

Induction of the Cytochrome P450 Gene CYP26 during Mucous Cell Differentiation of Normal Human Tracheobronchial Epithelial Cells

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Received April 4, 2000; accepted June 12, 2000

This paper is available online at <http://www.molpharm.org>

ABSTRACT

In this study, the expression of CYP26 is examined in relation to retinoid-induced mucosecretory differentiation in human tracheobronchial epithelial (HTBE) cells and compared with that in human lung carcinoma cell lines. In HTBE cells, retinoic acid (RA) inhibits squamous differentiation and induces mucous cell differentiation as indicated by the suppression of transglutaminase I and increased expression of the mucin gene MUC2. The latter is accompanied by increased expression of CYP26 mRNA. RA is required but not sufficient to induce RAR β , CYP26, and MUC2 mRNA because induction is only observed in confluent but not in logarithmic cultures, suggesting that additional factors are critical in their regulation. CYP26 mRNA can be induced by the RAR-selective retinoid 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-anthracenyl)-benzoic acid (TTAB) but not by the RXR-selective retinoid SR11217 or the anti-

activator-protein 1-selective retinoid SR11302. RAR α -, β -, and γ -selective retinoids are able to induce CYP26; this induction is inhibited by the RAR α -selective antagonist Ro41-5253. TTAB is able to induce CYP26 mRNA expression in only a few of the lung carcinoma cell lines tested. The lack of CYP26 induction in many carcinoma cell lines may relate to previously reported defects in the retinoid-signaling pathway. The induction of CYP26 correlated with increased metabolism of RA into 18-hydroxy-, 4-oxo-, and 4-hydroxy-RA. The latter metabolite was shown to be able to induce MUC2 and MUC5AC expression in HTBE cells. Our results demonstrate that in normal HTBE cells, CYP26 expression is closely associated with mucous cell differentiation and that many lung carcinoma cells exhibit increased RA metabolism and a defective regulation of CYP26.

Retinoids play a pivotal role in the regulation of cell growth and differentiation of epithelial cells in the respiratory tract during embryonic development and in the adult (Jetten, 1992; Zachman, 1995; Chytil, 1996). In the tracheobronchial epithelium, retinoids are crucial determinants for the maintenance of its normal function. During vitamin A-deficiency, the mucociliary epithelium is replaced by a squamous epithelium and normal differentiation and function is restored by the supplementation of vitamin A to the diet (Jetten, 1992). In culture, tracheobronchial epithelial cells mimic these responses to retinoids: in the absence of retinoids, cells undergo squamous differentiation and express squamous-specific genes, such as cornifin and transglutaminase type I (Jetten, 1992; Marvin et al., 1992; Medvedev et al., 1999). In the presence of retinoids, these cells express a normal mucosecretory phenotype as characterized by the synthesis and secretion of mucin glycoproteins (Rearick et al., 1987;

Rearick and Jetten, 1989; Koo et al., 1999). Retinoids also play an important role in the regulation of surfactant proteins in alveolar type II cells, reverse elastase-induced emphysema in rats, and inhibit the formation of second-primary tumors in humans (Benner et al., 1994; Massaro and Massaro, 1997; Grummer and Zachman, 1998).

Many of the effects of retinoids are mediated by nuclear retinoid receptors, which comprise two subfamilies, the retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Recent studies have indicated that the effects of retinoids in tracheobronchial epithelial cells are mediated through these nuclear receptors (Haq et al., 1991; Nervi et al., 1991; Sun et al., 1997; Koo et al., 1999a). Changes in the regulation and action of retinoid receptors have been implicated in lung tumorigenesis. The resistance of lung carcinoma cell lines to respond to retinoids has been related to defects in different steps in the retinoid-signaling pathway (Nervi et al., 1991; Zhang et al., 1994; Moghal and Neel, 1995; Sun et al., 1999; Xu et al., 1997).

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ABBREVIATIONS: RAR, retinoic acid receptor; RXR, retinoid X receptor; RA, retinoic acid; HTBE, human tracheobronchial epithelial; TTAB, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-anthracenyl)-benzoic acid; DMSO, dimethyl sulfoxide; RT-PCR, reverse transcriptase polymerase chain reaction; DCM, dichloromethane; RARE, RA response element.

Both the formation of active retinoids as well as the catabolism to inactive retinoids are important in the mechanism by which retinoids control physiological processes. Such enzymes could be involved not only in the regulation of the concentration of active retinoids but also determine the location and time of retinoid action. A number of different enzyme families that are able to catalyze retinoid metabolism have been identified and include various alcohol and aldehyde dehydrogenases and members of the cytochrome P450 family (Duester, 1996; White et al., 1996; Nadin and Murray, 1999; Napoli, 1999). Recently, a novel cytochrome P450 gene CYP26 (also named P450RAI) was identified (White et al., 1996, 1997; Ray et al., 1997; Abu-Abed et al., 1998) that metabolizes retinoic acid (RA) to 4-hydroxy-, 4-oxo- and 18-hydroxy-RA. CYP26 is induced by RA in a number of cell types and several functions for this enzyme in the regulation of development and differentiation have been proposed (White et al., 1997; Lane et al., 1999; Sonneveld et al., 1999).

In this study, we examine the expression of CYP26 in relation to the inhibition of squamous differentiation by RA in normal human tracheobronchial epithelial (HTBE) cells and the induction of differentiation into mucosecretory cells. In addition, we compare CYP26 expression between HTBE and various lung carcinoma cell lines. We demonstrate that the increase in CYP26 mRNA expression is closely associated with the induction of mucous differentiation suggesting a specific role for CYP26 in this process. We show that the presence of RA is not sufficient for the induction of CYP26 or mucin expression. These findings indicate that the control of CYP26 and MUC2 expression by RA is complex and that additional factors are critical in their regulation. Although RA is able to induce CYP26 in several human lung carcinoma cell lines, most cells are refractory to RA. The latter may, at least in part, be a reflection of defects in the retinoid signaling pathway observed previously in many lung carcinoma cell lines (Nervi et al., 1991; Zhang et al., 1994; Moghal and Neel, 1995).

Materials and Methods

Cell Culture. Normal HTBE were obtained from Clonetics Corp. (San Diego, CA). Cells were grown onto 24-mm permeable Transwell membranes Costar Corp. (Cambridge, MA) in serum-free BEGM medium (Clonetics) as described previously (Gray et al., 1996; Koo et al., 1999a,b). Cells were grown in the absence or presence of retinoids as indicated. SV40-T transformed HTBE cell lines BEAS-2B and BET-1A were obtained from the American Type Culture Collection (Rockville, MD) and also grown in bronchial epithelial cell growth medium. Human adenocarcinoma cell line NCI-H1355 was obtained from Dr. A. Gazdar (Austin, TX). All other carcinoma cell lines were purchased from American Type Culture Collection. NCI-H69 and H82 are small cell, NCI-H460 and H441 are adeno-, NCI-226 is a squamous cell, Calu-6 an anaplastic, and A549 an alveolar carcinoma cell line. All cell lines were mycoplasma-free. Carcinoma cell lines were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin, and streptomycin.

The RAR γ -selective retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene-carboxylic acid (AHPN, also referred to as CD437) was described previously (Sakaue et al., 1999) and obtained from Dr. U. Reichert (CIRD Galderma, Valbonne, France). The RAR pan-agonist SRI-6751-84 or 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-anthracenyl)-benzoic acid (TTAB), the RXR-pan-agonist SR11217 (4-[1-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-2-methylpropenyl]-benzoic acid), and anti-AP1-selective retinoid

SR11302 [(*E*)-3-methyl-9-(2,6,6-trimethyl-cyclohexenyl)-7-(4-methyl-phenyl)-2,4,6,8-nonatetraenoic acid] (Lehmann et al., 1992; Fanjul et al., 1994) were provided by Dr. M. Dawson (SRI, Menlo Park, CA). All-*trans*-RA, the RAR α -selective antagonist Ro41-5253 (6-[1-(4-carboxyphenyl)propen-2-yl]-3,4-dihydro-4,4-dimethyl-7-heptyloxy-2*H*-benzo-thio-pyrene-2,2-dioxide), the RAR α -selective agonist Ro40-6055 [4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-6-naphthalenyl carboxamido)benzoic acid], and the RAR β -selective agonist Ro48-2249 were obtained from Hoffmann-La Roche (Nutley, NJ). Retinoids were dissolved in dimethyl sulfoxide (DMSO). Control cells received vehicle only.

RNA Isolation and Northern Analysis. RNA from cultured cells was isolated using Tri-Reagent (Sigma) according to the manufacturer's protocol. Total RNA (10 μ g) was electrophoresed through a formaldehyde 1.2% agarose gel as described (Sakaue et al., 1999), blotted to a Nytran Plus membrane (Schleicher & Schuell, Keene, NH), and UV-crosslinked. Hybridizations were performed for 1 to 2 h at 68°C using QuikHybTM reagent (Stratagene, La Jolla, CA), blots were washed twice with 2 \times SSC/0.05% SDS for 15 min at room temperature, and in the final wash with 0.5 \times SSC/0.1% SDS for 30 min at 60°C. Autoradiography was carried out with Hyperfilm-MP (Amersham) at -70°C using double intensifying screens. A 0.4-base pair cDNA probe for human CYP26 was obtained by reverse transcriptase polymerase chain reaction (RT-PCR) using two CYP26-specific primers. The sequence of this PCR product was found to be identical with that of the published hCYP26 (White et al., 1997). Probes for transglutaminase type I and the nuclear RAR β were reported previously (Nervi et al., 1991; Medvedev et al., 1999).

RT-PCR. Methods to detect MUC2 and MUC5AC mRNA levels using quantitative RT-PCR have been reported elsewhere in detail (Koo et al., 1999a). Oligonucleotide amplimers for β 2 microglobulin were used as a control. In certain instances MIMIC (Clontech) was used as an internal standard (Koo et al., 1999a).

Analysis of RA Metabolism. Cells were pretreated with 10 nM TTAB for 16 h. Cells were then washed with serum-free medium and incubated with 10 nM all-*trans*-[³H]RA (55 mCi/mmol; Dupont). The purity of [³H]RA was checked by HPLC before each use. At the indicated times medium was removed and extracted with an equal volume of dichloromethane (DCM)/methanol (2:1, v/v). The aqueous phase containing hydrophilic metabolites and the organic phase containing hydrophobic metabolites and retinoic acid were separated by centrifugation. Retinoids were extracted from cells using methanol extraction. Metabolites were analyzed by HPLC using a Gilson 303 HPLC system and an ODS-2 column (25 \times 0.46 cm; Phenomenex). The metabolites were separated at a flow rate of 1 ml/min using the following gradient elution series of methanol and 60 mM ammonium acetate, pH 5.75: a 5-min isocratic gradient at 65% (v/v) methanol, followed by a 7-min convex gradient to 80% methanol, a 8-min linear gradient to 85% methanol, and a 10-min isocratic gradient at 99% methanol. Radioactivity was detected in an A500 Flow-One detector (Packard Instruments). Absorbance was monitored with an Applied Biosystems 785A absorbance detector. The standards RA, 13-*cis*-RA, 4-oxo-RA, 4-hydroxy-RA, and 18-hydroxy-RA were obtained from Hoffmann La Roche.

Results

Association of CYP26 mRNA Expression with Mucous Cell Differentiation. Previous studies have shown that the presence of retinoids is required for the differentiation of HTBE cells into mucous-secreting cells while in the absence of retinoids, HTBE cells undergo squamous differentiation when cultures reach confluence (Rearick et al., 1987; Rearick and Jetten, 1989; Koo et al., 1999a,b). In this study, we examined the expression of CYP26 mRNA in relation to these two differentiation programs. As shown in Fig. 1, HTBE cells grown to confluence in the absence of RA ex-

pressed little CYP26 mRNA. Subsequent treatment with RA caused a steady increase in the level of CYP26 expression. The CYP26 probe hybridized to two RNA species, approximately 1.9 and 2.4 kb in size. These different RNAs may be derived from the use of alternative polyadenylation signals. An increase in CYP26 mRNA could be observed as early as 24 h after the addition of RA and paralleled the increase in the expression of the mucin gene MUC2, an indicator of mucous cell differentiation. The induction of CYP26 mRNA was somewhat slower than the suppression of transglutaminase I mRNA expression, a squamous cell-specific marker (Jetten et al., 1992), and the induction of RAR β . RAR β has been reported previously to be up-regulated by RA in HTBE cells and its expression was found to be inversely related to squamous differentiation (Nervi et al., 1991; Koo et al., 1999a). Our results on CYP26 indicate that induction of mucous cell differentiation is associated with increased CYP26 mRNA expression.

Although retinoids are an absolute requirement for the induction of mucous differentiation, they are not sufficient to induce this differentiation pathway (Rearick et al., 1987). The latter is further illustrated by observations showing that RA is able to induce mucous differentiation and increase MUC2 (Fig. 2) and MUC5AC expression (not shown) only in confluent HTBE cultures and not in logarithmic cultures. The induction of CYP26 mRNA by RA was also found to be dependent on the cell density of the cultures because RA was able to induce CYP26 mRNA in confluent but not in exponential phase HTBE cell cultures (Fig. 2). These results suggest that additional signals, likely induced when HTBE cell cultures reach confluence, cooperate with RA in regulating mucous differentiation and CYP26 expression.

RAR-Mediated Induction of CYP26 in HTBE Cells. HTBE cells have been reported to express RAR α , RAR γ , RXR α , and low levels of RXR β and RXR γ , whereas RAR β is induced after retinoid treatment (Nervi et al., 1991; Koo et al., 1999a). To determine the role of these retinoid receptors, the effects of the RAR-selective retinoid TTAB, the RXR-selective retinoid SR11217, and SR11302, a retinoid with

reported selective anti-AP1 activity, on CYP26 induction were analyzed (Lehmann et al., 1992; Fanjul et al., 1994). As shown in Fig. 3, TTAB and RA were able to induce CYP26 mRNA whereas the RXR-selective retinoid and SR11302 were not. These results indicate that the induction of CYP26 mRNA in HTBE cells occurs through activation of RAR receptors. Treatment of HTBE cells with retinoids that selectively bind and activate the RAR α , β , or γ receptor also increased the level of CYP26 expression (Fig. 4A). The RAR α antagonist completely inhibited the induction of CYP26 mRNA by the RAR α agonist, whereas it only partially inhibited CYP26 expression induced by the RAR-selective retinoid TTAB (Fig. 4B). The latter result may indicate that although transactivation through RAR α is inhibited, TTAB can activate and induce CYP26 expression through other RAR receptors. These results suggest that in HTBE cells CYP26 mRNA expression can be induced through activation of either the RAR α , β , or γ signaling pathway.

The induction of CYP26 by TTAB in HTBE cells occurred in a dose-dependent manner (Fig. 5). TTAB was able to increase CYP26 mRNA expression at concentrations as low as 1 nM. At this concentration, TTAB seemed just as effective in inducing RAR β and MUC2 mRNA and inhibiting the expression of transglutaminase I.

CYP26 mRNA Expression Is Not Induced in Many Lung Carcinoma Cell Lines. In contrast to HTBE cells, lung carcinoma cell lines have been reported to be rather resistant to the growth-inhibitory action of RA (Geradts et al., 1993; Sun et al., 1997; Adachi et al., 1998). In addition, RA is unable to induce RAR β in many lung carcinoma cell lines. In many cases, the nonresponsiveness of these cells to RA is not related to the absence of retinoid receptors but to defects in the retinoid-signaling pathway (Nervi et al., 1991; Moghal and Neel, 1995). To determine whether such defects affect the induction of CYP26 by retinoids, we examined its induction in several human lung carcinoma cell lines. Previ-

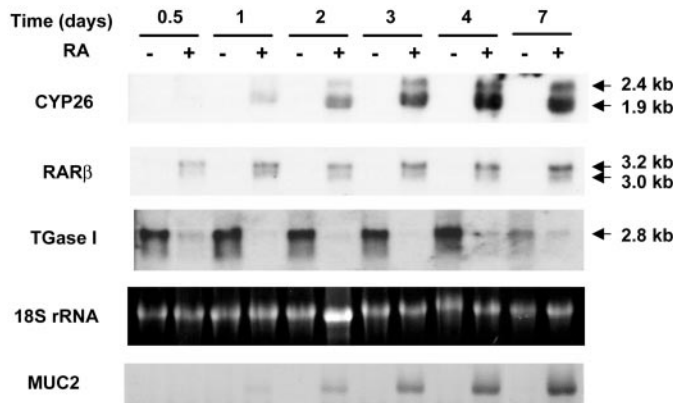


Fig. 1. Induction of CYP26 mRNA expression in HTBE cells by RA in relation to squamous and mucous cell differentiation. Cells were grown in Transwell dishes in the absence of retinoids for 7 days and then in the presence or absence of 1 μ M RA. At different time intervals, cells were collected and RNA was isolated. Total RNA (10 μ g) was examined by Northern blot analysis using 32 P-radiolabeled probes for CYP26, RAR β , and transglutaminase type I (TGase I). The 18S rRNA is shown to demonstrate equal loading. The level of MUC2 mRNA expression was analyzed in the same samples by RT-PCR as described under *Materials and Methods*. The size of the RNAs is indicated to the right.

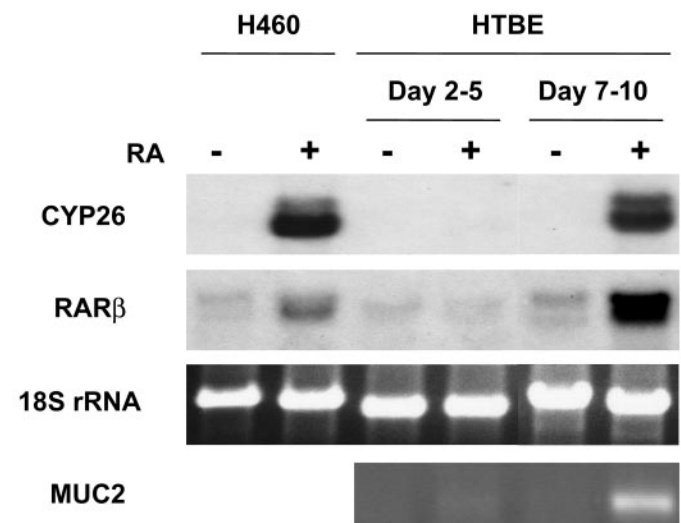


Fig. 2. Induction of CYP26 and MUC2 mRNA in HTBE cells by RA requires additional factors. Cells were grown in Transwell dishes in the absence of retinoids for 2 (early exponential phase) or 7 days (late exponential phase) and then for 3 more days in the presence or absence of 0.5 μ M RA. Total RNA (10 μ g) was isolated and examined by Northern blot analysis using a 32 P-radiolabeled probe for CYP26 and RAR β . The level of MUC2 mRNA expression was analyzed by RT-PCR. RA-treated and untreated human carcinoma H460 cells were included for comparison.

ous studies (Nervi et al., 1991; Moghal and Neel, 1995) reported that RA is able to induce RA response element (RARE)-mediated transcriptional activation in Calu-6 cells but not in H441 cells, suggesting that H441 cells exhibit a

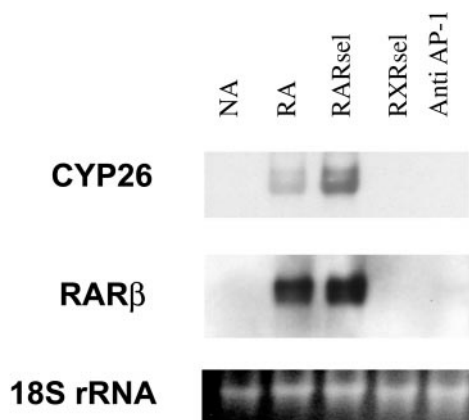


Fig. 3. Induction of CYP26 mRNA expression by retinoid receptor-selective retinoids. HTBE cells were cultured as described under *Materials and Methods*. At day 7, cells were treated either with the RAR-panagonist TTAB (100 nM; RAR_{sel}), the RXR-panagonist SR11217 (1 μM; RXR_{sel}), the anti-AP-1-selective retinoid SR11302 (1 μM; Anti-AP-1), or RA (1 μM). RNA was examined by Northern blot analysis using ³²P-radiolabeled probes for CYP26 and RARβ.

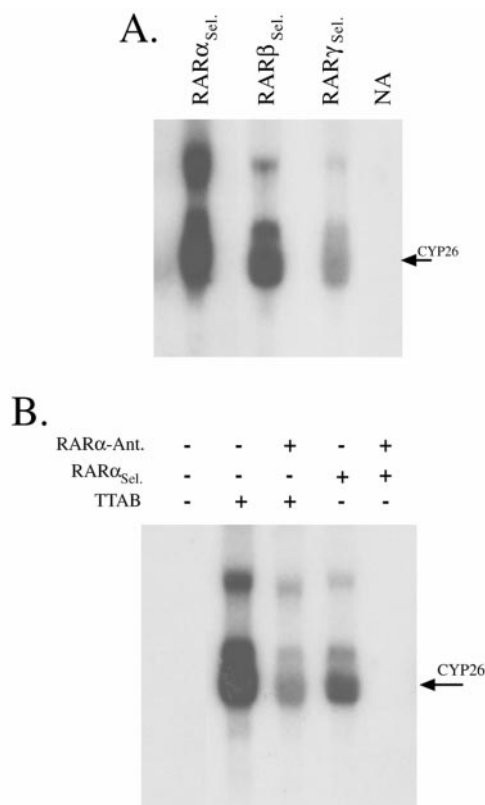


Fig. 4. A, induction of CYP26 mRNA expression by RARα, β or γ-selective agonists. HTBE cells were treated with different selective RAR agonists (0.1 μM) as described under *Materials and Methods*. RNA was isolated and examined by Northern analysis using a radiolabeled probe for CYP26. B, inhibition of TTAB and Am580 induced CYP26 expression by the RARα-selective antagonist Ro41-5253. HTBE cells were grown in the presence of the RAR pan-agonist TTAB (10 nM) and the RARα-selective agonist Am580 (50 nM; RARα_{sel}) in the presence or absence of Ro41-5253 (1 μM; RARα-Ant.). RNA was examined by Northern analysis using a radiolabeled probe for CYP26.

transcriptional defect. The ability of the RAR-selective retinoid TTAB to induce CYP26 and RARβ in Calu-6 cells and the lack of induction in H441 cells (Fig. 6A) are in agreement with these findings. The defect in RARE-dependent transcriptional activation in H441 may be responsible for the lack of CYP26 induction in these cells. TTAB was able to induce CYP26 expression in only two other lung carcinoma cell lines, adenocarcinoma H460 and alveolar carcinoma A549 cells. TTAB did not induce CYP26 in adenocarcinoma H1355 and H441 nor in squamous cell carcinoma H226, small cell carcinoma H82 and H69, mucoepidermoid carcinoma H292, or SV40-T transformed HTBE cells BET-1A and BEAS-2B. The largest induction was seen in H460 cells. In these cells, an increase in CYP26 mRNA expression could be observed as early as 4 h after the addition of TTAB (Fig. 6B). The fold-induction in CYP26 mRNA expression was comparable with that in HTBE cells (Fig. 2). In contrast to HTBE cells, CYP26 was induced in logarithmic cultures of H460 to the same extent as in confluent cultures (not shown). Moreover, in contrast to HTBE cells, the induction of CYP26 mRNA in H460 cells occurred faster and was transient, reaching a maximum at 16 h (Figs. 1 and 6B, and results not shown). These results indicate several differences in the mechanism by which RA controls CYP26 expression between normal HTBE cells and lung carcinoma cells.

Analysis of RA Metabolism. To examine whether increased expression of CYP26 led to increased RA metabolism, the rate of [³H]RA metabolism was determined in HTBE and several lung carcinoma cell lines. The level of radiolabeled polar metabolites in the aqueous-soluble fraction prepared from media of TTAB-treated and -untreated cell cultures was used as a measurement of the rate of metabolism (White et al., 1996). As shown in Fig. 7, the rate of RA metabolism in TTAB-pretreated HTBE cells was about 4-fold greater than in untreated HTBE. A similar increase was observed in sev-

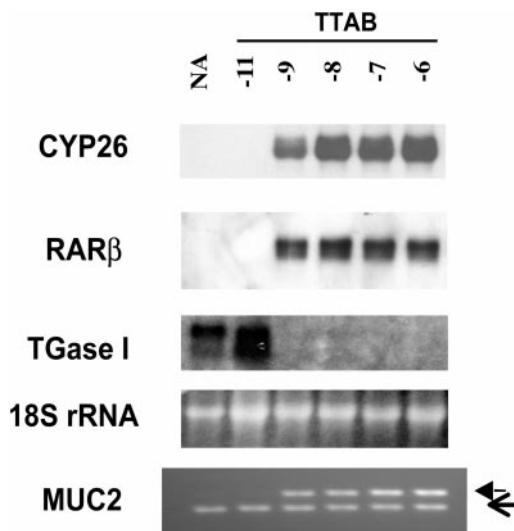


Fig. 5. Dose dependence of CYP26 mRNA induction by the RAR-panagonist TTAB in HTBE cells. HTBE cells were grown in Transwell dishes in the absence of TTAB for 7 days and then for another 7 days in the presence of the indicated concentration of TTAB. Total RNA was isolated and examined by Northern analysis using ³²P-radiolabeled probes for CYP26, RARβ, or TGase I. The level of MUC2 mRNA expression (solid arrow) was analyzed by RT-PCR in the presence of an internal standard (open arrow) as described under *Materials and Methods* (-6 to -11 refer to TTAB concentration 10⁻⁶ to 10⁻¹¹ M).

eral lung carcinoma cell lines. The increase in the rate of metabolism after TTAB treatment correlated rather well with the induction of CYP26 mRNA expression. In contrast to normal HTBE cells, most lung carcinoma cell lines exhibited a substantial rate of [3 H]RA metabolism in the absence of retinoid treatment. Moreover, several cell lines (e.g., H441) that lacked detectable levels of CYP26 mRNA were able to metabolize RA well. Although the presence of constitutive levels of CYP26 protein cannot be ruled out at this moment, because of the lack of a CYP26 antibody, this metabolism is likely to be caused by the presence of other P450 enzyme(s). Expression of members of the P450 family CYP2C, which have been demonstrated to be able to metabolize RA (Nadin and Murray, 1999), was detectable in some of these cell lines (not shown) and may be, at least in part, responsible for this metabolism. The high rate of RA metabolism in carcinoma cell lines may contribute to their resistance to the growth-inhibitory effects of RA. A time course of [3 H]RA metabolism in H460 cells is shown in Fig. 7B. Both TTAB-pretreated and untreated H460 cells metabolized RA; however, TTAB-treated cells exhibited about a 5- to 7-fold higher rate compared with untreated cells.

To identify the various metabolites, [3 H]RA metabolites formed in TTAB-treated and untreated H460 cells were analyzed by reverse-phase HPLC. TTAB itself was not metabolized by CYP26 (not shown). Figure 8 shows the HPLC analyses of labeled retinoids present in different fractions isolated from TTAB-pretreated and control H460 cell cultures incubated for 2 h with 10 nM [3 H]RA. HPLC analysis of the retinoids extracted from control cells showed that most of the radiolabeled retinoids consisted of a mixture of RA isomers, including 13-*cis*- and all-*trans*-RA (peaks 4 and 5), whereas the level of polar RA metabolites was very low (less than 5%) (Fig. 8A; arrows 1 to 3). The total amount of radiolabeled retinoids present in TTAB-pretreated cells was reduced by about 10-fold compared with control cells with approximately 20 to 25% of the radioactivity eluting with

4-oxo-, 4-hydroxy-, and 18-hydroxy-RA (Fig. 8B; arrows 1 to 3). Analysis of the radiolabeled retinoids in the organic phase of DCM-extracted media showed a dramatic increase in the percentage of polar metabolites (peaks 1–3) in the organic phase from TTAB-treated H460 cells compared with that from control cells (Fig. 8, C and D). The organic phase from untreated cells contained mostly RA (70 to 80%), whereas the majority of the labeled retinoids (about 70%) in the organic fraction of medium from TTAB-treated cells consisted of polar metabolites. The aqueous-soluble extract prepared from medium of control cells contained very low levels of polar metabolites in contrast to that of TTAB-treated cells (Fig. 8, E and F). These results demonstrate the rapid conversion of [3 H]RA into aqueous-soluble metabolites in TTAB-treated H460 cells. In these cells, most [3 H]RA was converted to polar metabolites within 2 h. The HPLC profiles of TTAB-pretreated and untreated HTBE cells were qualitatively very similar to those for H460 cells; however, in HTBE cells, the rate of RA conversion to polar metabolites was much smaller (not shown).

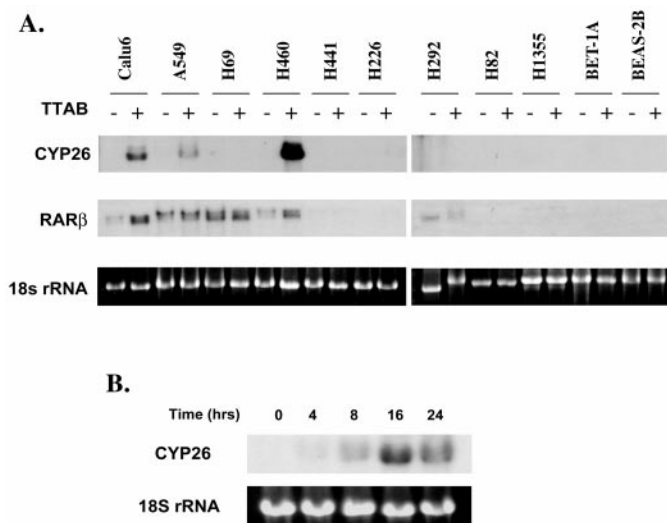


Fig. 6. A, induction of CYP26 mRNA expression by the RAR selective retinoid TTAB in several human lung carcinoma cell lines. Lung carcinoma cells were treated with 10 nM TTAB and after 16 h cells were collected and RNA was isolated. Total RNA (10 μ g) was examined by Northern blot analysis using radiolabeled probes for CYP26 and RAR β . B, time course of the induction of CYP26 mRNA by 10 nM TTAB in lung carcinoma H460 cells.

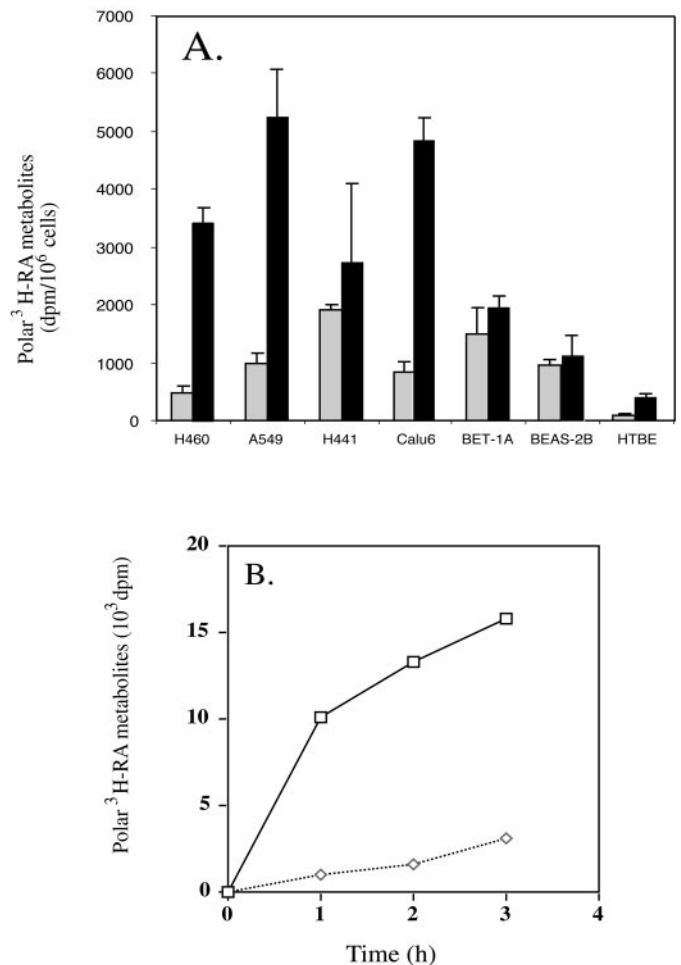


Fig. 7. A, comparison of the rate of [3 H]RA metabolism in various human lung carcinoma cell lines. Cell cultures pretreated for 16 h with 10 nM TTAB (solid box) or vehicle (hatched box) were incubated with 10 nM all-*trans*-[3 H]RA for 1 h, medium was then collected and extracted with DCM/methanol as described under *Materials and Methods*. The amount of radioactivity in the aqueous-soluble fraction, a measurement of RA metabolism, was then determined. Data were derived from triplicate dishes. B, time course of the release of aqueous-soluble [3 H]RA metabolites from TTAB-treated (solid line) and untreated (dashed lines) H460 cells.

Induction of Mucous Differentiation by 4-Hydroxy-RA. As shown in Fig. 9A, [3 H]RA-treated HTBE cells contained several polar RA metabolites that coeluted with 4-oxo-, 4-hydroxy-, and 18-hydroxy-RA. To determine whether these RA metabolites could play a role in the regulation of mucous cell differentiation, HTBE cells were treated with 4-hydroxy-RA and the effect on MUC2 and MUC5AC mRNA expression analyzed. As shown in Fig. 9B, 4-hydroxy-RA could effectively induce MUC2 and MUC5AC mRNA expression in HTBE cells, indicating that it is an active retinoid able to induce mucous cell differentiation.

Discussion

In this study, we demonstrate that the induction of CYP26 in HTBE cells by retinoids correlates closely with the induction of mucous cell differentiation. HTBE cells grown in the absence of retinoids express a squamous phenotype, as characterized by the expression of the squamous cell marker transglutaminase type I and do not express CYP26 mRNA. In the presence of retinoids, cells undergo mucous cell differentiation, as indicated by MUC2 and MUC5AC expression

and mucin secretion (Rearick et al., 1987; Rearick and Jetten, 1989; Koo et al., 1999a,b), and do express CYP26 mRNA. This increase in CYP26 mRNA by retinoids therefore parallels the inhibition of the squamous phenotype and the induction of mucous cell differentiation. Interestingly, the induction of CYP26 and mucous cell differentiation was only observed in confluent cell cultures and not in logarithmically growing cells. These results further strengthen the association between CYP26 expression and mucous cell differentiation. Moreover, these observations indicate that the presence of RA is required but not sufficient to induce CYP26, RAR β , and MUC2, suggesting additional requirements for the induction of mucous cell differentiation and the expression of these genes. In a number of cell systems, including HTBE cells, the confluent state of the culture has been demonstrated to generate changes that are critical for the induction of cellular differentiation. Several cytokines, mitogen-activated protein kinases, and cell surface signaling proteins have implicated in the regulation of differentiation in HTBE (Rearick et al., 1987; Rearick and Jetten, 1989; Jetten, 1992; Moghal and Neel, 1998). These factors likely play a role in the regulation of CYP26 expression as well. Although the transcriptional regulation of CYP26 by RA seems to be mediated by a RARE in the upstream promoter region of the CYP26 gene, several additional enhancer elements have been identified that are crucial in the transcriptional control of this gene (Petkovich, 1999). We are in the process of investigating the role of these elements in the regulation of CYP26 in mucous cell differentiation.

The induction of CYP26 by RA has been reported not to require de novo protein synthesis and is considered to be regulated at the transcriptional level (Abu-Abed et al., 1998; Sonneveld et al., 1998). Several studies have provided evidence indicating that this induction is mediated by nuclear retinoid receptors. Recent studies demonstrated that in RAR γ $^{-/-}$, RXR α $^{-/-}$, and RAR γ $^{-/-}$ /RXR α $^{-/-}$ F9 cells, the induction of CYP26 was dramatically reduced compared with parental F9 wild-type cells and it was enhanced in RAR α $^{-/-}$ F9 cells, suggesting that RAR γ is involved in the induction of

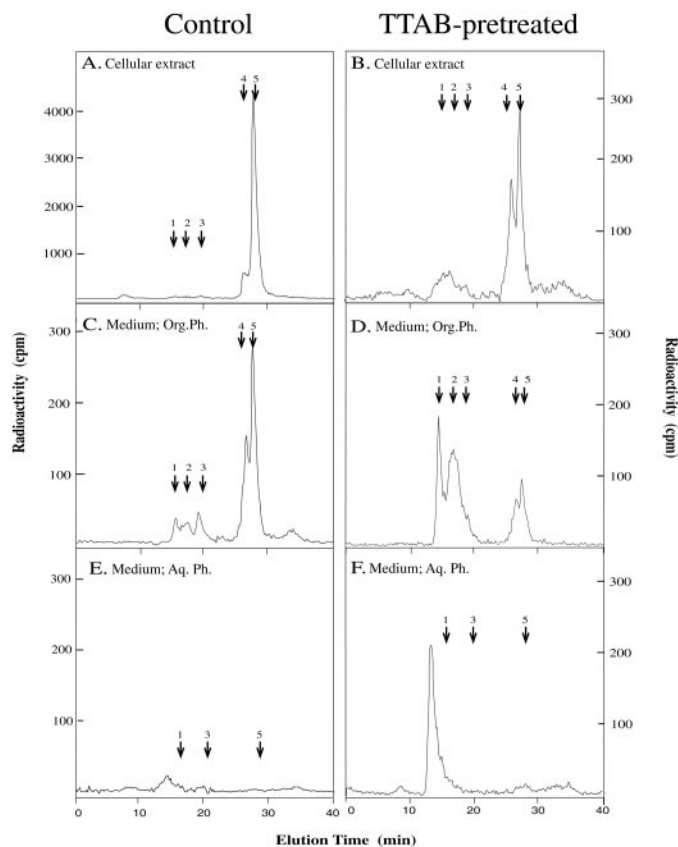


Fig. 8. Analysis of [3 H]RA metabolites from TTAB-pretreated and untreated lung carcinoma H460 cells. Cells were pretreated with 10 nM TTAB (B, D, F) or vehicle (DMSO; A, C, E) for 16 hrs. Cells were then washed and incubated with 10 nM [3 H]RA. After a 2-h incubation, medium was removed and extracted with DCM/methanol, and the organic and aqueous phase was isolated. The cells were rapidly washed in cold PBS and labeled retinoids extracted in methanol. The radiolabeled retinoids and its metabolites in the cellular extracts (A, B), and the organic (Org. Ph.; C, D) and aqueous phase (Aq. Ph.; E, F) from the medium were analyzed by reverse-phase HPLC as described under *Materials and Methods*. Arrows 1 to 5 indicate times at which the standards 4-oxo-RA, 4-hydroxy-RA, 18-hydroxy-RA, 13-*cis*-RA, and all-*trans*-RA, respectively, eluted.

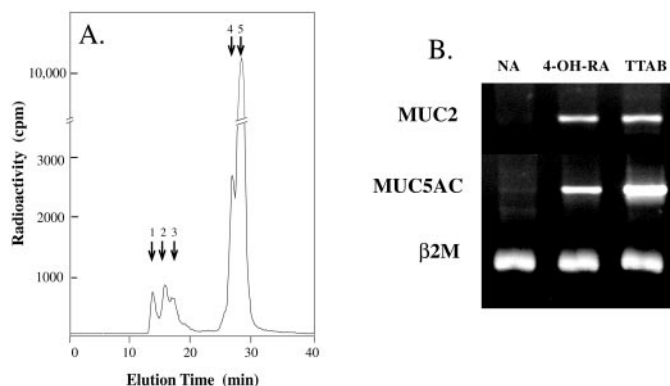


Fig. 9. A, synthesis of 4-oxo-RA and 4-hydroxy-RA by HTBE cells. Cultures of HTBE cells were grown to confluence and then treated with [3 H]RA (0.1 μ M, 5 mCi/mmol) for 3 days. The radiolabeled retinoids present in the cells were extracted in methanol and analyzed by HPLC. Arrows indicate the same standards as in Fig. 8. Arrow 1 and 2 indicate synthesis of 4-oxo-RA and 4-hydroxy-RA. B, induction of mucin gene expression by 4-hydroxy-RA (4-OH-RA) and TTAB in HTBE cells. HTBE cells were treated with 1 μ M 4-OH-RA or 0.1 μ M TTAB. After 72 h, cells were collected for RNA isolation and examined for the expression of MUC2 and MUC5AC mRNA by RT-PCR. β 2-Microglobulin (β 2 M) served as a control gene in RT-PCR.

CYP26 in F9 cells (Abu-Abed et al., 1998). A different study reported that RAR β ^{-/-} F9 cells also exhibited a dramatically reduced ability to induce CYP26 after RA treatment indicating a role for RAR β (Lane et al., 1999). In a colon carcinoma cell line containing low levels of RARs, induction of CYP26 could be restored by ectopic expression of either RAR α or γ , suggesting that both receptors can mediate induction of CYP26 in these cells (Sonneveld et al., 1998). HTBE cells have been reported to express RAR α , RAR γ , RXR α , and low levels of RXR β , RXR γ , and RAR β mRNA; the latter is increased after retinoid treatment (Nervi et al., 1991; Koo et al., 1999a). Activation of RXR is not sufficient to induce CYP26 or mucin expression. RAR α -, β -, and γ -selective retinoids can all induce CYP26 mRNA, suggesting that activation of either RAR α -, β -, and γ - could mediate the up-regulation of CYP26 in HTBE cells. This is supported by observations showing that the RAR α -selective antagonist Ro41-5253 totally blocks CYP26 induction by the RAR α -selective retinoid, whereas it only partially inhibits CYP26 induction by the RAR pan-agonist TTAB. The effects of various retinoid receptor agonists and antagonists on CYP26 expression are very similar to those reported for the mucin genes MUC2 and MUC5AC, suggesting that the control of these genes involve some common elements.

Lung carcinoma cell lines have been reported to be rather resistant to the growth-inhibitory effects of RA and to exhibit defects in the retinoid-signaling pathway (Haq et al., 1991; Nervi et al., 1991; Geradts et al., 1993; Zhang et al., 1994; Moghal and Neel, 1995; Sun et al., 1997). This is illustrated by observations showing that in many lung carcinoma cell lines, RA is unable to induce RAR β expression and RARE-dependent *trans*-activation. This resistance to retinoic acid has been linked to defects in different steps of the retinoid-signaling pathway (Nervi et al., 1991; Zhang et al., 1994; Moghal and Neel, 1995). In some instances, the defect is specific for the RAR β gene, whereas in other cases the resistance reflects a more general defect in the retinoid-signaling pathway. Comparison of the induction of CYP26 and RAR β mRNAs shows that TTAB is able to induce both RAR β and CYP26 in Calu-6 and H460 cells but that in the majority of cell lines, TTAB induces neither RAR β nor CYP26 mRNA. A recent study (White et al., 1997) reported a constitutive expression of CYP26 in non-small-cell lung cancer cell line SK-LC6. These studies point at an altered regulation of CYP26 expression in many human lung carcinoma cell lines. The inability of RA to induce CYP26 and RAR β mRNA expression in lung carcinoma cell lines, such as H441, is in agreement with the demonstration that many of these cell lines exhibit an intrinsic defect in the retinoid signaling pathway (Zhang et al., 1994; Moghal and Neel, 1995). It is interesting to note that compared with normal HTBE cells, lung carcinoma cells have a much higher rate of RA metabolism. This increased rate of metabolism is probably caused by expression of other P450 enzymes in lung carcinoma cells and may be responsible for the quick turnover of RA and the resistance of lung carcinoma cells to the growth-inhibitory effect of RA (Geradts et al., 1993; Sun et al., 1997; Adachi et al., 1998).

The precise role of CYP26 in retinoid action is not fully understood but several possible functions have begun to emerge. A role for CYP26 in catalyzing RA catabolism, thereby down-regulating the RA response or protecting cells

from excess RA, has been suggested (White et al., 1997; Iulianella et al., 1999; Swindell et al., 1999). In the early chick embryo, degradation of RA seems to be correlating with the presence of CYP26 (Swindell et al., 1999). Because the induction of CYP26 expression closely correlates with mucous differentiation and HTBE cells require retinoids for their normal function, a catabolic role for CYP26 in these cells may be a less attractive hypothesis. Several recent studies have proposed different functions for CYP26 in the regulation of cellular differentiation and embryonic development (Iulianella et al., 1999; Lane et al., 1999; Sonneveld et al., 1999). In murine embryonic stem cells, CYP26 has been reported to catalyze the conversion of retinol to 4-oxo-retinol, a metabolite that can bind and activate RARs effectively, resulting in induction of differentiation in these cells (Lane et al., 1999). In this light, it would be interesting to speculate on the role of CYP26 in HTBE cells, because retinol has been demonstrated to induce mucous cell differentiation in these cells. Preliminary studies have shown that 4-oxo-retinol is very effective in inducing mucosecretory differentiation and CYP26 in HTBE cells (J. S. Koo and A. M. Jetten, unpublished observations). Therefore, CYP26 could be involved in the generation of 4-oxo-retinol and as such may mediate the action of retinol in HTBE cells. A somewhat different role for CYP26 emerged from studies with embryonal carcinoma P19 cells, in which ectopic expression of CYP26 results in neuronal differentiation in the presence of low RA concentrations (Sonneveld et al., 1999). It was hypothesized that CYP26 expression could play a role in the generation of specific, active RA metabolites that, after binding to RARs, could regulate distinct functions/genes during neuronal differentiation. In this study, we provided evidence demonstrating that 4-hydroxy-RA, one of the metabolites generated from RA, is active and able to induce mucous cell differentiation in HTBE cells. Whether 4-hydroxy-RA or another metabolite generated by CYP26 has a specific function during mucous cell differentiation that is distinct from that of RA is an intriguing hypothesis. Studies are currently in progress to determine which of the proposed CYP26 functions is relevant to mucous cell differentiation in HTBE cells.

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