Requirement of Aryl Hydrocarbon Receptor Overexpression for CYP1B1 Up-Regulation and Cell Growth in Human Lung Adenocarcinomas

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Abstract

Purpose: CYP1B1 and CYP1A1 expression is up-regulated by activation of the aryl hydrocarbon receptor (AhR) through binding of ligands such as cigarette smoke components. We examined the association between AhR, CYP1B1, and CYP1A1 expression in noninvasive bronchioloalveolar carcinomas (BAC) and lung adenocarcinomas and investigated the effects of AhR overexpression on cell physiology.

Experimental Design: AhR, CYP1B1, and CYP1A1 expression was examined in 107 lung adenocarcinomas and 57 BAC by immunohistochemistry. AhR expression in lung adenocarcinoma H1355 cells was stably reduced by RNA interference (RNAi). AhR, CYP1B1, and CYP1A1 expression was examined using real-time reverse transcription-PCR. Cell physiology was evaluated by measuring anchorage-independent growth and intracellular reactive oxygen species.

Results: Expression of AhR and CYP1A1 was associated in smoking adenocarcinoma patients, whereas expression of AhR and CYP1B1 was associated regardless of smoking status. The level of CYP1B1, but not CYP1A1, was positively associated with AhR overexpression in BAC. 2,3,7,8-Tetrachlorobenzo-*p*-dioxin–induced *CYP1A1/1B1* expression was reduced in AhR RNAi clones. In the absence of 2,3,7,8-tetrachlorobenzo-*p*-dioxin, *CYP1B1* mRNA levels were reduced in AhR RNAi clones, whereas *CYP1A1* mRNA levels were barely detectable. Furthermore, anchorage-independent growth and intracellular oxidative stress were significantly reduced in AhR RNAi cells.

Conclusions: In the absence of exogenous AhR ligands (such as cigarette smoke components), AhR overexpression up-regulated the expression of CYP1B1 in the early stage of lung adenocarcinoma. Elevated AhR expression in lung adenocarcinoma cells could increase intracellular oxidative stress and promote cell growth, implying that disrupting AhR expression might prevent the early development of lung adenocarcinomas.

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Lung cancer is the most common cause of cancer incidence and mortality worldwide (1). The incidence of lung adenocarcinomas has been increasing worldwide, whereas the prevalence of cigarette smoking is declining (2). Thus, early prevention and treatment are important issues for lung adenocarcinoma. Identifying molecular changes in the early stages of lung adenocarcinoma will greatly facilitate these aims.

Cytochrome P450 1B1 (CYP1B1) belongs to the cytochrome P450 family 1 and is a major member of the extrahepatic xenobiotic-metabolizing CYP enzyme family (3). CYP1B1 has been shown to activate several human promutagens and procarcinogens (4), including 17β-estradiol, which is converted by CYP1B1 into a carcinogenic metabolite, 4-hydroxyestradiol (3). Several studies have shown that CYP1B1 is overexpressed in malignant cells in the kidney, breast, brain, and lung, but has only limited expression in the corresponding normal cells (5). Furthermore, CYP1B1 overexpression occurs in colorectal adenomas (6) and premalignant prostate tumors (7). Up-regulation of CYP1B1 in premalignant and malignant lesions might imply that CYP1B1 is involved in the early development of cancer. If this is the case, differential expression of CYP1B1 between

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cancerous and noncancerous cells may provide a potential chemopreventive and therapeutic strategy for CYP1B1-based prodrugs.

CYP1B1 expression is regulated by several genetic and epigenetic mechanisms (8). For example, CYP1B1 is transcriptionally activated via AhR and cyclic AMP pathways in a tissue-specific manner (8). It has been shown that CYP1B1 gene expression and activity are increased by ligands of aryl hydrocarbon receptor (AhR; ref. 3). AhR ligands include polycyclic aromatic hydrocarbons and halogenated aromatic hydrocarbons such as benzo(*a*)pyrene and 2,3,7,8-tetrachlorobenzo-*p*-dioxin (9). Liganded AhR translocates to the nucleus and heterodimerizes with AhR nuclear translocator to up-regulate several drug-metabolizing enzymes, including cytochrome *P*450 1A1 (CYP1A1) and CYP1B1 (10, 11). Exposure to cigarette smoke, which contains various polycyclic aromatic hydrocarbons, was shown to increase CYP1A1 and CYP1B1 expression in normal lung tissues and cells (12, 13). Previously, we reported that AhR is overexpressed in lung adenocarcinoma, which correlates with CYP1B1 expression (14, 15). However, more than 50% of lung adenocarcinoma patients in Taiwan are nonsmokers (16). To understand the mechanism of elevated CYP1B1 expression in lung adenocarcinoma cells, it is necessary to clarify the role of exposure to AhR ligands, such as cigarette smoke components, in CYP1B1 expression in lung adenocarcinoma.

Loss-of-function studies for AhR indicate that AhR might have physiologic functions (17). Expressing a constitutively active form of AhR by deleting a motif induces several adverse effects, such as stomach tumors and inflammatory skin lesions (18, 19). It is possible that high expression levels of normal AhR may also disturb the homeostasis of certain biological functions to initiate and/or augment certain pathologic changes.

Because a high level of CYP1B1 was detected in premalignant tumors, we hypothesized that AhR overexpression occurs in the early stages of lung adenocarcinoma and that this overexpression up-regulates CYP1B1 expression. Bronchioloalveolar carcinomas (BAC) are noninvasive and considered to be an early stage of adenocarcinoma (20). The objectives of this study were to evaluate this hypothesis by comparing AhR, CYP1B1, and CYP1A1 expression in BAC and lung adenocarcinoma, as well as to elucidate the function of AhR overexpression in the cell physiology of lung adenocarcinoma cells. In human lung adenocarcinoma tissues, CYP1A1 expression and a history of cigarette smoking were used as indicators for AhR ligand exposure. To study the role of AhR in CYP1A1 and CYP1B1 expression, AhR expression was modulated by RNA interference (RNAi) techniques in lung adenocarcinoma cells. Data generated from this study provide evidence that AhR and CYP1B1 are potential targets for chemoprevention or therapy of lung adenocarcinoma.

Materials and Methods

Subjects. One hundred seven lung tissue specimens were collected from the Department of Pathology, Chung Shan Medical University Hospital and Veterans General Hospital-Kaohsiung, Taiwan, between 1998 and 2004. The Chung Shan Institutional Review Board approved this study protocol and informed consent forms were obtained from all patients from whom specimens were collected. These specimens were obtained from lung cancer patients by wedge resection, lobectomy, or pneumonectomy. After formalin fixation, paraffin embedding, and routine staining with H&E, morphology was diagnosed according to the WHO classification (21). All cases were categorized as adenocarcinoma and by histologic subtype, including 22% acinar, 3% papillary, 4% solid, and 71% mixed subtype. Pure BAC specimens were not included in this study. Among these adenocarcinoma, 57 cases showed BAC mixed with invasive adenocarcinoma. A total of 58 males and 49 females were recruited. Smoking status as either smoker or nonsmoker was determined. A nonsmoker was defined as a subject who had never smoked. Individuals who currently or had smoked, regardless of smoking history, were counted as smokers. The majority (73 of 103, 71%) of patients were nonsmokers; smoking history was not available for four patients.

Immunohistochemistry. Immunohistochemistry was done as previously described (15). Briefly, the primary antibodies included anti-AhR (1:90 dilution; Biomol, Plymouth Meeting, PA), anti-CYP1B1 (1:1500 dilution; clone WB-1B1, Gentest Corp., Woburn, MA), and anti-CYP1A1 (1:100 dilution; clone H-70, Santa Cruz Biotechnology, Santa Cruz, CA). After incubation with the primary antibodies overnight at 4°C, a streptavidin-biotin peroxidase method was carried out according to the manufacturer's instructions (Universal LSAB2 kit, DakoCytomation, Glostrup, Denmark). Finally, these sections were counterstained with hematoxylin.

Evaluation of protein expression of AhR, CYP1B1, and CYP1A1 in lung tissue. Immunoreactivities of AhR, CYP1B1, and CYP1A1 were evaluated via immunostaining of the lung tumor and bronchiolar cells (Fig. 1) in the tissues examined. Because AhR is abundant in the basal cells of hyperplastic prostates (14) and CYP1B1 is constitutively expressed in the smooth muscle cells of the vascular and bronchiolar walls (15), vascular or bronchiolar walls in lung tissues and

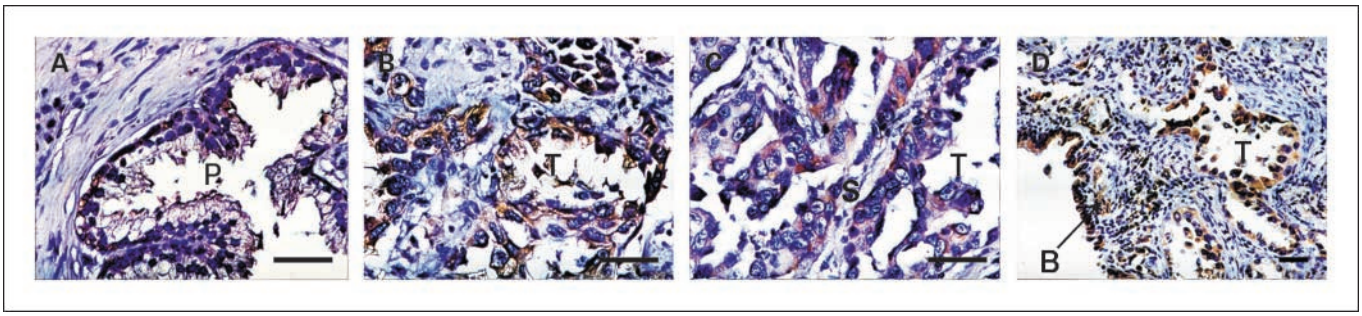

Fig. 1. Immunohistochemical analysis of AhR, CYP1A1, and CYP1B1 in lung adenocarcinoma. *A,* cytoplasmic AhR staining in the basal cells of hyperplastic prostate was used as a positive control. *B,* AhR expression was detected in the cytoplasm of adenocarcinoma cells at an intensity similar to the positive control. *C,* adenocarcinoma cells showed positive cytoplasmic CYP1A1 staining as compared with the CYP1A1-negative stroma. *D,* the cytoplasmic CYP1B1 immunostaining in these adenocarcinoma cells was similar to the adjacent bronchiolar wall; adenocarcinoma cells with this intensity of CYP1B1 immunostaining were defined as high expressers. The bronchiolar epithelial cells also showed high CYP1B1 expression. P, prostate; T, adenocarcinoma; S, stroma; B, bronchiolar wall. Bar, 50 μm.

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Table 1. Association between expression of AhR and CYP1B1 or CYP1A1 in lung adenocarcinoma stratified by smoking history

	AhR	CYP1B1 expression			CYP1A1 expression		
		Low	High	P^*	Negative	Positive	P^*
Total (n = 107)	Low	39	19	<0.001	43	15	0.009
	High	16	33		24	25	
Smokers (n = 30)	Low	9	5	0.024	13	1	0.002
	High	3	13		6	10	
Nonsmokers (n = 73)	Low	28	14	0.031	29	13	0.459
	High	12	19		18	13	

NOTE: AhR, CYP1B1, and CYP1A1 proteins were detected with immunohistochemical methods. Expression levels were calculated as of cases showing cells with cytoplasmic immunostaining scored according to staining intensity. For AhR immunohistochemistry, the specimen was scored as a high expresser when >10% of the adenocarcinoma cells exhibited cytoplasmic staining intensities that were equal to those of the prostate (positive control, Fig. 1A). For CYP1B1 immunohistochemistry, the specimen was scored as a high expresser when >10% of the adenocarcinoma cells exhibited cytoplasmic staining intensities that were equal to those of adjacent bronchiolar walls (bronchiolar wall, Fig. 1D). For CYP1A1 immunohistochemistry, the specimen was considered to be CYP1A1 positive when >10% of the adenocarcinoma cells exhibited cytoplasmic staining intensities that were stronger than those of the adjacent stroma (Fig. 1C). Four cases were missing a smoking history.
$^*P < 0.05$, Fisher's exact test.

hyperplastic prostate sections were used as positive controls for CYP1B1 and AhR immunostaining, respectively. Both AhR and CYP1B1 immunoreactivities in lung tumors and bronchiolar cells were graded against these positive controls. The intensities of immunoreactivity were scored by two independent pathologists in a double-blind system. The scoring results were then compared before conclusive ratings were made. When ≥10% of the cells in the tissues examined showed CYP1B1 immunointensities higher than those in the positive controls, they were rated as high expressers. Likewise, those tissues with CYP1B1 immunointensities less than those of the controls were rated as low expressers. For CYP1A1 evaluation, immunoreactivity of the tumor or bronchiolar cells was compared with those in the adjacent stoma that showed negative CYP1A1 immunoreactivity. CYP1A1 immunostaining intensity of the lung tumor or bronchiolar cells (>10% of cells in the tissues) being stronger than the adjacent stroma would be considered as a positive expresser. The percentage of high-expressing cells of at least 500 examined cells was then calculated.

Cell culture. Two adenocarcinoma cell lines, H1355 and H23, and three squamous cell carcinoma cell lines, H226, CH27, and Calu-1, were studied. H1355, H23, and H226 cells were a kind gift from Dr. C-M. Tsai (Veterans General Hospital-Taipei, Taiwan, ROC). Calu-1 cells were purchased from American Type Culture Collection (Manassas, VA). Information on H1355, H23, H226, and Calu-1 cells is available from the American Type Culture Collection. H1355, H23, H226, and CH27 cells were maintained in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum. Calu-1 cells were maintained in McCoy's 5A medium supplemented with 10% heat-inactivated fetal bovine serum.

Quantitative real-time reverse transcription-PCR assay. Total cell RNA was prepared using TRIzol reagent (Life Technologies, Rockville, MD) and the phenol-chloroform extraction method. Synthesis of cDNA was done with M-MLV Reverse Transcriptase (Promega, Madison, WI) with 2 μg total RNA mixed with 250 ng random primer (BioLabs, Beverly, MA). Quantitative PCR was carried out using the TaqMan PCR kit (AB Gene House, Epsom, Surrey, United Kingdom) and analyzed on an ABI PRISM 7700 Sequence Detector System (Perkin-Elmer Applied Biosystems, Foster City, CA). The primers and probe of AhR were from the Assay-on-Demand Gene Expression Assay Mix (Perkin-Elmer Applied Biosystems). The primer and probe sequences for *CYP1A1*, *CYP1B1*, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) have previously been described (22). The PCR reactions consisted of an initial step of 2 min at 50°C and a polymerase activation step for 10 min at 95°C, followed by 40 cycles of 95°C for 15 s, and 1 cycle at 60°C for 1 min. Quantitative values were obtained from the threshold cycle (Ct) number. The expression level of the target gene for each sample was normalized to its *GAPDH* mRNA content. Fold change = $2^{-\Delta Ct}$; $\Delta Ct = Ct_{\text{target gene}} - Ct_{GAPDH}$.

Western immunoblotting. Cytosolic homogenates were prepared for AhR Western immunoblotting and microsomes were prepared for CYP1B1 Western immunoblotting as previously described (15, 23). The samples were separated by 10% SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane. Anti-AhR (Biomol), anti-CYP1B1 (Gentest), anti–protein disulfide isomerase (Affinity BioReagents, Pittsburgh, PA), or anti-GAPDH (Santa Cruz Biotechnology) was used. Bands were visualized using an enhanced chemiluminescence kit according to the manufacturer's instructions (Amersham, Buckinghamshire, United Kingdom).

Aryl hydrocarbon hydroxylase activity assay. The aryl hydrocarbon hydroxylase activity assay was used to measure CYP1A1 and CYP1B1 enzyme activity (24). Cells were harvested by trypsinization, centrifuged at 10,000 × *g* for 30 min, and sonicated in 100 mmol/L HEPES, 1 mmol/L $MgCl_2$, pH 7.4. The formation of 3-hydroxy-benzo(*a*)pyrene is quantified. The protocol of the aryl hydrocarbon hydroxylase activity assay was modified as previously described (25).

RNAi. To perform RNAi, a stably expressed short hairpin RNA was driven by the human U6 promoter in an engineered pcDNA3.1/HU6 vector, a derivative of pcDNA3.1/myc-His(−)Neo (Invitrogen, San Diego, CA) with human U6 promoter (pcDNA/HU6). To generate short hairpin RNA template, two oligonucleotides, i-AhR-F (5′-GATCCGAATACTTCCACCTCAGTTGGCTTCAAGAGA-3′) and i-AhR-R (5′-AGCTTCCAAAAAAAATACTTCCACCTCAGTTGGCTCTCTTGAA-3′), which contain partial complementary sequence of the short hairpin RNA template with an overlapping loop, were synthesized and ligated with pcDNA3.1/HU6 (26, 27). The ligated product was then used to transform *E. coli* XL-10 cells. After sequencing, the vector containing the short hairpin RNA was transfected into H1355 cells to select for RNAi stable clones.

Cell growth assay. Cells were seeded in 24-well plates. After seeding, cell viability was determined on days 1, 2, 3, and 4 after plating. Cell viability was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 1 mg/mL, was added to medium and cells were incubated for an additional 4 h. Precipitated formazan was dissolved in 0.5-mL DMSO and the absorbance was measured at 535 nm.

Anchorage-independent growth assay. A total of 5×10^3 cells were suspended in RPMI 1640 containing 0.35% agarose and 10% fetal

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bovine serum and layered onto a 1.5-mL bed of RPMI 1640 containing 0.5% agarose and 10% fetal bovine serum in a six-well plate. Plates were incubated at 37°C for 3 weeks. Cell clones >50 μm in diameter were counted.

Reactive oxygen species measurement. Intracellular reactive oxygen species were detected using 2′,7′-dichlorofluorescein diacetate as previously described (28). A flow cytometer (Becton, Dickson and Company, San Jose, CA) was used to detect 2′,7′-dichlorofluorescein formed by the reaction of 2′,7′-dichlorodihydrofluorescein with intracellular peroxides. Relative levels of intracellular reactive oxygen species were determined by measuring the mean value of fluorescence per cell.

Statistical analysis. Trends for the expression levels in bronchiolar epithelium, BAC, and adenocarcinoma were determined by χ^2 for linear trend tests (Epi Info 2000 software statistical package, Centers for Disease Control and Prevention, Atlanta, GA). Differences in data were considered statistically significant at $P < 0.05$. Fisher's exact test was used for the expression of differences between groups (SPSS 8.0 software statistical package, SPSS, Inc., Chicago, IL). Data between groups in cell culture experiments were compared using the Student's t test.

Results

Association between AhR, CYP1B1, and CYP1A1 expression in human lung adenocarcinoma. Among 107 human lung adenocarcinoma, 49 were AhR high expressers, 52 were CYP1B1 high expressers, and 40 expressed CYP1A1 (Table 1). CYP1B1 and CYP1A1 expression each associated with AhR expression. After stratification by smoking status, the association between AhR and CYP1B1 remained for both smokers and nonsmokers. However, the association between AhR and CYP1A1 was only found in smokers. These results indicate that smoking status influenced the association between AhR and CYP1A1, but not between AhR and CYP1B1.

AhR, CYP1B1, and CYP1A1 expression trends in adjacent bronchiolar epithelia, mixed BAC, and invasive adenocarcinoma. BAC has been proposed to be a noninvasive neoplasia, eventually progressing to invasive adenocarcinoma. Among 107 adenocarcinoma, 57 cases were adenocarcinoma showing BAC mixed with invasive adenocarcinoma. We used these mixed-type specimens to investigate the association and trends of AhR, CYP1B1, and CYP1A1 expression among adjacent normal bronchiolar epithelia, BAC, and invasive adenocarcinoma. The fraction of cases with high expression levels of AhR and CYP1B1 was greatest (at 56% of the specimens showing high expression) for the invasive adenocarcinoma component of the mixed-type specimens (Table 2). In addition, elevated AhR and CYP1B1 expression was more common in the BAC (noninvasive) than in the adjacent bronchiolar epithelia. The percentage of specimens scored as expressing CYP1A1 in adenocarcinoma was 37%, only slightly higher than the adjacent bronchiolar epithelia. BAC exhibited the lowest CYP1A1 expression at 12%. Accordingly, the expression levels of AhR and CYP1B1, but not CYP1A1, increased in the order of adjacent bronchiolar

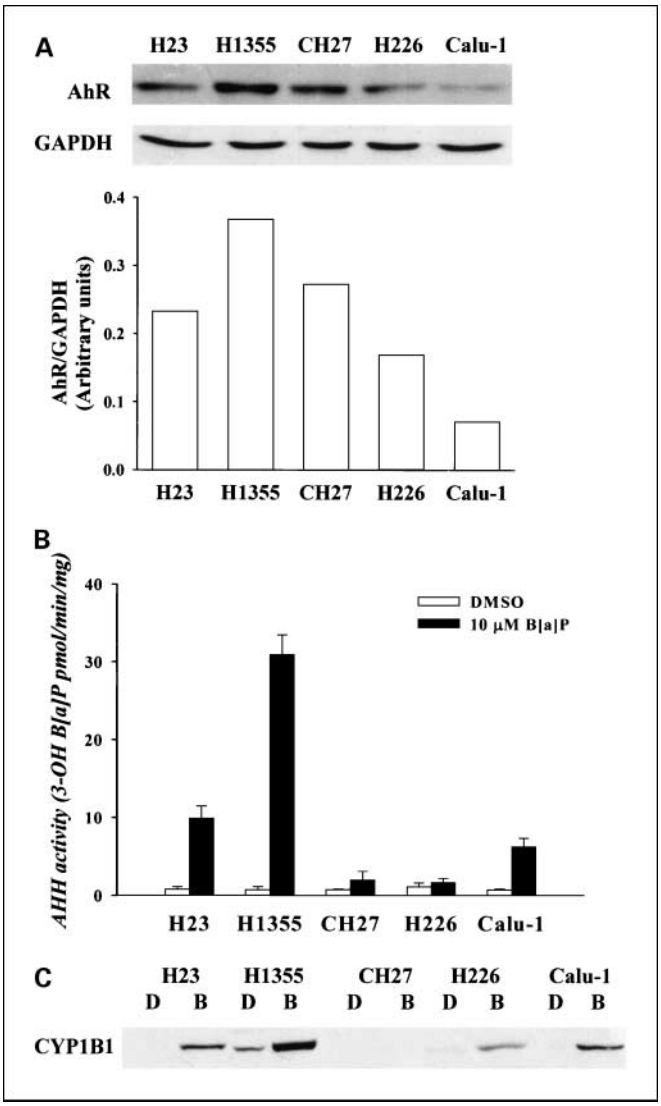

Fig. 2. AhR expression and activation in human lung cancer cells. *A,* AhR protein in cell homogenates was detected by Western immunoblotting. Protein levels were normalized to GAPDH. *B,* cells were treated with 0.1% DMSO or 10 μmol/L benzo(*a*)pyrene (*B[a]P*) for 24 h. AhR activation was determined by measuring increased aryl hydrocarbon hydroxylase (*AHH*) activity. Each analysis was replicated at least thrice. Columns, mean; bars, SD. *C,* cells were treated with 0.1% DMSO (*D*) or 10 μmol/L benzo(*a*)pyrene (*B*) for 24 h. CYP1B1 in microsomes was measured by Western immunoblotting.

Table 2. Expression of AhR, CYP1B1, and CYP1A1 in mixed BAC and invasive adenocarcinoma

Mixed adenocarcinoma (n = 57)	Cases* (%)		
	High AhR†	High CYP1B1†	Positive CYP1A1
Adenocarcinoma	32 (56)	32 (56)	21 (37)
BAC‡	27 (47)	29 (51)	7 (12)
Adjacent bronchiolar epithelium§	6 (11)	16 (28)	14 (25)

*Cases represent the number of cases with high AhR, high CYP1B1, or positive CYP1A1 expression in each group. AhR, CYP1B1, and CYP1A1 proteins were detected by immunohistochemical methods. The expression levels were calculated as the percentage of cases showing cells with cytoplasmic staining scored as previously described in Table 1 as well as in Materials and Methods.

†$P < 0.05$, χ^2 for trend test.

‡Six specimens of BAC were not done for CYP1A1 immunohistochemistry.

§Five specimens of adjacent bronchiolar epithelia were not done for CYP1B1 immunohistochemistry.

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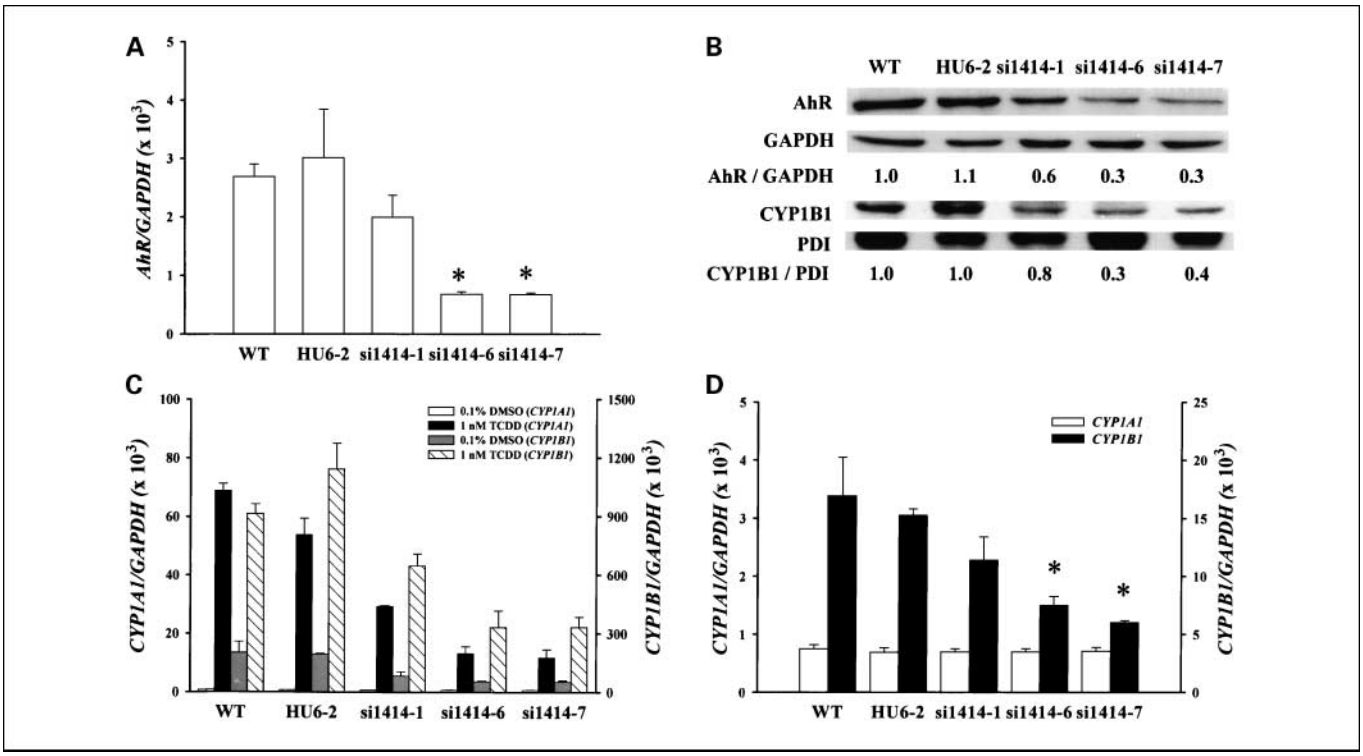

Fig. 3. Comparison of AhR expression and activation in control and AhR RNAi H1355 cells. *A,* AhR mRNA levels of the RNAi clones were determined with a real-time reverse transcription-PCR assay. *B,* AhR and CYP1B1 protein levels were determined by Western immunoblotting. AhR protein levels were normalized to GAPDH levels, and CYP1B1 protein levels were normalized to protein disulfide isomerase (*PDI*) levels. *C,* cells were treated with 0.1% DMSO or 1 nmol/L 2,3,7,8-tetrachlorobenzo-*p*-dioxin (*TCDD*) for 24 h. *CYP1A1* and *CYP1B1* mRNA levels were determined by real-time reverse transcription-PCR. *D,* constitutive *CYP1A1* and *CYP1B1* mRNA levels in these cells were determined by real-time reverse transcription-PCR. WT, wild-type H1355 cells; HU6-2, stable control clones. Si1414-1, si1414-6, and si1414-7 are AhR RNAi clones. AhR expression levels were normalized to GAPDH levels. Columns, mean of four replicates; bars, SD. *, $P < 0.05$, compared with wild-type cells.

epithelia < BAC < invasive adenocarcinoma (Table 2). These results suggest that AhR and CYP1B1 overexpression might be involved in the development of mixed BAC and adenocarcinoma.

Responsiveness of AhR to the ligand benzo(a)pyrene in lung adenocarcinoma H1355 cells. AhR expression and responsiveness to benzo(*a*)pyrene, an AhR ligand, were compared in several human lung cancer cell lines to choose an appropriate model cell line for further experimentation. Among the five lung cancer cell lines tested, lung adenocarcinoma H1355 cells expressed the highest level of AhR protein (Fig. 2A). Benzo(*a*)pyrene consistently induced the highest level of aryl hydrocarbon hydroxylase activity in H1355 cells (Fig. 2B). CYP1B1 protein, present at a detectable level in solvent-treated H1355 cells, was greatly increased in benzo(*a*)pyrene–treated H1355 cells (Fig. 2C). H1355 cells appeared to express high levels of AhR and were highly responsive to AhR ligands. Therefore, H1355 cells were used for subsequent RNAi experiments.

Isolation of stable AhR RNAi clones in H1355 cells with reduced AhR expression. We introduced a short hairpin RNA for AhR into H1355 cells to knock down AhR levels. To measure the reduction in AhR expression, AhR mRNA and protein levels as well as 2,3,7,8-tetrachlorobenzo-*p*-dioxin–induced *CYP1A1* and *CYP1B1* mRNA levels were measured. Three stable AhR RNAi clones were isolated: si1414-1, si1414-6, and si1414-7. HU6-2 clone was the vector control cells. AhR mRNA and protein levels in si1414-6 and si1414-7 cells were considerably reduced to 30% of wild-type and HU6-2 cell levels (Fig. 3A and B). However, AhR mRNA and protein levels in si1414-1 cells were only reduced to 60% of wild-type and HU6-2 cell levels (Fig. 3A and B). Consistent with reduced AhR expression, 2,3,7,8-tetrachlorobenzo-*p*-dioxin–induced *CYP1A1* and *CYP1B1* expression was greatly reduced in si1414-6 and si1414-7 cells and moderately reduced in si1414-1 cells (Fig. 3C).

Effects of AhR RNAi on constitutive CYP1B1 and CYP1A1 expression. Some components of cigarette smoke are AhR agonists, and cigarette smoke has been shown to induce CYP1A1 and CYP1B1 expression in lung cells and tissues (12, 13). However, we observed a positive association between AhR and CYP1B1 expression in nonsmoking patients. Therefore, it was important to evaluate the role of AhR expression in basal expression levels of *CYP1B1* and *CYP1A1*. In the absence of exogenously added AhR ligands, CYP1B1 mRNA and protein levels correlated positively with AhR levels. Both si1414-6 and si1414-7 cells showed reduced CYP1B1 expression of ~30% of control cells, and CYP1B1 expression in si1414-1 cells was reduced to 80% of control cells (Fig. 3B and D). However, constitutive *CYP1A1* mRNA levels were extremely low and similar between control cells and AhR RNAi clones (Fig. 3D). These results suggest that AhR expression regulates CYP1B1 expression in lung adenocarcinoma cells when exogenous ligands are absent.

We attempted to identify CYP1A1 and CYP1B1 in lung tissues by Western blotting. CYP1B1 was readily detected (15),

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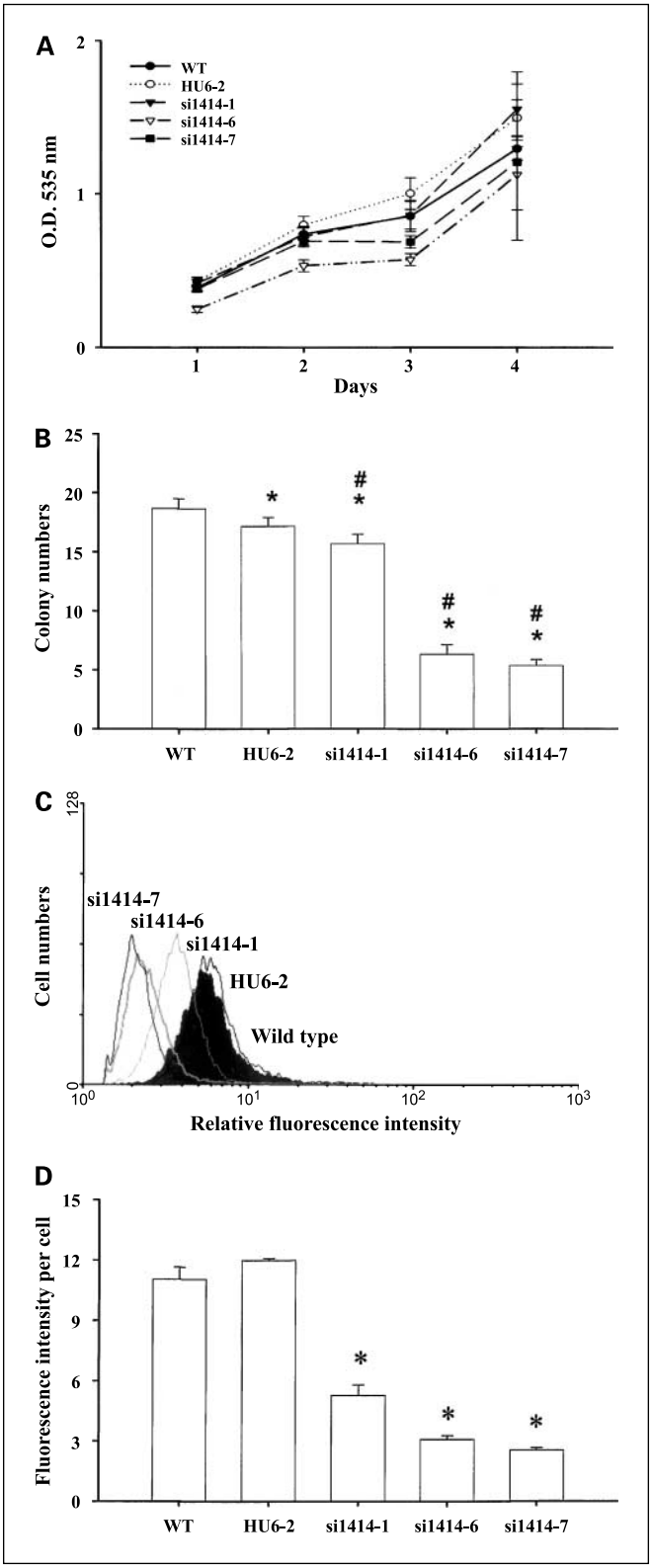

Fig. 4. Effects of the introduction of AhR RNAi in H1355 cells on cell physiology. *A,* cells were seeded on day 0 and cell viability was determined on days 1 to 4 with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. *B,* cells were seeded in soft agar and, after 3 wk, the colonies were counted as a measure of anchorage-independent growth. *C,* intracellular reactive oxygen species levels were determined by flow cytometry. The results were quantified and are presented in (*D*). Columns, mean; bars, SD. *, $P < 0.05$, compared with wild-type cells. #, $P < 0.05$, compared with HU6-2 cells.

but CYP1A1 was only weakly detected with the antibody used in our study. Similarly, CYP1A1 was barely detectable in the lung cancer cell lines with Western immunoblotting. Comparing the real-time reverse transcription-PCR data, we believe that the levels of CYP1A1 were much lower than those of expressed CYP1B1. Furthermore, the anti-CYP1A1 used in our study might be less sensitive than anti-CYP1B1.

Roles of AhR expression in the cell physiology of lung adenocarcinoma H1355 cells. Our previous (14) and present studies showed that AhR is overexpressed in lung adenocarcinoma. Some possible roles of AhR in lung adenocarcinoma cells were evaluated by measuring cell growth rate and anchorage-independent growth as well as intracellular reactive oxygen species. We found that cell growth rate was only slightly reduced in AhR RNAi–transfected clones (si1414-6 and si1414-7; Fig. 4A). Correlating with AhR levels, anchorage-independent growth was significantly reduced in si1414-1 cells (with a median level of AhR expression) and greatly reduced in si1414-6 and si1414-7 cells (with low levels of AhR expression; Fig. 4B). Importantly, levels of intracellular reactive oxygen species also strongly correlated with AhR levels in that they were moderately reduced in si1414-1 cells and substantially reduced in si1414-6 and si1414-7 cells (Fig. 4C and D). These results suggest that elevated AhR expression may enhance anchorage-independent growth and increase intracellular reactive oxygen species levels in lung adenocarcinoma.

Discussion

Whereas CYP1B1 is overexpressed in lung adenocarcinoma, its expression in lung is also induced by cigarette smoke. It remains to be clarified whether CYP1B1 overexpression in lung adenocarcinoma is a consequence of cigarette smoking. After controlling for history of cigarette smoking and when compared with CYP1A1 expression, we concluded that CYP1B1 expression was not dependent on cigarette smoking in lung adenocarcinoma. By comparing the AhR expression in lung adenocarcinoma and modulating AhR expression in adenocarcinoma cells, we further showed that AhR overexpression up-regulated CYP1B1 expression in lung adenocarcinoma.

Unlike CYP1B1 overexpression in a variety of cancers, AhR overexpression has only been reported in lung adenocarcinoma (14) and pancreatic cancer (29). We did not observe AhR overexpression when CYP1B1 was overexpressed in colorectal cancer (6). Therefore, AhR overexpression may be characteristic of only a few cancers including lung adenocarcinoma. Some of our data suggest that high levels of AhR in lung adenocarcinoma are not constitutively active but inducible. First, we observed increased immunostaining for AhR in the cytoplasm of tumor cells. Second, AhR expression correlated with CYP1A1 expression in smokers. Third, AhR was highly inducible, as measured by *CYP1A1* induction, in lung adenocarcinoma cell lines. The effects of AhR overexpression, anchorage-independent growth and reactive oxygen species accumulation, were partially elucidated in this study. It has been shown that reactive oxygen species generation and accumulation are elevated in cancer cells (30), and this may increase cell proliferation. Uncontrolled cell growth or hyperproliferation is an early phenotype of tumor cells. In this study, we found that AhR overexpression occurred in BAC, which is an early stage of adenocarcinoma. These data suggested that AhR

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overexpression is involved in the early development of lung adenocarcinoma. Furthermore, AhR overexpression was beneficial to tumor cell growth.

Cytochrome *P*450 members catalyze the oxidation of endogenous and exogenous lipophilic compounds. Poor coupling of the *P*450 catalytic cycle yields active oxygen species, such as superoxide anion (31, 32). Because CYP1B1 expression is positively correlated with AhR expression in lung adenocarcinoma tissues and cells, it is plausible that elevated CYP1B1 activity enhances reactive oxygen species generation in cells highly expressing AhR. Further studies are needed to verify this hypothesis. In addition to cytochrome *P*450, AhR also regulates phase II drug-metabolizing enzymes and cell growth–related genes (33, 34). Expression of reactive oxygen species–related genes should also be altered following AhR overexpression.

Both CYP1B1 and AhR are potential targets for chemoprevention. AhR-mediated CYP1A1 and CYP1B1 activities convert polycyclic aromatic hydrocarbons into carcinogenic epoxide metabolites. Many flavonoids have been suggested as chemopreventives because they antagonize AhR activation, inhibit CYP1A1/CYP1B1 activity, or prevent polycyclic aromatic hydrocarbon–induced genotoxicity (35–39). Moreover, CYP1B1 also provides a therapeutic target for potential anticancer drugs that can be metabolically activated by CYP1B1 (40). Here, we report that AhR is overexpressed in lung adenocarcinoma. Some AhR agonists inhibit the growth of pancreatic cancer cells that express high levels of AhR (29). Therefore, AhR may also be considered to be a therapeutic target for lung adenocarcinoma in the future.

In our study, AhR reduction diminished the basal level of *CYP1B1* mRNA, but not that of *CYP1A1*, in H1355 cells. Furthermore, AhR expression failed to correlate with CYP1A1 expression in lung adenocarcinoma among nonsmoking patients. The intrinsic properties of enhancers at *CYP1A1* and *CYP1B1* promoters might be responsible for the differences in gene expression in response to the exogenously ligand-free AhR activity. Several coactivators have been identified that provide a bridge between AhR molecules at the enhancer and general transcription factors at the promoter of *CYP1A1* (41). In addition to the xenobiotic response element for AhR, an enhancer consisting of steroidogenic factor 1 elements and a cyclic AMP-response element also regulates human *CYP1B1* expression (42). These elements have not been assessed in the *CYP1A1* enhancer. In the absence of AhR ligands, cyclic AMP was shown to trigger nuclear translocation of AhR (43). Addition of 8-bromo-cyclic AMP or the adenyl cyclase agonist forskolin increased *CYP1B1* expression (44). Therefore, AhR might interact with different coactivators and transcription factors to differentially regulate *CYP1A1* and *CYP1B1* expression.

Few studies have investigated the relationship between AhR expression and oncogenesis. Chesire et al. (45) reported that overexpression of a hyperactive mutant of β-catenin by transfection increases AhR expression in prostate cancer cells. Although β-catenin mutation or β-catenin nuclear translocation is rare in lung cancer, it is still possible that the downstream signaling pathway of β-catenin may be involved in AhR overexpression in lung adenocarcinoma.

In summary, AhR overexpression up-regulated CYP1B1 expression in lung adenocarcinoma tissues and cell lines in the absence of exogenous ligands. Abolishing AhR expression reduced intracellular reactive oxygen species and tumor cell growth in lung adenocarcinoma. Therefore, AhR and CYP1B1 might be potential therapeutic targets for lung adenocarcinoma.

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