Communications

Cloning of Human Retinoic Acid-metabolizing Enzyme (hP450RAI) Identifies a Novel Family of Cytochromes

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Retinooids, including all-trans-retinoic acid (RA) and its stereoisomer 9-cis-RA, play important roles in regulating gene expression, through interactions with nuclear receptors, during embryonic development and in the maintenance of adult epithelial tissues (Chambon, P. 1995 Rec. Prog. Horm. Res. 50, 317–32; Mangelsdorf, D. J., and Evans, R. M. 1995 Cell 83, 841–50; Petkovich, M. 1992 Annu. Rev. Nutr. 12, 443–471). Evidence suggests that 4-hydroxylation of RA inside the target cell limits its biological activity and initiates a degradative process of RA leading to its eventual elimination. However, 18-hydroxylation and glucuronidation may also be important steps in this process. In this paper, we describe the cloning and characterization of the first mammalian retinoic acid-inducible retinoic acid-metabolizing cytochrome P450 (hP450RAI), which belongs to a novel class of cytochromes (CYP26). We demonstrate that hP450RAI is responsible for generation of several hydroxylated forms of RA, including 4-OH-RA, 4-oxo-RA, and 18-OH-RA. We also show that hP450RAI mRNA expression is highly induced by RA in certain human tumor cell lines and further show that RA-inducible RA metabolism may correlate with P450RAI expression. We conclude that this enzyme plays a key role in RA metabolism, functioning in a feedback loop where RA levels are controlled in an autoregulatory manner.

Regulation of retinoid signaling may be controlled by a number of coordinated mechanisms, including retinoid synthesis, cell-specific expression of retinoid-binding proteins and nuclear receptors, and metabolism of retinoids (for review see Refs. 1–3). The generation of RA1 from its precursors, retinol and retinaldehyde, and its catabolism to more polar hydroxylated forms such as 4-OH-RA, 4-oxo-RA, and 18-OH-RA are counter-balanced metabolic pathways that regulate RA levels in RA-sensitive tissues (4, 5). Cellular retinoid acid-binding proteins may also play a role in establishing this balance by sequestering high levels of RA (6). There is considerable evidence to suggest that 4-OH-, 4-oxo-, and 18-OH-RA are polar intermediates in the catabolism and eventual elimination of RA (5, 7, 8). Thus both sequestration and metabolism may function to protect RA-sensitive tissues from deleterious concentrations of RA.

We have cloned and characterized cDNAs corresponding to a retinoic acid-inducible gene encoding a human cytochrome P450-related hydroxylase (P450RAI) responsible for generation of multiple hydroxylated products of RA. hP450RAI appears to be the human ortholog of the previously characterized zebrafish P450RAI (zP450RAI) (9), indicating that this important cytochrome is highly conserved structurally and functionally across species. We also demonstrate that hP450RAI is inducible by RA in a number of different cell types. We speculate that this enzyme plays a key role in determining the metabolic fate of endogenous retinoids and may also be implicated in the clearance of exogenous retinoids administered therapeutically.

MATERIALS AND METHODS

Cloning Library Screening—A NTERA2-D1 cDNA library (Stratagene) was screened according to the manufacturer's directions. Briefly, 1.0 × 10⁶ independent plaques were screened using a random-primed, α-[³²P]dATP-labeled full-length zP450RAI cDNA. Filters were prehybridized for 4 h at 37 °C in 50% formamide, 5 × SSPE, 1 × Denhardt's (without bovine serum albumin), 0.2 mg/ml denatured salmon sperm DNA. Hybridization was performed overnight at 37 °C. Filter were washed two times for 20 min in 2 × SSC, 0.05% SDS at room temperature followed by one 10-min wash in 1 × SSC, 0.1% SDS, and then exposed to Kodak XAR film overnight at −70 °C. Positive plaques were rescreened until purified. Bluescript-containing colonies, generated using the in vivo excision protocol (Stratagene) were plated onto LB + ampicillin plates and grown overnight at 37 °C. Plasmid DNA was purified using the Qiagen Mini-Plasmid prep kit (Qiagen) and sequenced using the T7 sequencing kit (Pharmacia Biotech Inc.). Sequence data analyses were performed with the Geneworks software package (Intelligenetics).

Analysis of RA Metabolism in Cultured Cells—Cells were transfected with 3 μg of hP450RAI in pTL1 or the empty vector control pTLI, together with 1 μg each of ferrodoxin and ferrodoxin reductase expression vectors (10). Media from transfected cells incubated with 575 μM [11,12-⁵¹ᵐCi]HRA (Fig. 2, A and B) or 1 μM RA (Fig. 2C) for 24 h were acidified with 0.1% acetic acid. All incubations were performed in the presence of fetal calf serum. For the analysis of RA metabolism, untransfected MCF7 (Fig. 3C) and MCF10A cells (Fig. 3B) were incubated in the presence of 10⁻⁶ M RA or vehicle (0.1% ethanol) for 24 h, washed with phosphate-buffered saline containing 1% bovine serum albumin, five times to remove as much RA as possible, and then analyzed for their ability to metabolize [11,12-³¹⁸F]HRA as described below, except that cells were incubated with [11,12-⁵¹ᵐCi]HRA for only 4 h. Lipid-soluble metabolites were separated from aqueous-soluble metabolites using a total lipid extraction of the medium (11). Metabolism of [11,12-³¹⁸F]HRA to total aqueous-soluble metabolites was measured using β-scintillation of aliquots of the aqueous phase of media extracts (Figs. 2B and 3D). Lipid-soluble extracts were evaporated to dryness under a stream of nitrogen acid; 4-OH-RA, 4-hydroxyretinoic acid; 4-oxo-RA, 4-oxo-retinoic acid; 18-OH-RA, 18-hydroxy-retinoic acid; HPLC, high performance liquid chromatography; DMEM, Dulbecco's modified Eagle's medium; MEM, minimal essential medium.

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Northern analysis was done as described previously (12) using 2.5–4.5
between hP450RAI and zP450RAI (Fig. 1A). A high degree of amino acid identity (68%) was observed be-
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P450 (zP450RAI) previously shown to be involved in RA-induc-
species, with zP450RAI exhibiting 68% amino acid identity to its hu-
RA metabolism experiments were performed at least three times and each

mRNA Expression in Human Cell Lines—Cells were grown to about
80% confluence in T75 flasks in the following media: HEK293, DMEM +
160% FCS + glutamine; EL-E, RPMI 1640 + 7% FCS; MCF10A,
15% horse serum; EL-E, RPMI 1640
10% FCS; HepG2, MEM
5% FCS; MCF7, MEM
6 M all-
metabolites is observed hP450RAI transfected cells (B).

RESULTS AND DISCUSSION

Cloning Human P450RAI—We probed a panel of mRNAs from human cell lines with cDNA from zebrafish cytochrome
P450 (zP450RAI) and other cytochromes listed in GenBank™, sug-
5/95 H2O/methanol (2 ml/min.). The putative 4-oxo-RA peak con
mRNA Expression in Human Cell Lines—Cells were grown to about
80% confluence in T75 flasks in the following media: HEK293, DMEM +
4-oxo-RA (2 ml/min.). The putative 4-oxo-RA peak comigrated with standards
3.5-fold increase in aqueous-soluble me-
 identities (CR1-CR4, Fig. 1A) with amino acid identity

P450RAIs and other cytochromes listed in GenBank™, sug-
A high degree of amino acid identity (68%) was observed be-
between hP450RAI and zP450RAI (Fig. 1A). We also have re-
cently cloned the murine P450RAI, which is 89% conserved
with the human counterpart (data not shown). In contrast,
there was less than 30% amino acid identity between the

Fig. 1. Amino acid alignment between human P450RAI and
zebrafish P450RAI A. Comparisons between the human and zebrafish
P450RAIs indicate that these proteins are highly conserved across
species, with zP450RAI exhibiting 68% amino acid identity to its hu-
man counterpart. B, Kyte-Doolittle hydrophobicity (14) plot comparing
the human and zebrafish P450RAIs. The profiles are almost identical ex-
cept for a short nonconserved region between CR3 and CR4.

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erved with RNA from RA-treated teratocarcinoma cells
(NTERA2-D1) (13) (data not shown). A cDNA library from RA-treated
NTERA2-D1 (Stratagene) was screened with the
zP450RAI cDNA, and a full-length human cDNA was isolated.
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Human RA-metabolizing Enzyme

We have studied the expression of hP450RAI mRNA in a variety of human cell lines by Northern blot analysis (Fig. 3A). Poly(A)⁺ mRNAs from RA-treated and control cells show several different expression profiles: constitutive expression (embryonic kidney HEK293 and small cell lung carcinoma SK-LC6 (19)), inducible expression (nonsmall cell lung carcinoma LC-T, breast adenocarcinoma cell lines MCF7 and MCF10A, breast epithelial cell lines EL-E, MCF10A, and MCF1, human fibroblasts fibroblasts 293, and myelomonocytic leukemia cell line U937, inducible RA-metabolizing enzyme activity (16–18). hP450RAI in the presence of RA, exhibits a metabolite profile similar to that observed in hP450RAI-transfected COS-1 cells, inducing the production of 4-oxo- and 4-OH-RA. RA treatment of MCF7, but not MCF10A, induces an increase in total aqueous-soluble RA metabolites. The standards RA, 4-OH-RA, and 4-oxo-RA eluted at 4.65, 10.28, and 11.86 min, respectively. Results shown are representative of data from four (B–D) individual experiments. Error bars in D are standard deviation of the mean from triplicate samples.
carcinoma MCF7, acute promyelocytic leukemia-derived NB4 (20), and hepatocarcinoma HepG2), or a complete lack of expression (breast carcinoma-derived EL-E and nontransformed breast MCF10A). Inducible P450RAI expression was also observed in the human keratinocyte-derived cell lines HPK1A and HPK1A-ras (21) (data not shown). These findings suggest that P450RAI expression is regulated by multiple factors in a cell-specific manner. It is notable that P450RAI expression is highly inducible in the acute promyelocytic leukemia-derived cell line NB4 since 1) increased RA metabolic activity of the type demonstrated by P450RAI has been implicated in the acquired clinical resistance to RA (22), and 2) NB4 cells have been shown to have inducible RA metabolism following pretreatment with RA (23). The diversity of cell types exhibiting P450RAI expression suggests that if P450RAI induction is indeed responsible for conferring clinical RA resistance, induced metabolism in both the leukemic cells and other tissues where P450RAI is induced would contribute collectively to lowering the therapeutic efficacy of RA.

Correlation between hP450RAI mRNA Expression and RA Metabolism—RA-inducible RA metabolism has been previously characterized in a number of cell lines and tissues, including the breast epithelial cell line MCF7 (24). To determine whether the inducibility of P450RAI expression, which we have observed in this cell line (see Fig. 3A), correlates with RA metabolic activity, MCF10A and MCF7 were incubated with or without RA for 24 h and then tested for their ability to metabolize [11,12-3H]RA. Interestingly, in MCF10A, there is no inducible expression of hP450RAI, there is also no detectible production of 4-oxo- or 4-OH-RA in either control or RA-treated cells (Fig. 3B). In contrast, MCF7 cells, which contain highly inducible P450RAI expression, do produce these metabolites in response to RA (Fig. 3C). Analysis of the aqueous-soluble fractions shows a 3.5-fold increase in aqueous-soluble radioactivity only in the induced MCF7 cells (Fig. 3D), which correlates with the ability of the cells to convert RA to 4-hydroxylated derivatives. 4-Hydroxylation of RA may be an obligatory step in the production of these aqueous soluble products. However, we note that the 4-OH-RA and 4-oxo-RA peaks observed in MCF7 cells are broad, suggesting that they may contain multiple metabolic products. These products may arise from alternate hydroxylations or isomers, suggesting that if P450RAI is responsible for all the metabolism in MCF7 cells that it can perform metabolic steps other than 4-hydroxylation. These experiments are consistent with the possibility that P450RAI plays a major role in RA metabolism.

In summary, we have cloned and characterized a human cytochrome P450, which is very highly conserved between zebrafish and human, constituting a novel family, CYP26, of cytochromes P450. P450RAI is able to metabolize all-trans-RA, and Northern blot analysis of its expression in a number of human cell lines shows that it can be induced in response to RA treatment, expressed constitutively, or not expressed at all, suggesting its regulation is complex and may be tissue type-dependent. Several additional findings (not presented in this paper) support our hypothesis that this enzyme is specific for RA and is responsible for RA catabolism. Preliminary enzyme kinetic studies on whole cells indicate a relative K_m less than 1 μM in accordance with the K_m of micromolar cytochrome preparations shown to hydroxylate RA in vitro (5, 25) and in the same range as retinoid metabolizing activity defined in T47D cells (18). In addition, we find that 9-cis-RA is a good substrate for hP450RAI, while retinol, even at micromolar concentrations, is not. In our studies we find that expression of the P450RAI message and RA metabolic activity appear to be correlated implicating P450RAI in the observed RA metabolism.

The identification of P450RAI, its inducibility by RA, and its RA metabolic activity define a feedback loop, which may be critical in regulating both normal and therapeutic RA levels. Other autoregulatory feedback loops, such as that described for conversion of retinol to retinoic acid, contribute to controlling overall levels of active retinoid (26). This emphasizes the importance of maintaining stable physiological levels of RA. Inhibitors designed to block P450RAI function may therefore be useful in elevating normal tissue RA levels or maintaining high therapeutic levels of RA (22, 27). Since RA has proven useful in the treatment of a number of cancers, premalignancies, and skin disorders, an understanding of the role of hP450RAI in regulating RA levels in normal and disease states will be important clinically.

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