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Expression of the Retinoic Acid-Metabolizing Enzyme CYP26A1 Limits Programmed Cell Death

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ABSTRACT

Vitamin A deficiency has been associated with increased incidence of certain types of cancer; however, the mechanisms by which vitamin A depletion promotes tumorigenesis are poorly understood. In addition all-*trans*-retinoic acid (RA), the most active form of vitamin A metabolites, has been shown to limit carcinogenesis in animal models and to trigger programmed cell death (apoptosis) in certain types of tumor cells. On the other hand, we show here that various cell lines overexpressing CYP26A1, a cytochrome P450 enzyme specifically involved in the catabolic inactivation of RA, exhibit increased resistance to various apoptogenic factors, including death receptor ligands such as tumor necrosis factor-related apoptosis-inducing ligand. This resistance could be reversed by pretreatment with ketoconazole, a broad-spectrum inhibitor of cytochrome P450 enzymes. In addition, synthetic retinoids Am80 (4[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbamoyl]benzoic acid) and Am580 [4(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphtamido)benzoic acid], which are resistant to CYP26A1 metabolism, can restore the sensitivity of these cells to apoptogens. Thus, these findings support the idea that CYP26 expression levels may play a role in determining cellular commitment to apoptosis, and increased RA metabolism may be at least partially responsible for these observed effects.

The regulation of programmed cell death (also known as apoptosis) is a process critical for both normal embryonic development and turnover of healthy tissue in the adult. In a number of disease states, however, the pivotal balance between proapoptotic and cell survival signals is disrupted, leading to loss of healthy cells as in neurodegenerative disorders or failure to eliminate genetically damaged cells, leading to cancer (Evan and Vousden, 2001; Green and Evan, 2002). The mechanisms controlling cellular self-destruction programs are tightly controlled, and many physiological growth control signals that govern cell proliferation and tissue homeostasis are linked to apoptosis (Evan and Vousden, 2001; Gozani et al., 2002; Green and Evan, 2002). Apoptosis pathways can be activated in a cell-specific manner by a diversity of distinct triggers, including death receptor ligands such as tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), Fas and TNF-α, retinoids, genotoxins, oxidative stress, nutrient deprivation, and anoikis (anchorage dependence-mediated cell death).

The vitamin A metabolite all-*trans*-retinoic acid (RA) is an essential signaling molecule in embryonic development and throughout life; a potent regulator of cell differentiation, proliferation, and apoptosis in various cell types (Durston et al., 1989; Means and Gudas, 1995). RA acts through specific RA nuclear receptors (RARα, β, and γ) and their heterodimeric counterparts, the retinoid-X-receptors (α, β, and γ) to positively or negatively regulate expression of RA target genes by binding to their respective response elements (Kastner et al., 1995; Mangelsdorf and Evans, 1995). Disruptions in retinoid signaling through mutations in nuclear receptors RARs and retinoid-X-receptors have been found in certain types of tumor cells (de Thé, 1996). Vitamin A deficiency has been linked to increased susceptibility to carcinogenesis in animal models and seems to be causally associated with tumor formation and progression (first reported by Wolbach and Howe, 1925; for review, see Lotan, 1996).

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ABBREVIATIONS: TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; RA, all-*trans*-retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; APL, acute promyelocytic leukemia; FBS, fetal bovine serum; PCR, polymerase chain reaction; EGFP, enhanced green fluorescent protein; PBS, phosphate-buffered saline; DAPI, 4,6-diamidino-2-phenylindole; PBST, phosphate-buffered saline/0.5% Tween 20; wt, wild-type; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Am80, 4[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbamoyl]benzoic acid; Am580, 4(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphtamido)benzoic acid; IAP, inhibitor of apoptosis protein.

Although essentially all cell types express nuclear retinoic acid receptors, cellular responsiveness is determined by RA bioavailability regulated by the coordinated balance between vitamin A nutritional status and RA biosynthesis and catabolism. We and others have previously demonstrated that expression of the RA-metabolizing cytochrome P450s CYP26A1, B1, and C1 protects cells and tissues from exposure to RA (generated by retinaldehyde dehydrogenase enzymes) during embryogenesis by restricting RA access to transcriptional machinery by converting RA into rapidly excreted oxoderivatives (4-OH RA, 4-oxo RA, 18-OH RA) (White et al., 1997; Abu-Abed et al., 2001; MacLean et al., 2001; Tahayato et al., 2003; Taimi et al., 2004). CYP26 enzymes may play a similar but separate role in limiting the consequences of fluctuations in nutritional vitamin A. The possibility that pathological conditions such as cancer might involve aberrant expression of CYP26A1 has recently emerged from several studies. Elevated CYP26A1 expression and RA catabolic activity have been detected in breast epithelial adenocarcinoma cells in culture (Van Heusden et al., 1998), leukemic cells from patients with acute promyelocytic leukemia (APL) (Ozpolat et al., 2002), and cells derived from squamous cell carcinoma from head and neck cancers (Klaassen et al., 2001); however, the relevance of elevated CYP26A1 activity in tumor cells remains to be fully clarified. In the present study, by overexpressing CYP26A1 in various cell lines, we wanted to generate a state of RA deficiency in cultured cells to examine whether RA was an obligatory component of apoptosis signaling pathways. We demonstrate that CYP26A1 expression can modify cellular sensitivity to various apoptogens.

Materials and Methods

Cell Line and Culture. All cell lines were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Burlington, ON, Canada) supplemented with 10% FBS, 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified 5% CO_2 atmosphere unless otherwise specified.

Construction of Expression Vector and Transfection. Full-length human CYP26A1 cDNA was amplified by reverse transcriptase-polymerase chain reaction (PCR) from 1 μg of total RNA extracted from RA-treated MCF-7 cells, which was reported to be CYP26A1-inducible after 1 μM RA treatment (White et al., 1997), using an EcoRI site-tagged forward primer (5′-GAATCCATGGGGCTCCCGGCGCTGCT-3′) and a BamH1 site-tagged reverse primer (5′-GGATTCTCAGATTTCCCCATGGAAAT-3′). After subcloning into a TA-cloning vector (pCR II) using a TA cloning kit (Invitrogen), the digested 1505-base pair EcoRI-BamHI fragment was ligated into the response plasmid pEGFP-C1 (BD Biosciences Clontech, Palo Alto, CA), designated as pEGFP-C1-CYP26A1 plasmid. The integrity of the final pEGFP-C1-CYP26A1 construct was confirmed by sequence analysis. As a control vector, pCMS-EGFP-CYP26A1 plasmid, which has separate transcription cassettes containing EGFP and CYP26A1 cDNA, was made by the same method except in using an EcoRI site-tagged forward primer (5′-GAATCCGCCGCCACCATGGGGCTCCCGGCGCTGCT-3′) and an XbaI-tagged reverse primer (5′-TCTAGATCAGATTTCCCCATGGAAAT-3′) for PCR and pCMS-EGFP as an expression vector (BD Biosciences Clontech).

pEGFP-C1-CYP26A1 alone (5 μg) or plasmid mixture of pCMS-EGFP-CYP26A1 (5 μg) plus pcDNA3.1(−) (1 μg) was transfected into a number of different cell lines containing HeLa, human cervical cancer cells; MCF-7, human breast cancer cells; A549, nonsmall cell lung cancer cells; and Hep3B, human hepatocellular carcinoma cells, using LipofectAMINE reagent (Invitrogen). G418 (Geneticin) (800 μg/ml; Sigma Diagnostics, Oakville, ON, Canada)-resistant clones were screened by immunofluorescence microscopy followed by culturing on glass coverslips (Fisher Scientific Co., Toronto, ON, Canada) to confirm expression of EGFP or EGFP fusion proteins. Stably transfected clones were also screened both by Northern blot analysis for CYP26A1 mRNA expression and metabolic assay for measuring the metabolism of radiolabeled RA ([^{3}H]RA) as described previously (White et al., 1997).

Cell Death Analyses. Cells were plated at 1×10^5 cells per six-well plates and incubated for 24 h before 5-min pulse treatment of 0.1 μM RA or various concentrations as indicated and were washed twice with phosphate-buffered saline (PBS) followed by replacement with Dulbecco's modified Eagle's medium supplemented with 10% charcoal-dextran-treated FBS. To avoid the significant accumulation of RA metabolites, RA treatment was very short (5 min), which is known to be sufficient to switch on the retinoic acid response element-driven transcriptional machinery. Thereafter, apoptosis was stimulated with TRAIL (R&D Systems, Minneapolis, MN) at a dose range of 0 to 20 ng/ml for 0.5 h up to 24 h. These cells were also treated with various death-inducing agents, including agonistic Fas antibody (0.025–10 μg/ml, clone CH-11; Panvera, Madison, WI), TNF-α (1–100 ng/ml; R&D Systems), H_2O_2 (50 or 100 μM), cisplatin (10 μg/ml), etoposide (VP16; 50 μM), heat shock at 42°C for 30 min, and γ-irradiation (20 Gy). In some experiments, ketoconazole (0.5–10 μM; Sigma Diagnostics) was added 4 h before apoptotic stimulation and then maintained throughout the experiments.

Apoptotic assays and quantification were described in detail previously (Osanai et al., 1997). In brief, floating dead cells were pelleted by centrifugation and washed with PBS. The pellet was resuspended in lysis buffer containing 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 0.5% Triton X-100, and centrifuged at 12,000*g* for 10 min at 4°C. Each supernatant, which preferentially contains low molecular weight cellular DNA, was once extracted with phenol/chloroform (1:1), and the fragmented DNA was ethanol-precipitated. Gel electrophoresis of the DNA samples was performed on 2.5% agarose gels, and ladder formation caused by fragmentation was visualized with ethidium bromide under ultraviolet illumination.

The DNA fragmentation rate was also quantified by the method described in Osanai et al. (1997). In brief, total cells in the tissue culture wells were harvested, washed with PBS, and lysed with lysis buffer containing 5 mM Tris-HCl, pH 8.0, 20 mM EDTA, and 0.5% Triton X-100 for 15 min at 4°C. The cells were then centrifuged at 12,000*g* for 20 min at 4°C to separate intact chromatin in the pellet from DNA fragments in the supernatant. The DNA amounts in each pellet and supernatant were determined using a diphenylamine reagent, and the fragmentation rate (percentage) was expressed as (fragmented DNA/total DNA) × 100.

DAPI Staining. Cells grown on glass coverslips for confocal microscopy were washed once with PBS and then incubated in 4,6-diamidino-2-phenylindole (DAPI) (1 μg/ml; Molecular Probes, Eugene, OR) solution for 15 min at 37°C.

Cell Counting, Giemsa Staining, and Mitotic Index Measurement. These assays were performed as described in detail previously (Hsu et al., 1999). The mitotic index was estimated by counting at least 1000 cells of each treatment at 400× magnification by light microscopy. The percentage of mitotic cells was calculated.

Cell Cycle Analysis by Flow Cytometry. Cells on 100-mm tissue culture dishes were treated with 0.5 μM RA for 5 min before harvesting by centrifugation, and permeabilized with ice-cold 70% ethanol for at least 30 min. After washing with PBS, cells were treated with PBS containing 100 μg/ml DNase free-RNase A at 37°C for 30 min. After centrifugation, cells were suspended in PBS containing 50 μg/ml propidium iodide and stained at 37°C for 30 min. DNA content was analyzed by FACScan (BD Biosciences, San Jose, CA).

Western Blot Analysis. EGFP and EGFP fusion protein, Bcl-X_L, caspase-3, cytochrome *c*, p21^{Waf1}, and β-actin protein levels were

examined by Western blot analysis. Cell fractionation and mitochondrial isolation were performed as reported previously (Yang et al., 1997). From 1×10^7 intact cells for each sample, mitochondrial supernatants (called cytosolic fraction) and the resulting pellets containing mitochondria (designated as mitochondrial fraction) were aliquoted and subjected to Western blot analysis for cytochrome *c*. Whole cell lysates or fractionated lysates were run on 12 to 15% polyacrylamide gels containing SDS and electroblotted onto nitrocellulose filters. These were then blocked with 5% nonfat dry milk in PBS with 0.5% Tween 20 (PBST) and then immunoblotted with antibodies against EGFP (BD Biosciences Clontech), Bcl-X_L (BD Biosciences PharMingen, Mississauga, ON, Canada), caspase-3 (BD Biosciences PharMingen), cytochrome *c* (BD Biosciences PharMingen), $p21^{Waf1}$ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and β-actin (Santa Cruz Biotechnology, Inc.) protein. After extensive washing in PBST, the filters were reacted with peroxidase-labeled corresponding secondary antibodies in PBST and again washed. Finally, the immunoreactions were visualized by using an enhanced chemiluminescence system (Amersham Biosciences Inc., Barie d'Urfé, PQ, Canada).

For densitometric analysis, signals in Western blot from at least triplicate independent experiments were quantitated using Scion Image 1.62 (Scion Corporation, Frederick, MD), and the data are presented as mean ± S.D.

Semiquantitative Reverse Transcriptase-PCR and Southern Analysis. Total RNA (1 μg) from each specimen was reverse-transcribed using poly(T) oligonucleotide and Moloney murine leukemia virus reverse transcriptase (Roche Diagnostics, Tokyo, Japan). For analysis of RARα expression, RARα was amplified from 100 times diluted cDNA using sense (5′-CAAATCATCCGGCTACCACT-3′) and antisense (5′-TTGAGGAGGGTGATCTGGTC-3′) primers for 25 cycles at 96°C for 10 s, 52°C for 5 s, and 72°C for 30 s. Electrophoresed DNA from the agarose gel was transferred to the Hybond N^+ nylon membrane (Amersham Biosciences Inc., Tokyo, Japan). Full-length cDNA of RARα was used as a probe labeling with [^{32}P]dCTP to a specific activity of approximately 1×10^9 cpm/μg using a random priming-based cDNA labeling kit (Nippon Gene, Tokyo, Japan). We examined various cycling parameters for each PCR experiment to define optimal conditions for linearity to allow for semiquantitative analysis of signal intensity (data not shown).

cDNA Array Analysis. Total RNA was extracted from wt HeLa cells and its CYP26A1 transfectants using TRIzol reagent (Invitrogen), and aliquots of RNA (5 μg) were processed to generate cDNA probes. cDNA microarray analysis was performed to analyze the apoptosis-specific signaling pathway by GEArray according to a manufacturer's instructions, and mRNA levels were quantitated by GEArrayAnalyzer software (SuperArray Inc., Bethesda, MD). The relative amount of a given gene was normalized to the signals derived from GAPDH on the same membrane and expressed in arbitrary units (therefore the expression of GAPDH is 1 arbitrary unit), to consider the possible variation in RNA quantification between samples. Finally, average signal intensities of each gene were calculated from at least two independent experiments, and the gene expression levels in CYP26A1-overexpressing cells were divided by the values from that observed in wt cells to represent the change of gene expression in the event of the CYP26A1 overexpression.

Statistical Analysis. Unless otherwise specified, all data represent the mean ± S.D. of at least three independent experiments, each in triplicate wells. Statistical differences were analyzed using the paired *t* test and were considered statistically significant when $p < 0.05$.

Results

Increased Resistance of TRAIL-Induced Cell Death by CYP26A1 Expression. In the present study, we established a human cervical cancer cell line HeLa, constitutively overexpressing human CYP26A1, designated as $HeLa^{CYP26A1}$. To facilitate detection of CYP26A1 expression, we used a fusion construct comprising the entire coding region of the CYP26A1 cDNA linked to the EGFP encoding gene (pEGFP-C1-CYP26A1). We first determined the differential sensitivity of these cells to a 24-h exposure to TRAIL, which activates cell death response through the death receptor-mediated pathway. Before treatment with TRAIL, cells were briefly exposed to a pulse of 0.1 μM RA. RA increased the sensitivity of HeLa cells to TRAIL-induced apoptosis in wt cells; however, $HeLa^{CYP26A1}$ cells showed significant resistance to synergistic apoptotic effects of TRAIL in the range of 0.025 to 20 ng/ml with 0.1 μM RA (Fig. 1A, left). To exclude the possible artifacts of the EGFP-CYP26A1 fusion protein, we also examined the control vector, pCMS-EGFP-CYP26A1, which expresses EGFP and CYP26A1 from different transcription cassettes. Cells transfected with this plasmid showed effects similar to those obtained with the pEGFP-C1-CYP26A1 construct (Fig. 1A, right). We also observed TRAIL resistance in the $HeLa^{CYP26A1}$ cells in experiments performed with serial time points after TRAIL treatment (Fig. 1B). Furthermore, $HeLa^{CYP26A1}$ cells required approximately 10-fold higher concentrations of RA to observe the same degree of death in wt cells in the range from 0.01 to 10 μM (Fig. 1C). DNA fragmentation analysis and morphological studies at 24 h also confirmed time-dependent activation of apoptosis by TRAIL in wt cells and reduced apoptotic induction in $HeLa^{CYP26A1}$ cells (Fig. 1, D and E).

To evaluate how general the suppressive effect of CYP26A1 expression was on TRAIL signaling, we stably transfected other cell lines, including MCF-7, A549, and Hep3B cells, with the same construct to establish overexpressing clones. Although these cell types were responsive to TRAIL-mediated apoptosis, CYP26A1 expression had similar suppressive effects on cell responsiveness with or without pretreatment of RA (Fig. 1F; data not shown).

We also overexpressed another cytochrome P450, CYP24, involved in the metabolism of 1α,25-dihydroxyvitamin D3, in both HeLa and A549 cells; however, we were unable to detect any effect of CYP24 on the apoptotic potential of these cells (data not shown), suggesting that the antiapoptotic effect is CYP26 specific.

Apoptotic Suppression by CYP26A1 Is Not Specific for TRAIL-Mediated Apoptosis. We next determined whether the suppressive role played by CYP26A1 was applicable to other apoptogenic stimuli, including oxidative stress, heat shock, genotoxic agents, and γ-irradiation. CYP26A1 expression resulted in the appreciable inhibition of apoptotic cell death against these stimuli (Fig. 2, A and B), suggesting that the protective role of CYP26A1 against apoptosis could be observed after exposure to a broad range of cell stressors.

Direct Interactions between RA Metabolism and the Susceptibility of Apoptotic Cell Death. Ketoconazole, a broad-spectrum inhibitor of cytochrome P450s that we have previously shown can inhibit CYP26A1 activity (Chithalen et al., 2002), was next used to establish whether the effect of CYP26A1 on cell death inhibition was mediated by its role in conversion of RA into polar metabolites. Expression data of $HeLa^{CYP26A1}$ cells is shown, in which CYP26A1-EGFP fusion protein could be clearly detected by Western blot analysis using EGFP antibody, revealing strong catabolic activity of RA that was inhibited by ketoconazole treatment (Fig. 3, A

and B). Ketoconazole alone did not significantly affect cell viability. Although other members of the TNF family such as Fas and TNF-α could induce apoptosis in wt cells, the cells overexpressing CYP26A1 were also protected from these apoptogenic factors (Fig. 3C). Pretreatment of CYP26A1 transfected cells with ketoconazole, however, restored sensitivity of these cells to cell death stimuli, suggesting that the catalytic activity of this enzyme was important for its anti-apoptotic effect on these cells.

The RARα-selective compounds Am80 and Am580, which are resistant to metabolism by CYP26A1, were reported to stimulate apoptosis in susceptible cells as efficiently as RAR-panactive retinoids, including RA (Schneider et al., 2000; Luu et al., 2001). We used these reagents to further demonstrate the direct relationship between CYP26A1-mediated RA catabolism and apoptosis resistance. In a dose range from 0.5 to 10 nM, a clear dose dependence of apoptosis was found in cells that were treated with Am80 and Am580 (data not

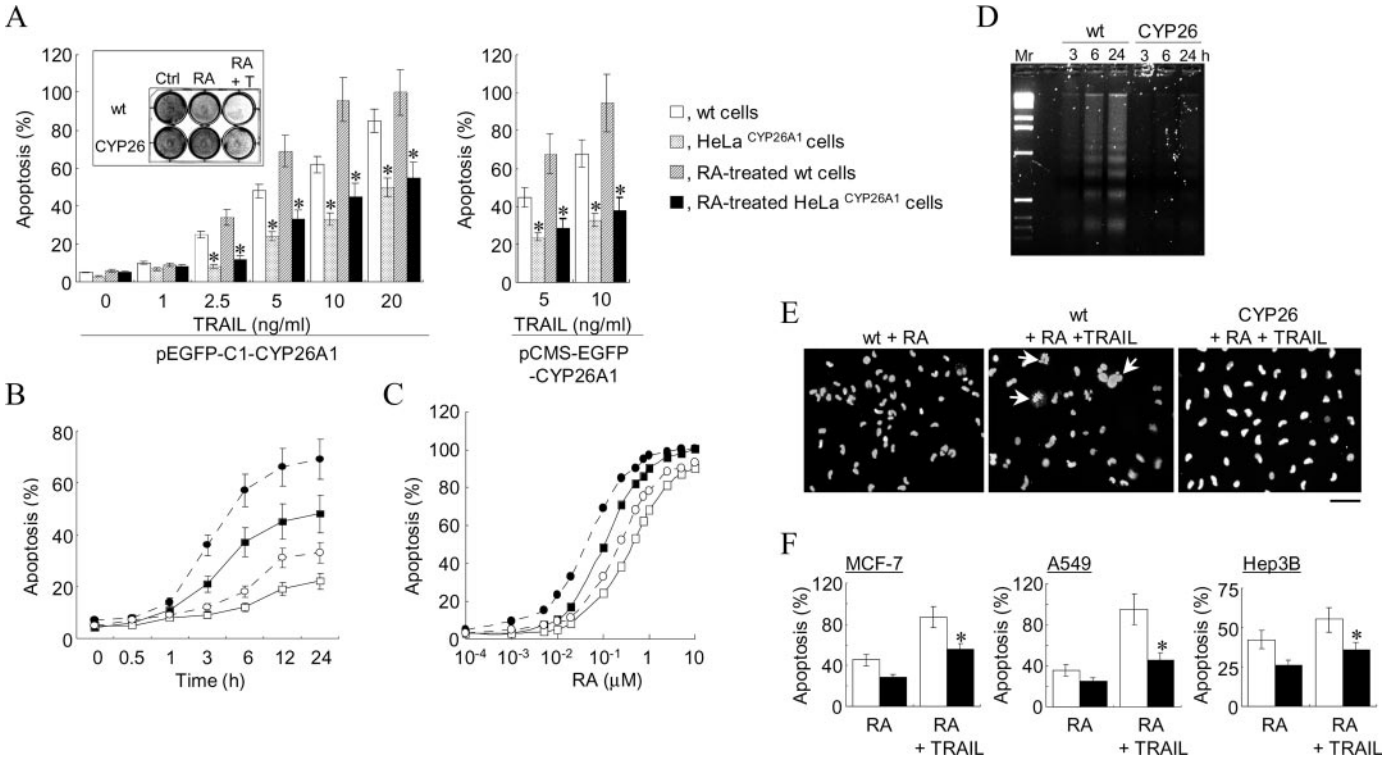


Fig. 1. CYP26A1 overexpression provides resistance to TRAIL-induced apoptosis. A, cell death was examined after 24 h in the presence or absence of various concentrations of TRAIL with or without 0.1 μM RA pretreatment in wt and HeLa cells transfected with two different CYP26A1 expression constructs (pEGFP-C1-CYP26A1 and pCMS-EGFP-CYP26A1). Inset shows the Giemsa staining of cell culture plate treated for 24 h with either 5 ng/ml TRAIL (T) and/or 0.1 μM RA in wt and HeLa^{CYP26A1} (CYP26) cells. *, $p < 0.05$ versus wt cells with or without RA treatment. B and C, death of wt (filled symbol) and HeLa^{CYP26A1} (open symbol) cells with (circle) or without (square) 0.1 μM RA pretreatment at the indicated time points up to 24 h (B) and various concentrations of RA after 24 h (C) in the presence of 5 ng/ml TRAIL. D, DNA laddering analysis to examine the type of cell death at the indicated time points after 5 ng/ml TRAIL treatments in wt and HeLa^{CYP26A1} (CYP26) cells. Molecular marker is shown in the leftmost lane (Mr). E, nuclear morphology of wt and HeLa^{CYP26A1} (CYP26) cells stained by DAPI after culturing glass coverslips with or without either 5 ng/ml TRAIL or 0.5 μM RA. Arrow indicated the fragmented nuclei, note the differential background cell densities. Bar, 50 μm. F, other cell lines, including MCF-7, A549, and Hep3B overexpressing CYP26A1 were assayed the sensitivity of cell death against stimulation of 0.1 μM RA and/or 5, 10, or 25 ng/ml TRAIL, respectively. White column, wt; black column, CYP26A1-overexpressing cells. *, $p < 0.05$ versus wt cells.

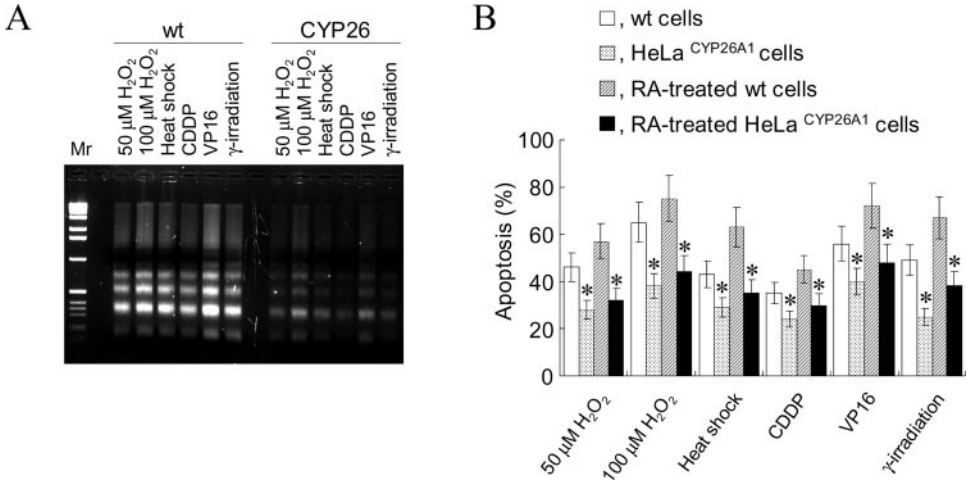


Fig. 2. CYP26A1 expression protects cells from various proapoptotic cell stresses. A, representative gel image to examine the differential sensitivity of wt and HeLa^{CYP26A1} (CYP26) cells in the response to various apoptogenic stimuli. B, cell deaths were quantitated after 24 h in the presence or absence of 0.1 μM RA pretreatment with or without various death stimuli used in A. *, $p < 0.05$ versus wt cells with or without RA treatment.

shown). The treatment of cells with these reagents in combination with death receptor ligand abrogated the inhibitory effect of CYP26A1 expression on TRAIL-, Fas-, or TNF-α-mediated apoptosis in HeLa^{CYP26A1} cells (Fig. 3D). Am80 was not as effective as Am580 in inducing apoptosis in HeLa cells, requiring approximately 5-fold higher concentrations to produce similar effects. These data strongly indicate that RA metabolism is associated with the suppression of apoptotic responsiveness in CYP26A1-expressing cells.

To confirm that the proapoptotic effects of RA were indeed mediated through a RAR signaling pathway, we examined the expression level of RARα. HeLa and HeLa^{CYP26A1} cells were clearly expressed RARα, but not RARβ or RARγ, and RA could induce mRNA expression in wt HeLa cells approximately 6-fold (Fig. 3E; data not shown), which is consistent with previous literature (Daly et al., 1989; Geisen et al., 1997). Together with our observation that the cells respond comparably to RA under all conditions even when CYP26A1 is overexpressed as well as Am80/Am580, this result indicates that the gain-of-function effects of CYP26A1 on apoptosis could be mediated through an RAR mechanism of action.

The apoptosis-sensitizing effect of RA has been demonstrated in a number of different experiments. Although the effect may be less than that observed for Am80 and Am580, it is consistent and significant. We have observed the RA effect on proapoptogenic agents in many different cell lines and observed different levels of response. The difference between the RA-response observed in Fig. 3C (comparing top and bottom) reflects differences in cell type. Why some cell lines respond better than others may reflect inherent differences in the induction of endogenous CYP26A1, B1, and/or C1. HeLa cells, for example, are known to express CYP26A1 and B1, and both enzymes are inducible by RA (our unpublished data).

CYP26A1 Overexpression Mediates Aberrant Mitotic Progression. Many lines of evidence indicate that apoptosis is linked to cell cycle events (Evan and Vousden, 2001). To determine the potential association of CYP26A1 expression with cell cycling, we investigated the differences of cell growth rates, cell cycle analysis, and mitotic index between CYP26A1 expressing and nonexpressing cells (Fig. 4). CYP26A1 expression had a slight promoting effect on cell proliferation (Fig. 4A), but it had a much greater effect on mitotic index (Fig. 4B). The effect of RA was minimal on cell growth; however, mitotic index was significantly suppressed by RA treatment in wt cells. By contrast, a decrease in mitotic index induced by RA was significantly abrogated in HeLa^{CYP26A1} cells, even when both TRAIL and RA were added to the cultures (Fig. 4B). Cell cycle analyses clearly indicated that RA-induced G_1 arrest and a concomitant decrease in S-phase fractions were partially abrogated in HeLa^{CYP26A1} cells, suggesting that CYP26A1-induced RA catabolism involves the G_1/S-phase transition and allows cells to escape from RA-mediated specific cell cycle checkpoints (Fig. 4C).

Altered Gene Expression Profile Provides Antiapoptotic State in CYP26A1-Expressing Cells. To explore the molecular mechanisms of the apoptotic suppression caused

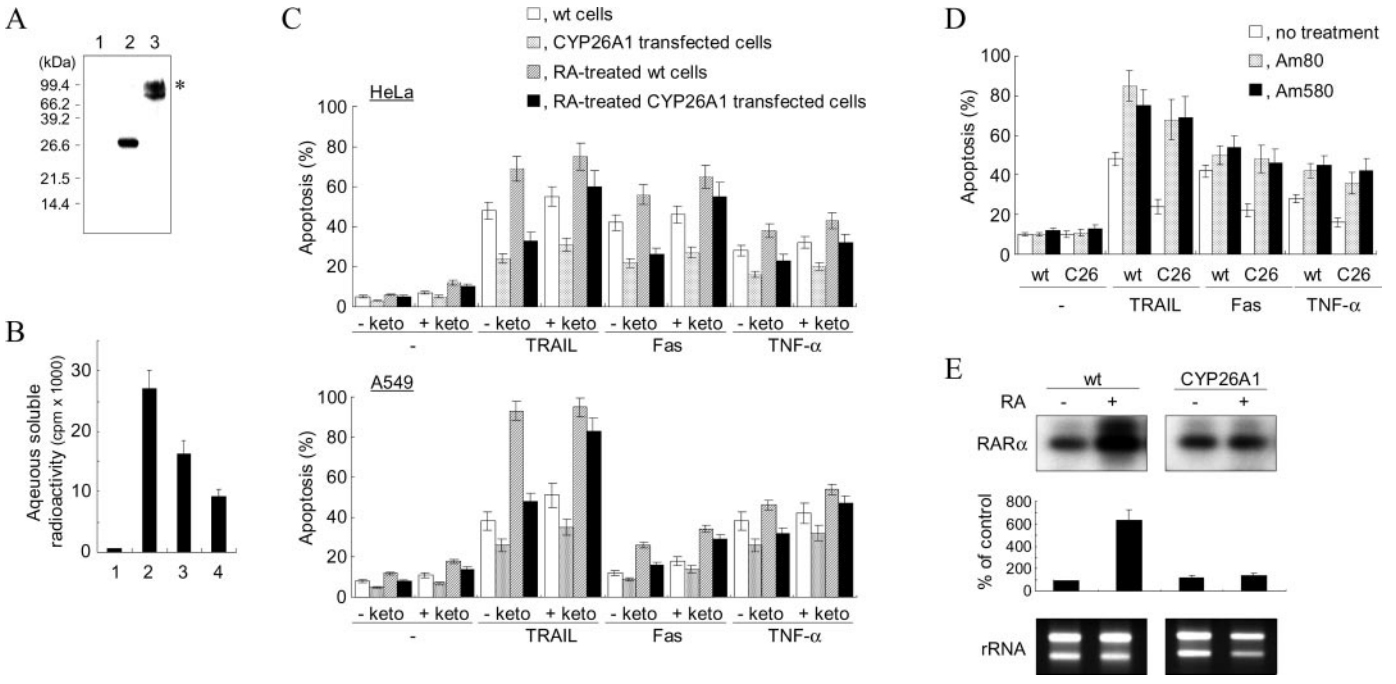


Fig. 3. Preferential interactions between RA metabolism and the susceptibility of apoptosis. A, HeLa^{CYP26A1} cells express EGFP-CYP26A1 fusion protein (83 kDa, lane 3), which has different motility in SDS-polyacrylamide gel electrophoresis compared with the cells transfected with EGFP expressing vector only (27 kDa, lane 2) or wt cells (lane 1). Asterisk (*) denotes nonspecific band. B, metabolic assay in HeLa and HeLa^{CYP26A1} cells using [^{3}H]RA as a substrate showed that EGFP-CYP26A1 fusion protein has a catabolic activity to detect conversion of RA to water-soluble metabolites (lane 2) compared with wt cells (lane 1). This metabolic activity was suppressed with the pretreatment of 1 μM (lane 3) or 5 μM (lane 4) ketoconazole, a chemical inhibitor of CYP26A1. C, partial resensitization of CYP26A1-mediated apoptotic suppression by 5 μM ketoconazole (keto) in the response to either TRAIL (5 ng/ml), Fas (2.5 μg/ml), or TNF-α (25 ng/ml) alone or in combination with 0.1 μM RA in HeLa^{CYP26A1} and A549 ^{CYP26A1} cells. D, absence of inhibitory effect of TRAIL-, Fas-, or TNF-α-mediated apoptosis in the presence of either Am80 (10 nM) or Am580 (2.5 nM) alone or in combination with TRAIL (5 ng/ml), Fas (2.5 μg/ml), or TNF-α (25 ng/ml) in wt and HeLa^{CYP26A1} (C26) cells. E, RARα expression in HeLa and HeLa^{CYP26A1} cells with or without 1 μM treatment. Equal loading is demonstrated by ribosomal RNA. We also show the densitometric analysis of the RARα signals on the blot from triplicate independent experiments. *, $p < 0.05$ versus cell without RA treatment.

by CYP26A1 expression, we screened the gene-expression profile with the GEArray system. Total RNA was isolated from wt and HeLa^{CYP26A1} cells that had been cultured for 24 h with or without 1 μM RA, and aliquots of 5 μg of total RNA were reverse-transcribed into cDNA, labeled with ^{32}P and hybridized to GEArray apoptosis pathway-specific gene array membranes. Some differences of gene expression profile between wt and HeLa^{CYP26A1} cells were observed without RA stimulation; however, the differences were more apparent in the presence of RA (Fig. 5A). Although CYP26A1 overexpression induces up-regulation of apoptosis-inhibitory genes such as *bcl-x*, *bcl-w*, and *bcl-2* and conversely down-regulation of apoptosis-inducing genes such as *bad*, *bax*, and *bak*, we observed more marked changes in inhibitor of apoptosis protein (IAP) family genes, including survin, IAP-1 and IAP-2, and XIAP. CYP26A1 expression also up-regulates a number of TNF ligand family members; however, its expression concomitantly decreases a larger set of apoptosis-associating genes, including caspase family (caspase-1–10, -13, and -14), TNF receptor family (TNFR2, CD40, OX40, Fas, DR3, DR5, DcR1, and DcR2), death domain family (CRADD, FADD, and DAP kinase 2), death effecter domain family (CASPER), and p53 and ATM pathway genes (ATM, Rad53, mdm2, p63, and p53). These gene expression alterations are consistent with CYP26A1-mediated apoptotic suppression in HeLa cells.

To further investigate the potential role of *bcl-2* family members played in mediating the antiapoptotic activity of CYP26A1 overexpression, we focused on the Bcl-X_L and investigated protein expression levels by Western blot analysis (Fig. 5B). Bcl-X_L protein was detectable in wt HeLa cells and both RA and H_2O_2-mediated protein decreases were observed after 24 h treatment with H_2O_2 in combination with or without RA in wt cells. In contrast, RA and H_2O_2, either alone or in combination could induce up-regulation of Bcl-X_L protein levels in HeLa^{CYP26A1} cells. No cleaved forms of Bcl-X_L were observed under any condition. These results are consistent with findings using the gene expression array analysis and suggest that certain Bcl-2 family proteins are modulated by CYP26A1 expression.

Cytochrome *c* is normally located in the intermembrane space of mitochondria, and its release is known to be one of the upstream mitochondrial events that can contribute to caspase activation (Ferri and Kroemer, 2001). Once the cytochrome *c* is released into the cytosol in response to apoptotic stimulation, it initiates a caspase activation cascade (Ferri and Kroemer, 2001). We next examined whether CYP26A1 expression affects cytochrome *c* release (Fig. 5B). We found that cytochrome *c* was largely located in the mitochondria in both wt and HeLa^{CYP26A1} cells. This protein was slightly increased in the mitochondrial compartment after RA treatment but further enhanced by cotreatment with H_2O_2. In wt cells, this protein shifted from the mitochondria to cytosol and rapidly accumulated to significant levels by 24 h of treatment. However, we observed less alteration in cytochrome *c* protein levels and/or minimal compartmental shifts in response to either RA or H_2O_2 in HeLa^{CYP26A1} cells. These results suggest that the abrogation of RA-induced Bcl-X_L up-regulation in CYP26A1-expressing cells may at least partially contribute to the suppression of cytochrome *c* induction and/or redistribution.

Caspase-3 is a key effector caspase in the proteolytic cascade that results in cell death (Ferri and Kroemer, 2001). Caspase-3 expression was also examined by Western blot analysis (Fig. 5B). Both RA- and H_2O_2-induced apoptosis was accompanied by activation of caspase-3, indicated by the presence of activated caspase-3 p17 fragment, at 24 h after treatment. Only the inactive procaspase (p32) was detectable in HeLa^{CYP26A1} cells, suggesting that the expression of CYP26A1 could inhibit the activation of effector caspases through the modulation of the death signals upstream of caspase-3. This result is also supported by the data obtained from the gene expression signature of HeLa^{CYP26A1} cells, showing that death signaling was significantly suppressed at both the receptor and effector levels, which resulted in the inhibition of caspase cascade activation.

Apoptosis may be linked to cell cycle events, and several cell cycle regulators might be involved in responses that lead to apoptosis (Evan and Vousden, 2001). Because our preliminary study showed that expression of p21^{Waf1}, a cyclin-dependent kinase inhibitor involved in the regulation of apoptosis in a number of carcinoma cells, was altered because of

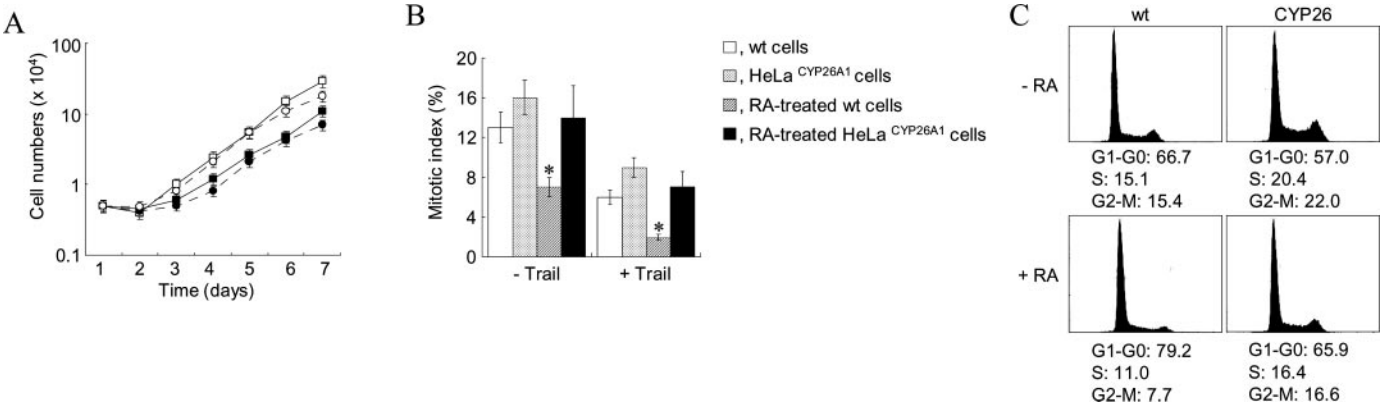
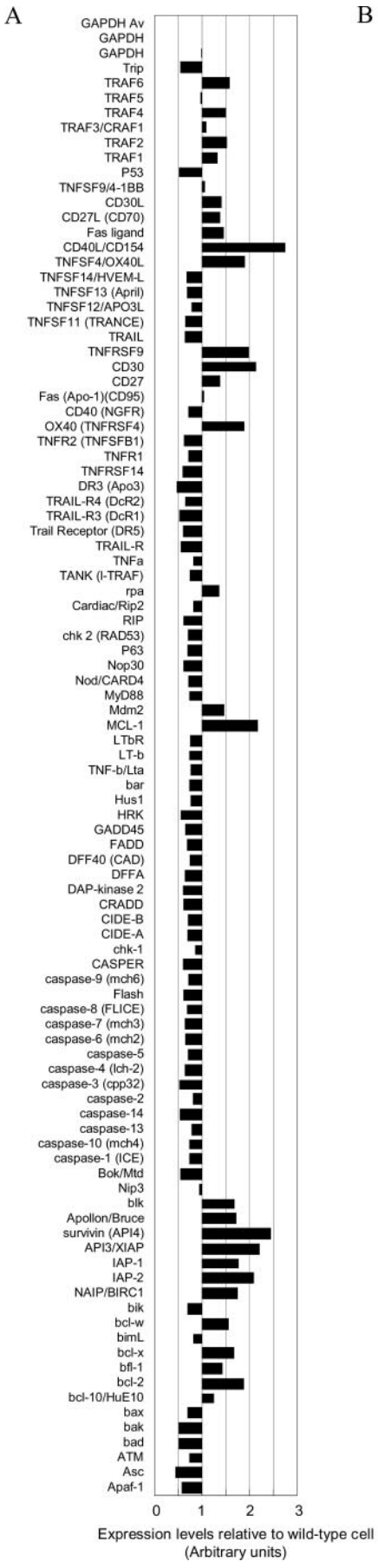


Fig. 4. RA depletion-induced aberrant mitotic progression by CYP26A1 overexpression. A, cell proliferation assay in wt (filled symbol) and HeLa^{CYP26A1} (open symbol) cells with (circle) or without (square) 0.5 μM RA treatment at the indicated time points up to day 7. Both types of cells were plated at day 1 (5×10^3 cells), and cell numbers were counted every day. B, mitotic index estimated by counting at least 1000 cells of each treatment at 400× magnification by light microscopy, and the percentage of mitotic cells was calculated. wt and HeLa^{CYP26A1} cells were subjected to this assay in the presence or absence of 2.5 ng/ml TRAIL for 3 h with or without 0.1 μM RA. *, $p < 0.05$ versus cells without RA treatment. C, flow cytometry analysis of propidium iodide-labeled wt (left column) and HeLa^{CYP26A1} (right column) cells after 5 min of pretreatment with (bottom) or without (top) 0.1 μM RA. The percentage of cells in each cell cycle fraction is indicated below the histogram.



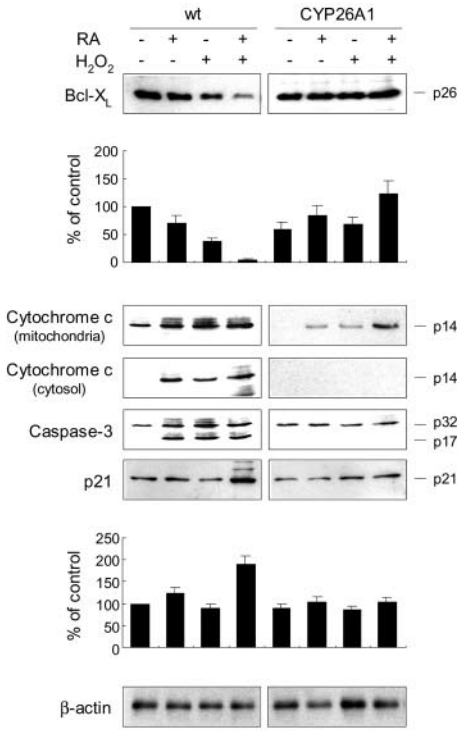


Fig. 5. CYP26A1 overexpression is sufficient to induce antiapoptotic state in HeLa cells. A, total RNA was extracted from the wt and HeLa^{CYP26A1} cells that had been exposed 1 μM RA for 24 h, and aliquots of 5 μg of RNA were reverse-transcribed to cDNA in the presence of [^{32}P]dATP. Labeled cDNA was hybridized to apoptosis pathway-specific gene expression array membranes. After normalization of the mRNA levels of a given gene with average value of GAPDH (GAPDH Av) on the same membrane, gene-expression levels in CYP26A1-overexpressing cells were calculated in arbitrary units divided by the values from that in wt cells. Data represent mean value from independent two-hybridization experiments from two independent RNA samples. B, Western blot analysis of apoptosis-related proteins, including Bcl-X_L, cytochrome *c*, caspase-3, and p21^{Waf1}, in wt and CYP26A1-overexpressing HeLa cells treated with 25 μM H_2O_2 for 24 h in combination with or without of 1 μM RA in the media supplemented with 10% charcoal-dextran-treated FBS. We also examined the subcellular localization of cytochrome *c* to separate into mitochondria and cytosolic fractions. Equal loading is demonstrated by β-actin expression. We also show the densitometric analysis of Bcl-X_L and p21^{Waf1} signals on the blot from triplicate independent experiments.

CYP26A1 overexpression, we next examined corresponding protein expression levels. Western blot analysis showed that protein levels of $p21^{Waf1}$ were increased in wt cells challenged with cell stressors such as RA and H_2O_2. This upregulation was abrogated by the overexpression of CYP26A1 in HeLa cells, even in the presence of both RA and H_2O_2 (Fig. 5B), which is consistent with the data of growth properties of $HeLa^{CYP26A1}$ cells, showing the marked abrogation of cell stress-mediated mitotic index decrease even in those initiated by synergistic effects of RA and H_2O_2 (Fig. 4).

Discussion

Apoptosis is an evolutionarily conserved process in normal embryogenesis, tissue homeostasis, and regulation of the immune system (Fisher, 1994; White, 1996). Many studies have implicated RA as an important regulator of this process, and certain types of tumor cells undergo apoptosis after RA treatment (Fisher, 1994; White, 1996). RA has been shown to enhance sensitivity of certain cells to a number of proapoptogenic factors, including death receptor ligands such as TRAIL. Our present studies show that the expression of the RA-metabolizing enzyme CYP26A1 significantly abrogates the proapoptotic activity of RA and its synergistic activity with apoptosis-inducing factors, resulting in the suppression of cellular response to several differently acting triggers of apoptosis. We also observed a reversal of the cytostatic effects of RA in CYP26A1-overexpressing cells as measured by changes in cell growth and mitotic index. Cultured cells overexpressing CYP26A1 gain significant resistance to apoptosis signaling, possibly because of a state of reduced RA bioavailability caused by metabolic inactivation of RA. However, we cannot exclude the possibility that forced expression of CYP26A1 may have some additional interesting but as yet uncharacterized effects on the cells.

One possible alternative explanation for the effect of CYP26A1 overexpression on apoptosis is that RA metabolites generated by CYP26A1 may have antiapoptotic activities. However, this is unlikely because ketoconazole can partially restore the RA-enhanced sensitivity to proapoptotic agents, suggesting that RA, but not its metabolites, is probably responsible for this enhancement effect. In addition, synthetic retinoids (such as Am80 and Am580) that are not metabolized by CYP26A1 can also enhance sensitivity to proapoptotic agents. These findings support one possibility that the catalytic activity of CYP26A1 is causally related to the altered response of CYP26A1-overexpressing cells to combinations of RA and proapoptotic treatments. However, we noted here that ketoconazole treatment did not fully restore apoptosis in $HeLa^{CYP26A1}$ cells to the level seen in wt. This observation was consistent in three different clones of $HeLa^{CYP26A1}$ as well as in A549 cells (Fig. 3C). We cannot presently explain this observation; however, one possibility is that the period of time required for clonal expansion in RA-free conditions (because of CYP26A1 overexpression) may have limited the ability of these cells to respond to apoptotic stimuli presented in the media. Moreover, Western blot analysis of apoptosis-relating proteins collectively indicates that there may be an effect of CYP26A1 expression on the levels of certain proteins, in the absence of exogenously added RA. It is possible that there may be a secondary effect of CYP26A1 overexpression because of possible changes in cell phenotype associated with clonal expansion of CYP26A1-expressing cells; such cells are grown under conditions that would be avoid of any RA or its precursor retinal. Although genetic studies support that RA is clearly a substrate for CYP26A1, there may be other, as-yet-unidentified compounds in the cell that are activated or catabolized by CYP26A1. No direct proof exists to support this latter possibility.

Loss of responsiveness to inducers of cell death is a contributing factor in the progression of a cell toward a malignant phenotype, because it is now thought that impaired or decreased susceptibility to respond to various apoptotic signals has been associated with oncogenic transformation rather than enhanced cell growth (Evan and Vousden, 2001; Gozani et al., 2002; Green and Evan, 2002). CYP26A1 overexpression is sufficient to desensitize cells to a number of different apoptogenic agents, an effect that is not seen in cells overexpressing CYP24. If increased RA metabolism is solely responsible for the apoptogen-desensitizing effect we have observed, our results would imply that in a state of RA deficiency, cells may be less susceptible to apoptosis after the acquisition of DNA damage. As a corollary, persons suffering from vitamin A deficiency might accumulate DNA damage with higher frequency. This is consistent with observations in experimental animals indicating a link between vitamin A deficiency and increased tumor formation. This may also provide a plausible explanation for the increased incidence and higher risk of development of cancer observed in persons who are deficient in vitamin A (Wolbach and Howe, 1925; Lotan, 1996). In addition, enhanced RA metabolism has been observed in several types of cancer, including head and neck squamous cell carcinomas along with elevated levels of CYP26 enzymes in a number of cancer cell types (Van Heusden et al., 1998; Klaassen et al., 2001; Ozpolat et al., 2002). Whether RA metabolism contributes to tumor formation and/or progression remains to be determined.

Because of the strong antiproliferative and differentiation and apoptosis-inducing effects on cancer cells both in vitro and in vivo, retinoids show promise in the treatment and chemoprevention of epithelial carcinogenesis and in cancer differentiation therapy (Lotan, 1996; Hong and Sporn, 1997; Hansen et al., 2000). In this regard, the most striking chemical use of retinoids is for differentiation therapy of APL based on the ability of retinoids to induce differentiation of leukemic promyelocytes, and a growing body of evidence indicates that a high proportion of patients with APL achieve complete remission after short-term treatment with RA (Huang et al., 1988; Muindi et al., 1992; Fenaux and Degos, 1997). However, this high rate of remission is brief and relapse occurs in most cases; patients gain progressive clinically acquired resistance to further treatment with RA (Fenaux and Chomienne, 1996). Several lines of evidence suggest that clinically acquired resistance to RA treatment may be caused in part by increased RA metabolism, and we have also previously shown that NB4 cells derived from APL patients exhibit inducible expression of CYP26A1 after RA pretreatment (White et al., 1997). Increased RA catabolism may not only reduce cell sensitivity to RA but also consequently reduce effectiveness of proapoptotic agents whether endogenous or therapeutic; thus, blocking RA catabolism in conjunction with treatment with proapoptotic agents may be an effective therapeutic strategy to treat malignancies where CYP26 enzymes are expressed. Imidazole derivatives (keto-

conazole and liarozole) have been shown to potentiate the apoptotic effects of cell death-inducing agents in a variety of cancers and showed clinical effectiveness in treatment of cancer patients, although the underlying mechanisms of how these agents could induce proapoptotic effects are not fully understood (Mahler et al., 1993; Wang et al., 2002). Whether at least part of the effectiveness of combining P450 inhibitors with apoptogenic agents is caused by blocking RA catabolism remains to be determined. Because we have shown that synthetic retinoids resistant to metabolism can restore sensitivity of CYP26A1-expressing cells to apoptogenic agents, such retinoids might also be useful for therapeutic strategies involving combination therapy. These studies also demonstrate for the first time that retinoids can synergize with γ-irradiation, suggesting that retinoids and/or RA metabolism inhibitors may also be used to enhance the efficiency of radiation therapy.

Gene expression profiles have demonstrated that a large number of signaling pathways are associated with the suppression of apoptotic cell death caused by CYP26A1-mediated RA depletion. Previous studies have shown that two major pathways exist in transmitting the death signals to the apoptosis-executing compartment (Scaffidi et al., 1998). In "type I" cells, triggering an initiator caspase (e.g., caspase-8) as a consequence of death receptor ligation can kill cells by stimulating the production of an effector caspase such as caspase-3 (extrinsic pathway). The "type II" cell types show optimal formation of death ligand-death ligand receptor complex, initiating a caspase cascade directly through activation of the mitochondrial pathway controlled by Bcl-2 family members (intrinsic pathway). Our results may be explained by the possibility that CYP26A1 expression affects both of these pathways. In addition, Bcl-2 is a member of an expanding family of related proteins. Some of these are antiapoptotic (Bcl-2 and Bcl-X_L), some are proapoptotic (Bax and Bak). The importance of Bcl-2 family stemmed from observations that one mechanism for inducing apoptosis is associated with down-regulated expression of Bcl-2 or the altered equilibrium between these arbitrary two subsets to form homo- and heterodimers, which helps determine the susceptibility of the cells to death signal. One model is proposed that antiapoptotic Bcl-2 molecules are "guarding the mitochondria gate" from the proapoptotic Bcl-2 members that might otherwise "gain access" after a death signal (Gross et al., 1999). Highlighting the importance of Bcl-2 family in CYP26A1-mediated apoptotic inhibition, we found that CYP26A1 overexpression is clearly involved in the signaling cascades from death receptors, which converge on the Bcl-2 family as a target relevant to epithelial cell survival.

Finally, our present study shows that many of the genes involved in apoptotic pathways are modulated to favor cell survival when CYP26A1 is overexpressed. It is possible that both during embryogenesis and in the adult, CYP26 enzymes may play a role in modulating cell sensitivity to RA and thus apoptogenic factor, which influence the sculpting process of tissue morphogenesis. This is supported by CYP26A1 knockout mouse experiments, which show animals lacking CYP26A1 suffering severe caudal agenesis and embryonic lethality (Abu-Abed et al., 2001). The gain-of-function effect of CYP26A1 on apoptosis is also demonstrated through the analysis of CYP26A1 null embryo, which revealed enhanced apoptosis at a very early stage of tailbud development (our unpublished observation). On the other hand, recapitulation of CYP26A1 expression in cancer cells would provide a selective growth advantage to these cells and thus evade normal apoptotic mechanisms. Therefore, strategies that decrease activity of CYP26A1 may be an important means of increasing the sensitivity of cancer cells to proapoptotic therapies.

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