 μL total RT reaction), 1 mmol/L of each primer, 200 mmol/L of each deoxynucleotide; 10 mmol/L Tris HCl (pH 8.3), 50 mmol/L KCl, 2.5 mmol/L MgCl₂, and 5 μg Tag DNA polymerase (Perkin Elmer-Cetus, Norwalk, CT). PCR products (10 μL) were electrophoresed on 2% NuSieve/1% SeaKem agarose (FMC, Rockland, ME) gels and visualized by staining with ethidium bromide. The specificity of PCR amplification was also examined by Southern blotting of the amplified cDNA with hybridization to nonradioactive probes (ECL 3'-oligolabeling and detection system; Amersham).

Assays for aryl hydrocarbon (benzo[α]pyrene) hydroxylases in leukemic cells. Microsomal fractions of leukemic cells were purified as previously described, and protein content was determined by using bovine serum albumin as the standard.²⁶ Incubation mixture consisted of nicotinamide adenine dinucleotide phosphate (0.8 mmol/L), glucose 6-phosphate (6 mmol/L), glucose 6-phosphate dehydrogenase (1 IU), MgCl₂ (6 mmol/L), NaKPi buffer (pH 7.4; 100 mmol/L), EDTA (0.1 mmol/L), leukemic cell microsomes (0.88 mg), and 0.1 mmol/L benzo[α]pyrene as a substrate in a total volume of 1 mL. The reaction was started by adding β-nicotinamide adenine dinucleotide phosphate dehydrogenase-generating system and was reacted at 37°C for 60 minutes. The activity of aryl hydrocarbon hydroxylases (AHHs) was quantitated using synthetic 3-hydroxybenzo[α]pyrene as the standard.²⁶
RETINOID RESISTANCE IN LEUKEMIC CELLS

Control  All-trans RA  CTM  VP  All-trans RA  All-trans RA

HL-60

APL

RS HL-60

RS APL

Fig 1. Morphological changes induced in RA-resistant HL-60 (RS HL-60) cells and fresh retinoid-resistant leukemic cells from APL patients (RS APL) by ATRA (10^{-8} mol/L) and either the cytochrome P450 inhibitor, clotrimazole (CTM; 10^{-6} mol/L), or the P-glycoprotein antagonist, verapamil (VP; 10^{-6} mol/L). RS HL-60 and RS APL cells were cultured for 4 days in serum-free medium with indicated agents. Also, RA-sensitive HL-60 (HL-60) cells and APL cells were treated with ATRA, CTM, or VP alone for 4 days. Cytospin slides were prepared and stained with Wright-Giemsa. Original magnification, × 1,000. The CTM (10^{-6} mol/L) or VP (10^{-6} mol/L) alone had no affect on the morphology of the cells.

RESULTS

Morphological changes induced in RA-resistant leukemic cells cultured with ATRA and either clotrimazole or verapamil. The RA-resistant HL-60 cells were cultured for 4 days in serum-free medium containing 10^{-8} mol/L ATRA with or without clotrimazole (10^{-6} mol/L) or verapamil (10^{-6} mol/L). Exposure of RA-resistant HL-60 cells to ATRA and either clotrimazole or verapamil resulted in differentiation towards mature granulocytes (Fig 1). The leukemic cells from retinoid-resistant APL patients also differentiated to mature granulocytes but only after being cultured with ATRA (10^{-8} mol/L) and either the cytochrome P450 inhibitor or the P-glycoprotein antagonist (Fig 1). However, neither clotrimazole nor verapamil alone induced differentiation of RA-sensitive or RA-resistant leukemic cells into either mature granulocytes or monocytes (Fig 1).

Functional evidence for differentiation of RA-resistant leukemic cells to granulocytes. Induction of differentiation of RA-resistant HL-60 cells into mature granulocytes by ATRA and either clotrimazole or verapamil was assessed by the expression of CD11b and CD14 antigens (Fig 2A). Each of the chemicals individually did not alter the expression of CD11b antigen by fluorescence-activated cell sorter (FACS) analysis as compared with that of control cells. In marked contrast, the combination of various concentrations of ATRA plus 10^{-6} mol/L clotrimazole or verapamil increased the expression of CD11b antigen by 6- to 10-fold as compared with that with either chemical alone (Fig 2A). However, expression of CD14 antigen was unchanged after exposure to each of the chemicals alone or to the combination of ATRA and either clotrimazole or verapamil. Clotrimazole or verapamil alone did not affect the expression of CD11b and CD14 antigens in RA-sensitive HL-60 cells (Fig 2B). These results were consistent with the morphological changes in RA-resistant leukemic cells.

Modulation of c-myc and MRP-8/14 mRNA expression by ATRA and either clotrimazole or verapamil in RA-resistant leukemic cells. Studies have shown that exposure of HL-60 cells to ATRA decreased accumulation of c-myc mRNA; thus, c-myc is one of the genetic markers for cellular differentiation. Therefore, we examined the abilities of the combination of ATRA and either clotrimazole or verapamil to modulate expression of c-myc mRNA in RA-resistant HL-60 cells (Fig 3). RA-resistant HL-60 cells were cultured for 4 days in the presence of ATRA (10^{-8} mol/L) and 10^{-6} mol/L of either clotrimazole or verapamil. Accumulation of c-myc mRNA decreased after the cells were exposed to ATRA and either verapamil or clotrimazole. The combination of the above three chemicals had the most potent effect in decreasing levels of c-myc mRNA (Fig 3, lane 7).

We have previously reported that the expression of MRPs and their transcripts were regulated during the differentiation of myeloid cells. Therefore, we also examined the modulation of MRP-8/14 mRNAs in RA-resistant HL-60 cells (Fig 3). Untreated or ATRA-, clotrimazole-, or verapamil-treated cells did not express detectable levels of either MRP-8 or...
A : RS HL-60

Fig 2. Expression of CD11b and CD14 antigens by FACS analysis (A) RA-resistant HL-60 cells (RS HL-60) were cultured with chemicals for 4 days as follows: 1, control; 2, ATRA (10^{-8} mol/L); 3, clotrimazole (CTM; 10^{-6} mol/L); 4, verapamil (VP; 10^{-6} mol/L); 5 to 7, CTM (10^{-6} mol/L) plus ATRA (10^{-6}) (10^{-6}, and 10^{-5} mol/L, respectively); and 8 and 9, VP (10^{-6} mol/L) plus ATRA (10^{-6} and 10^{-5} mol/L, respectively). Right panel represents the percentage of CD11b-positive cells. (B) RA-sensitive HL-60 (HL-60) cells were also cultured for 4 days with either chemical alone as follows: 1, control; 2, ATRA (10^{-8} mol/L); 3, CTM (10^{-6} mol/L); and 4, VP (10^{-6} mol/L). Cells were incubated for 60 minutes with human AB serum to block Fc receptors and then stained with two-color direct immunofluorescence using FITC-conjugated mouse antihuman CD14 and phycoerythrin-conjugated anti-human CD11b antibodies. Control studies were performed with a nonbinding control mouse IgG1 and IgG2 isotype antibodies.

B : HL-60

MRP-14 mRNA. However, MRP-8 and MRP-14 mRNAs were upregulated in the presence of ATRA and cytochrome P450 inhibitor and/or P-glycoprotein antagonist (Fig 3). These results indicated that MRP mRNAs were expressed during granulocytic differentiation of RA-resistant HL-60 cells.

Effects of ATRA and clotrimazole and/or verapamil on cell survival and proliferation of RA-resistant leukemic cells. The RA-resistant HL-60 cells were cultured for 4 days in serum-free medium containing 10^{-8} mol/L of ATRA and 10^{-6} mol/L of verapamil or clotrimazole or both of them. When added separately to culture, neither ATRA (10^{-8} mol/L) nor 10^{-6} mol/L of either clotrimazole or verapamil affected either the number of viable cells or the absorbance of the MTT assay, which is a nonradioactive cellular proliferation assay system (Figs 4A, B, columns 2 through 4). However, the combination of ATRA (10^{-8} mol/L) and either clotrimazole or verapamil (10^{-6} mol/L) inhibited the cellular proliferation of the RA-resistant HL-60 cells (Figs 4A and B, columns 5 and 6). Moreover, the combination of clotrimazole, verapamil, and ATRA showed additive effects in inhibiting cellular proliferation (Figs 4A and B, column 7).

Cytochrome P450 contents in leukemic cells. To detect the cytochrome P450 in leukemic cells, we measured cytochrome P450 activity as AHH activity. Studies have reported that no detectable basal AHH activity occurs in HepG2 hepatoma cells; however, activity of this enzyme was markedly induced in these cells after treatment with 3-methylcholanthrene. After isolation of microsomes, measurements showed that levels of AHH activity in both wild-type and RA-resistant HL-60 cells were 2.29 pmol/mg/h and 2.88 pmol/mg/h, respectively (Fig 5A).

We next examined the expression of cytochrome P450 (CYP) gene in leukemic cells using the RT-PCR assay. Expression of the CYP1A1 gene was only detected in HepG2 cells treated with 3-methylcholanthrene (positive control) (Fig 5B). Neither the wild-type and RA-resistant HL60 cells nor the fresh APL cells expressed detectable CYP1A1.
RETINOID RESISTANCE IN LEUKEMIC CELLS

Recently, ATRA has been used to induce complete remission of a high proportion of patients with APL, although their clinical remissions are usually of brief duration and they develop RA-resistant disease. Relapse and acquired resistance to treatment with ATRA in the patients with APL has been hypothesized to result from alteration of drug metabolism and from increased expression of several proteins related to RA metabolism. In this study, we showed that RA-resistant HL-60 cells and fresh RA-resistant leukemic cells from APL patients differentiated to mature granulocytes when cultured with both ATRA and either a cytochrome P450 inhibitor or a P-glycoprotein antagonist. Therefore, these two proteins (cytochrome P450, P-glycoprotein, or related proteins) may have an important role for the altered metabolism of RA in the development of resistance of leukemic cells.

Several possible mechanisms have been proposed to explain in vitro resistance to retinoids. One possible mechanism is the result of molecular alterations of the RA receptor (RAR) genes. Several RA-resistant HL-60 subclones have been investigated and reported to have a point mutation of the RAR-α gene. Both subclones have the same point mutation, a C to T transition in codon 416, in the ligand-binding domain of the RAR-α gene. However, some of the mRNA (Fig 5B). These results were also confirmed by Southern blotting of the RT-PCR products using a probe specific for CYP1A1 gene (Fig 5B).

Expression of P-glycoprotein and MDR-1 mRNA in leukemic cells. To examine the expression of P-glycoprotein recognized by monoclonal antibody MRK16, FACS analysis was performed. Expression of P-glycoprotein was low in both RA-resistant (RS HL-60) and wild-type HL-60 cells; however, it was slightly higher in RS HL-60 as compared with that in wild-type HL-60 cells (Fig 6). To increase the sensitivity of detection of expression of the MDR-1 gene in leukemic cells, we examined the expression of MDR-1 mRNA by RT-PCR technique. The MOLT-3/TMQ200 cells were a positive control of MDR-1 gene (Fig 6B). As shown in Fig 6B, RA-resistant HL-60 cells and leukemic cells from two RA-resistant APL patients were positive for MDR-1 transcripts; however, we could not detect MDR-1 mRNA in wild-type HL-60 cells and RA-sensitive APL cells.

DISCUSSION

Fig 3. Modulation of expression of c-myc and MRP-8/14 mRNAs by ATRA and either clotrimazole or verapamil in RA-resistant HL-60 cells. Cells were cultured with chemicals for 4 days and then total RNA was extracted and analyzed by Northern blot technique. Hybridization was performed with [32P]-labeled c-myc and MRP-8/14 probes, as described in Materials and Methods. RA-resistant HL-60 cells were treated with chemicals as follows: lane 1, control; lane 2, ATRA (10^-8 mol/L); lane 3, clotrimazole (10^-8 mol/L); lane 4, verapamil (10^-8 mol/L); lane 5, ATRA (10^-8 mol/L) plus verapamil (10^-8 mol/L); lane 6, ATRA (10^-8 mol/L) plus clotrimazole (10^-8 mol/L); lane 7, ATRA (10^-8 mol/L) plus clotrimazole (10^-8 mol/L) and verapamil (10^-8 mol/L).

Fig 4. Effects of ATRA, clotrimazole, and verapamil on cell survival and proliferation of RA-resistant HL-60 cells in liquid culture. (A) Viable cell number. RA-resistant HL-60 cells (2 x 10^6/mL) were cultured in the presence of chemicals for 4 days, and viable cells were counted by trypan-blue dye exclusion. Results are the mean ± SD from three experiments. (B) MTT assay. RA-resistant HL-60 cells (1 x 10^4 cells) were incubated in 96-microtiter well plates with chemicals for 4 days, then 10 mL/well of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added according to the manufacturer’s protocol. After 4 hours at 37°C in a humidified 5% CO2 atmosphere, the reaction was stopped by adding 100 μL of 0.04 N HCl in isopropanol. The absorbance at 570 nm (OD570) was recorded using an enzymelinked immunosorbent assay plate reader. Results are expressed as percent of control absorbance of four experiments, and SD was within 10% of the mean. Each number of the column indicates as follows: 1, control; 2, ATRA (10^-8 mol/L); 3, clotrimazole (10^-8 mol/L); 4, verapamil (10^-8 mol/L); 5 ATRA (10^-8 mol/L) plus verapamil (10^-8 mol/L); 6, ATRA (10^-8 mol/L) plus clotrimazole (10^-8 mol/L); 7, ATRA (10^-8 mol/L) plus verapamil (10^-8 mol/L) and clotrimazole (10^-8 mol/L).
Aryl Hydrocarbon (Benzo[a]pyrene) Hydroxylase (AHH) activity

Expression of CYP1A1 mRNA by RT-PCR

Fig 5. Detection of cytochrome P450 in leukemic cells. (A) Aryl hydrocarbon (benzo[a]pyrene) hydroxylase activity in HL-60 cells and RA-resistant HL-60 (RS HL-60) cells. The procedures used for the measurements of the activities are described in the Materials and Methods. Data represent the mean of triplicate experiments, the SD was within 10% of the mean. (B) Expression of CYP1A1 mRNA in leukemic cells by RT-PCR methods. Total RNA was extracted from various samples as follows: HepG2, human hepatoblastoma cells; HepG2/MC, HepG2 cells treated with 10^{-6} mol/L 3-methylcholanthrene (MC) for 24 hours; HL-60, wild-type HL-60 cells; RS HL-60, RA-resistant HL-60 cells; and leukemic cells from retinoid resistant APL patients (RS APL; no. 1 and 2) and RA-sensitive APL patients (no. 3 to 5). The 432-bp product indicates the presence of CYP1A1 mRNA. RT-PCR products were electrophoresed in agarose gel, transferred, and hybridized to CYP1A1-specific probe (middle panel). Lower panel represents RT-PCR of β-actin (838 bp) using the same RT product as for CYP1A1.

APL patients who relapsed after a remission was induced by ATRA could achieve a second remission by retinoid. Moreover, the point mutations of the RAR-α gene detected in the RA-resistant HL-60 cells developed under the artificial conditions of the continuous exposure to high concentrations of RA (≥10^{-6} mol/L). In contrast, the clinically administered ATRA is rapidly cleared from plasma, with a half-life of about 40 minutes and peak concentrations of less than 10^{-6} mol/L for only 1 to 2 hours.

Another potential mechanism for clinical resistance to RA is pharmacological alterations in the metabolism of ATRA. One possible explanation for accelerated clearance is the induction of cellular RA-binding proteins (CRABPs), resulting in binding cytoplasmic ATRA and lowering plasma and intracellular levels of active retinoids. Recently, Delva et al have reported that CRABP-II expression was increased at the time of relapse of APL compared with the levels before initiating ATRA treatment.

One important route of RA metabolism consists of the hydroxylation at position 4 of the cyclohexenyl ring to form

A. Flow Cytometric Analysis

B. RT-PCR

Fig 6. Expression of P-glycoprotein and MDR1 transcript. (A) FACS analysis. HL-60 and RA-resistant HL-60 (RS HL-60) cells were initially incubated with human AB serum to block Fc receptors and then were stained with monoclonal antibody MRK16, followed by staining with goat antimouse second antibody conjugated to FITC. Results are expressed as a fold-increased expression of P-glycoprotein antigen as compared with that of wild-type HL-60 cells. Data represent the mean of triplicate experiments; the SD was within 10% of the mean. (B) Southern blot of RT-PCR products of leukemic cells. RT-PCR products were electrophoresed, transferred, and hybridized with MDR1— and β-actin–specific probes.
4-hydroxy-RA, which is oxidized readily to 4-keto-RA. The 4-hydroxylation of RA is mediated by a cytochrome P450-dependent mono-oxygenase system in vitro. Thus, induction of cytochrome P450 enzymes may result in lowering intracellular levels of active retinoids in leukemic cells. Treatment with cytochrome P450 inhibitors such as ketoconazole and liarozole could reduce the accelerated catabolism of retinoids and increase the mean plasma ATRA concentrations in patients with APL and other cancers. We showed that the combination of ATRA and nonspecific cytochrome P450 inhibitor, clotrimazole, could induce differentiation of RA-resistant HL-60 cells and fresh RA-resistant APL cells into mature granulocytes in vitro. Also, this combination markedly decreased cellular proliferation of the cells. However, contrary to expectations, we could not detect the increased levels of cytochrome P450 activity as measured by AHH activity in RA-resistant leukemic cells. Moreover, levels of AHH activity in wild-type HL-60 cells were very low and almost equal to those present in RA-resistant cells. The cytochrome P450 superfamily consists of several enzymes, and at least 19 forms are responsible for the metabolism of foreign substrates in humans. A recent study has shown that an RAR-response element is present in the promoter region of the CYP1A1 gene; thus, by RT-PCR method, we examined the expression of the CYP1A1 gene in the leukemic cells. No CYP1A1 transcripts were detectable in either RA-sensitive or RA-resistant leukemic cells, although CYP1A1 is considered to be a form expressed ubiquitously in body tissues. These results suggest that AHH activity is not related to RA metabolism and the CYP1A1 gene is not a major factor in the rapid catabolism of retinoids in RA-resistant leukemic cells. Further studies will be needed to identify the isoform of cytochrome P450 related to RA metabolism by using specific cytochrome P450 inhibitors and more detailed molecular analyses in leukemic cells.

The other catabolic pathway of RA may involve an interaction of lipid hydroperoxides in cancer patients. Recent studies have shown that RA can be oxidized to inactivate metabolites by lipid hydroperoxides. Thus, the imidazoles such as clotrimazole generate the lipid hydroperoxides that may be involved RA metabolism.

Studies have shown that resistance to multiple chemotherapeutic agents is related to the expression of P-glycoprotein, a transmembrane drug efflux pump that is encoded by MDR. In addition, the P-glycoprotein is an energy-dependent efflux pump for various lipophilic compounds; thus, we speculated that retinoids might be involved in this transportation system and decreased intracellular accumulation of RA may occur in RA-resistant leukemic cells. Combination of ATRA and the P-glycoprotein antagonist, verapamil, but neither of them alone, induced morphological and functional differentiation of RA-resistant leukemic cells. Although we could detect only low level of P-glycoprotein in both RA-sensitive and RA-resistant HL-60 cells, the RA-resistant leukemic cells expressed MDR1 mRNA, but the RA-sensitive leukemic cells did not. To measure the low and heterogenous levels of expression of MDR1/P-glycoprotein, the PCR-based method might be more reliable and sensitive.

A recent study showed significantly lower P-glycoprotein expression in APL than in other types of AML. These results suggest that a metabolic pathway of retinoids might exist that depends on P-glycoprotein and whose abnormal expression may be responsible in part for retinoid resistance in leukemic cells.

From our results and previous studies, several possible mechanisms of retinoid resistance may occur in leukemic cells (Fig 7). Based on these findings, a couple of strategies are possible to overcome RA resistance in APL patients. To date, most approaches have not yet been successful in overcoming RA resistance in vivo. This might indicate a redundancy and complexity of the biological mechanisms of retinoid resistance. Therefore, the combination of several approaches may be effective for the treatment of patients with RA-resistant APL. For example, we have shown that the simultaneous exposure of a cytochrome P450 inhibitor and a P-glycoprotein antagonist with ATRA showed surprisingly potent ability in inhibiting proliferation and inducing differentiation of RA-resistant leukemic cells.

In summary, retinoid resistance is a serious problem of differentiation-inducing therapy for patients with APL, as well as for patients with other cancers. The data presented in this report strongly suggest that acquired resistance to RA may be explained in part by the drug metabolism in leukemic cells. Moreover, the combination of ATRA and cytochrome P450 inhibitors and/or P-glycoprotein antagonists might be able to overcome the resistance to RA in APL and a variety of cancers.
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