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Differential Toxicogenomic Responses to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin in Malignant and Nonmalignant Human Airway Epithelial Cells

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In humans, exposure to high levels of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is associated with chronic obstructive pulmonary disease and lung cancer. While several studies have shown that the lung is a target organ for TCDD toxicity, little is known on the specific biological pathways altered by TCDD. Studies have shown that the transcriptional response of TCDD (*in vivo* and *in vitro*) is complex, and exhibits cell type and tissue specificity. Thus, the purpose of this study was to look at global and concentration-dependent effects of TCDD on gene expression in human lung cells. Gene expression profiling of both a nontumorigenic (HPL1A) and a malignant, tumorigenic lung cell line (A549) was performed by microarray dual fluorescence hybridizations in cells treated with increasing concentrations of TCDD (0, 0.1, 1, 10 nM) for 24 h. Real time RT-PCR was used to verify alterations in specific genes. Results showed that 68 out of 2091 genes were changed in each cell line, and 15 of those genes were found altered in both cell lines. Common gene responses altered by TCDD were identified and included known xenobiotic metabolizing genes, genes known to alter cell cycle, as well as genes that are involved with cell signaling and that mediate cell motility or communication. Cell line specific differences in gene expression were found that indicate the nonmalignant HPL1A cells are retinoic acid responsive. In addition, TCDD altered specific immunomodulatory genes in the HPL1A cells. These data show that TCDD alters multiple integrated networks of signaling pathways associated with pulmonary disease, particularly that of lung cancer.

Key Words: **toxicogenomics; microarray analysis; dioxin; TCDD; AhR; lung cells; A549; HPL1A; real time RT-PCR.**

The environmental contaminant, 2,3,7,8-tetrachlordibenzo-*p*-dioxin (TCDD) is a known carcinogen when tested in rodent bioassays. The induction of tumors by TCDD is tissue, sex, and species specific (Della Porta *et al.,* 1987; Kociba *et al.,* 1978; NTP, 1982; Rao *et al.,* 1988). In humans, the carcinogenic risk associated with TCDD exposure is increased for all cancers combined (Kogevinas, 2000) with epidemiological studies that support increased risk for specific types of cancer in highly exposed populations. These include non-Hodgkin's lymphoma, soft-tissue sarcoma, rectal cancer, and lung cancer (Bertazzi *et al.,* 2001; Kogevinas *et al.,* 1997; Steenland *et al.,* 1999). In humans, TCDD exposure is also associated with chronic obstructive pulmonary disease (Bertazzi *et al.,* 2001; Pesatori *et al.,* 1998; Steenland *et al.,* 1999).

Previous studies in our laboratory (Tritscher *et al.,* 1999, 2000) as well as rodent bioassays (NTP, 1982), confirm that the lung is a target organ for TCDD. In rats, TCDD disrupts the normal cellular proliferation/differentiation milieu resulting in alveolar-bronchiolar metaplasia and hyperplasia of the bronchiolar epithelium (Tritscher *et al.,* 1999), adenomatous hyperplasia (NTP, 1982), and keratinizing squamous cell carcinoma of the lung (Kociba *et al.,* 1978). There is evidence that TCDD causes tumor promotion by interfering with intracellular signal transduction pathways related to growth factors and cytokines such as transforming growth factor and interleukin-1β (IL1β; Abbott and Birnbaum, 1990; Sutter *et al.,* 1991). Investigations aimed at examining signaling pathways in lung are needed since the mechanisms whereby dioxins induce pulmonary diseases and/or cancer is largely unknown.

Multiple studies have identified a number of genes whose expression is altered by TCDD (Frueh *et al.,* 2001; Kurachi *et al.,* 2002; Lai *et al.,* 1996; Puga *et al.,* 2000b; Sutter and Greenlee, 1992). TCDD alters many of these genes by a direct receptor-mediated mechanism, involving a basic helix-loop-helix (bHLH) protein known as the aryl hydrocarbon receptor (AhR; Denison and Heath-Pagliuso, 1998; Okey *et al.,* 1994; Rowlands and Gustafsson, 1997). The AhR is maintained in a ligand-binding state by association with cytosolic proteins that include heat shock protein 90 (HSP90) dimer, c-SRC (Enan and Matsumura, 1996), AIP1 (also known as ARA9 or XAP2; Ma and Whitlock, 1997), and p23 (Kazlauskas *et al.,* 1999).

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Upon ligand binding, the AhR dissociates from the HSP90, is transferred into the nucleus where it forms a heterodimer with another bHLH protein, the aryl hydrocarbon nuclear translocator (ARNT). The AhR/ARNT heterodimer binds to the DNA xenobiotic responsive element (XRE, also known as the AhRE or DRE) in the promoter region of Ah responsive genes. Coactivators and general transcription factors are recruited with the result being transactivation of specific AhR-responsive genes. While this is a common mechanism, not all genes altered by exposure to dioxin necessarily have XRE in their DNA. It is becoming increasingly clear that the AhR is involved in a number of complex protein–protein interactions. For example, the AhR can sequester ARNT preventing other known ARNT-dependent intracellular events to occur (Chan *et al.*, 1999). Alternately, it can bind directly to other proteins that interact with other transcription factors (Ge and Elferink, 1998; Puga *et al.*, 2000a). Regardless of the downstream mechanism responsible for gene expression changes, the initial event is TCDD binding to the AhR.

Given the central role of the AhR in mediating the effects of TCDD, the identity of genes altered by activation of the AhR is crucial to understanding the pathological effects of TCDD. Early studies using differential hybridization screening of a human keratinocyte cell line identified novel target genes of TCDD, including cytochrome P450 1B1 (CYP1B1), plasminogen activator inhibitor-2 (PAI2), and IL1β (Sutter *et al.*, 1991). More recently, the complexity of the transcriptional response of human liver cells (HepG2) to TCDD have been reported (Frueh *et al.*, 2001; Puga *et al.*, 2000b). This study and others showed using cDNA microarray analysis that multiple genes are altered transcriptionally by TCDD, both directly and indirectly (Kurachi *et al.*, 2002; Thomas *et al.*, 2001). In mice, Park *et al.* (2001) also used cDNA microarray analysis, to identify *in vivo* novel TCDD targets with putative roles involved in toxicity of TCDD in the thymus. Tissue and cell specificity is a crucial factor in the biochemical and toxic effects of TCDD. With this in mind, the lung as a target organ for TCDD toxicity has widely been ignored although some reports of genes altered in the lung are available (DeVito and Birnbaum, 1995; Santostefano *et al.*, 1996; Vogel *et al.*, 1994, 1998). The lung is a known target organ for TCDD and other polyhalogenated aromatic hydrocarbons (PHAHs) in both rodent and humans. The fundamental mechanism for altered gene transcription is via activation of the AhR, hence there clearly is a need to identify the signaling pathway networks that are altered by TCDD and other PHAH compounds.

The majority of pulmonary adenocarcinomas are bronchogenic in origin, and some are derived from the periphery of the lung, that possesses type II pneumocyte characteristics. We were interested in the sensitivity of type II pneumocytes to TCDD since hyperplasia of these cells is linked to chronic lung disease, and this cell type is a possible precursor of pulmonary adenocarcinoma (Mori *et al.*, 1998). Type II pneumocytes represent 3–5% alveoli surface area, are critical in maintaining normal alveolar function, and believed to play a role in the development of chronic lung diseases. These cells are a major source of surfactant that reduces surface tension in the lung providing protective functions for the peripheral lung. They undergo proliferation and transform into type I pneumocytes upon cellular injury and subsequent repair. To assess the effects of TCDD on type II pneumocytes, two cell lines were investigated; the HPL1A and the A549 cell lines. We chose the A549 cell line because it is a well-established and characterized human lung adenocarcinoma (type II pneumocytes) that expresses a number of cytochromes P450 including CYP1A1, a well-characterized TCDD-inducible gene (Hukkanen *et al.*, 2000). The A549 cells have proven valuable in elucidating some aspects of TCDD-regulation of cytochrome P450 responses in lung cells (Vogel *et al.*, 1994). However, as a malignant cell line it may not be the most appropriate model for assessing effects of TCDD on gene expression in "nonmalignant" human lung epithelium. The HPL1A cells were used to represent a "normal" cell line. They are a recently established immortalized human peripheral lung (HPL) epithelial cell line (Masuda *et al.*, 1997) that retains morphological and biochemical characteristics of peripheral epithelial cells (type II pneumocytes and Clara cells). These characteristics include cytokeratin staining, multivesicular bodies, incomplete multilamellar body-like structures, and expression of Clara cell specific genes (Masuda *et al.*, 1997).

The objective of this present study was to identify and compare concentration-dependent effects of TCDD on gene expression in a malignant tumorigenic and a nonmalignant lung cell line. To accomplish this, gene expression profiling by microarray dual fluorescence hybridizations was performed in cells treated with increasing concentrations of TCDD for 24 h. We identified novel genes altered by TCDD that are involved with differentiation and immune regulation. These genes represent integrated networks of signaling pathways that may be associated with pulmonary disease, particularly that of lung cancer.

MATERIALS AND METHODS

Cell culture. All cell culture reagents unless otherwise stated were purchased from Life Technologies (Life Technologies, Rockville, MD). A549 cells (ATCC No. CCL-185) were purchased from American Type Culture Collection (Manassas, VA) and plated on 20-cm^2 dishes (Nalge Nunc International, Naperville, IL) at 4.2×10^4 cells/ml in F12 nutrient mixture (HAM) with glutamine and supplemented with 10% defined FBS (HyClone, Logan, UT), 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, 25 μg/ml amphotericin B, and 1 mM HEPES. HPL1A cells were plated at 5.4×10^4 cells/ml in media as described previously (Masuda *et al.*, 1997). A stock solution of TCDD was prepared in FBS as reported previously (Spencer *et al.*, 1999) and diluted to working concentrations with FBS for treatments. Forty-eight h after incubation, half of the culture media was removed and spiked with the TCDD/FBS or vehicle/FBS to achieve final concentrations of 0, 0.1, 1, and 10 nM TCDD. Twenty-four h after culture in the presence or absence of TCDD, cells were washed with phosphate buffered saline (pH 7.4), lysis buffer was added, and cells were scraped from plate. The cell lysate was then placed

into 50-ml sterile polypropylene tubes (Becton Dickinson Labware, Franklin Lakes, NJ) on dry ice until all pooled lysates from each treatment group were collected. Samples were kept at –70°C until RNA was extracted. TCDD concentrations used in these were not cytotoxic judged by both cell morphology and LIVE/DEAD assay (Molecular Probes, Eugene, OR; data not shown).

RNA extraction. Total RNA was prepared using Qiagen RNeasy midiprep columns (Qiagen, Valencia, CA) according to the manufacturer recommendations, with the following minor modifications: twice as many columns as recommended were used and RNA was eluted using maximal amount of H_2O. Total RNA was concentrated using Microcon 30 spin columns (Millipore Corp., Bedford, MA), quantitated by UV spectroscopy at 260 nM, and stored in RNase-free H_2O at –70°C. Samples were separated by electrophoresis on formaldehyde denaturing agarose gels stained with Sybergreen dye to confirm the integrity of the 18S and 28S ribosomal RNAs.

cDNA microarray labeling. A detailed protocol for the synthesis of labeled cDNA used can be found at the following website: http://dir.niehs.nih.gov/microarray/methods.htm. Briefly, total RNA (35 μg for Cy3 label, 75 μg for Cy5 label) was annealed to the oligo d $(T)_{12–18}$ primer at 70°C for 10 min using: oligo $d(T)_{12–18}$, 500 μg/ml (Amersham-Pharmacia BioTech, Piscataway, NJ), and RNase Inhibitor, 10 U/μl (Life Technologies, Rockville, MD). The RNA was then reverse transcribed at 42°C for 3 h in a 40 μl total volume of first strand buffer (1X); Stanford dNTP mix, (0.75 mM dATP, 0.75 mM dGTP, 0.75 mM dCTP, 0.66 mM dTTP; Amersham-Pharmacia, Piscataway, NJ); FluoroLink Cy3-dUTP, 2.5 nM FluoroLink Cy5-dUTP, 2.5 nM (Amersham-Pharmacia BioTech, Piscataway, NJ); 0.1 M DTT (Life Technologies, Rockville, MD); SuperScript II RNase H- Reverse Transcriptase (18 U/μl; Life Technologies, Rockville, MD). RNA was then degraded using 0.1 M NaOH for 30 min at 70°C and neutralized with 0.1 M HCl. 1X TE buffer, (pH 7.5) and the fluorescent DNA probe pairs were combined and washed 3 times using a Microcon-30 filter (Millipore Corp., Bedford, MA) using 1X TE. During the last wash the following blockers were added to the TE buffer: 20 μg Human COT-1 DNA (Life Technologies, Rockville, MD), 20 μg yeast tRNA (Sigma, St. Louis, MO), 20 μg Poly dA (Amersham-Pharmacia BioTech, Piscataway, NJ). The probes were concentrated to 28 μl and 20X SSC (3 M NaCl, 0.3 M NaCitrate; 3X final conc.), 25X Denhardt's (2X final conc.) and 10% SDS (0.3% final) were added as the hybridization solution. The mixture was heated at 100°C for 2 min, maintained at room temp (RT) for 10 min, then filtered through a Millipore Ultrafree-MC 0.45 μm filter unit (Millipore Corp., Bedford, MA).

cDNA microarray hybridization. Labeled cDNAs were hybridized to the NIEHS Human ToxChip v1.0 (http://dir.niehs.nih.gov/microarray/chips.htm). DNA clones used for the Human ToxChip were obtained from Research Genetics (Research Genetics, Huntsville, AL), amplified by polymerase chain reaction and then spotted onto Poly L lysine-treated glass slides using a microarray printer (Beecher Instruments, Silver Spring, MD). All hybridizations were carried out in triplicate, control RNA was labeled in two hybridizations with Cy5 and in the third hybridization, a reciprocal fluorescent labeling was used where the control RNA was labeled with Cy3. Hybridizations were carried out for 16–24 h at 65°C in a sealed, humidified chamber (National Health Genomic Research Institute). Following hybridization, slides were washed at room temperature (4 washes, 16 min each) using 0.5 × SSC, 0.01% SDS, and air-dried.

Microarray data acquisition and analysis. Fluorescence signals were obtained using a Genepix array scanner (Axon Instruments, Inc., Union City, CA). Cy3 and Cy5 fluorescence were individually scanned using a 532 and 635 nM laser. The photomultiplier tubes were auto-adjusted to obtain equal signal strength of the two channels. Raw scanned images were processed initially using IPLab (Scanalytics, Inc.) with the ArraySuite extensions (originally developed at the National Human Genome Research Institute). Scatterplots of the channel intensities were individually examined and the intensity cut-off levels adjusted to limit dye-specific labeling artifacts.

Cy3 and Cy5 grayscale images were overlaid, to obtain a pseudo-color image, and the analysis software (Arraysuite v1.3; Scanalytics, Arlington VA) defined the spots in the ratio image by accessing the gene in plate order (GIPO) file. The GIPO file is a database that describes which gene is at that location on the microarray. The software shows spots having a ratio < 1 as green, those having a ratio > 1 as red, and with ratios $= 1$ as yellow (equal amounts of red and green). The distribution of $\log_2$-normalized spot ratio values was normalized to 84 "housekeeping" genes that were included on the chip. All spots whose ratio were outside of the 99% confidence interval of the population distribution for all genes on the chip were classified as statistically significant gene changes in this specific hybridization. The methods of the image analysis and quantitation are previously described (Chen *et al.,* 1997). The statistical information generated for each spot was uploaded to the file server for further analysis at other workstations.

Expression profiles from the differentially expressed genes were managed in the MicroArray Project System (MAPS) relational database (Bushel *et al.,* 2001): http://www.niehs.nih.gov/Connections/2000/feb/maps.htm. A binomial distribution (Casella and Berger, 1990) was computed with the gene expression profiles obtained from the compound treatment to determine the probability of randomly detecting genes altered by treatment. That is for $k = 0, 1, 2, \ldots, n$

$$P_{k \text{ out of } n} = \frac{n!}{k!(n-k)!}(p^k)(q^{n-k})$$

where p is the probability that k will occur, q is $1 - p$ and n is the number of chances k can occur. A two-dimensional gene expression data matrix with genes as the objects (rows) and sample treatments as the attributes (columns) was generated using the sets of genes that were consistently differentially expressed and had a low probability chance occurrence ($p < 0.01$) of being randomly detected at the 99–95% confidence level (Chen *et al.,* 1997). Ratio pixel intensity values for the validated gene set were log base-2 transformed.

Real Time RT-PCR

Reverse transcription. Total RNA (100 ng) was reverse transcribed using 1X RT, $MgCl_2$ (2.2 mM), dNTP (2.0 mM), RNAsin (0.2 U/μl), random hexamer primers (0.5 mM), and MMLV reverse transcriptase (0.3 U/μl) in 10 μl reactions using a 3-step cycle: 25°C, 10 min; 48°C, 30 min; and 95°C, 5 min. Reverse transcription reagents were purchased from PE Applied Biosystems (Foster City, CA). The resulting cDNA was used in subsequent real time PCR reactions.

Sybergreen detection. Real time fluorescence detection was carried out using an ABI Prism 7700 Sequence Detection System. Reactions were carried out in microAmp 96 well reaction plates, PCR buffer 1X (containing Sybergreen), $MgCl_2$ (5 mM), dATP, dCTP, dGTP, dUTP (0.2 mM each), Taq Polymerase (0.25 units/μl; PE Applied Biosystems, Foster City, CA), forward and reverse primers (0.2 μM each, Research Genetics, Huntsville, AL) and cDNA (10 μl) in a final PCR reaction volume of 50 μl. Amplification parameters were: denaturation at 94°C 10 min, followed by 40 cycles of 95°C, 15 s; 60°C, 60 s. All primers and probes were designed using PrimerExpress Software (PE Applied Biosystems, Foster City, CA) and can be found in Table 1. Samples were analyzed in triplicate, and actin was used as an endogenous control. Fold induction was calculated using the formula $2^{-\Delta\Delta}C_T$, where ΔC_T = target gene C_T – actin C_T, and $\Delta\Delta C_T$ is based on the mean ΔC_T of respective control (non-TCDD treated). The C_T value is determined as the cycle at where the fluorescence signal emitted is significantly above background levels and is inversely proportional to the initial template copy number. Amplification products using Sybergreen detection were initially checked by electrophoresis on ethidium bromide stained agarose gels. The estimated size of the amplified products matched the calculated size for transcript by visual inspection.

Taqman probe detection. Real time RT-PCR using a 5′ nuclease "Taqman" probe was carried out essentially the same as the Sybergreen detection with the following exceptions: PCR buffer 1X (containing ROX, to normalize emissions), a FAM-labeled *CYP1A1* or *CYP1B1* probe (see Table 1). Absolute quantitation of specific RNA levels were determined using standard curves of 5 10-fold serial dilutions of a total RNA "standard." This RNA was generated from MCF-7 cells that were also treated for 24 h with TCDD (10 nM). RNA

TABLE 1
Primer Pairs Used for Real Time RT-PCR

Primer name	Forward primer (5′-3′)	Reverse primer (5′-3′)	Probe (5′-3′)
Taqman specific, absolute quantitation			
ACTIN	cctggcacccagcacaat	gctgatccacatctgctggaa	atcaagatcattgctcctcctgagcgc
CYP1A1	ccaagaggagctagacact	ccatagcttctggtcatggttga	cccacagcacaacaagagacacaagtttgaa
CYP1B1	gccagccaggacaccct	gatccaattctgcctgcactc	cgctgcagtggctgctcctcct
Sybergreen detection, relative fold change compared to control			
ALDH1A3	gcatgagcccattggtgtct	cgcaggcttcaggaccat	- - - - - - - - - - - -
CDH1	tgagtgtcccccggtatcttc	cagtatcagccgctttcagatttt	- - - - - - - - - - - - -
CDKN1A	cctaatccgcccacaggaa	aagatgtagagcgggcctttg	- - - - - - - - - - - - -
CRYZ	gagctgccatcggcattc	aacactctctccagctttcacaca	- - - - - - - - - - - - -
CTNNB1	cccactggcctctgataaagg	acgcaaaggtgcatgatttg	- - - - - - - - - - - - -
CRCX4	cagtggccgacctcctctt	ggactgccttgcataggaagtt	- - - - - - - - - - - - -
EGR1	gcctgcgacatctgtggaa	cgcaagtggatcttggtatgc	- - - - - - - - - - - - -
GLRX	gattggagctctgcagtaacca	caatgccatccagctcttga	- - - - - - - - - - - - -
HSPA2	cgaggtggccgttagttgac	gggcagacatcctgactgaaa	- - - - - - - - - - - - -
IFNGR1	ccaggttggacaaaaagaatct	tgatatccagtttaggtggtccaat	- - - - - - - - - - - - -
IL8	ccggaaggaaccattctcact	atcaggaaggctgccaagag	- - - - - - - - - - - - -
IRF4	acccgcagatgtccatgag	gtggcatcatgtagttgtgaacct	- - - - - - - - - - - - -
ISG15	gggacctgacggtgaagatg	acgccaatcttctgggtgat	- - - - - - - - - - - - -
KLF4	accaggcactaccgtaaacaca	ggtccgacctggaaaatgct	- - - - - - - - - - - - -
MX1	cagcacctgatggcctatca	acgtctggagcatgaagaactg	- - - - - - - - - - - - -
NCOA2	agctgcctggaatggatatga	caactggcttcagcagtgtca	- - - - - - - - - - - - -
PDK4	ccccgagaggtggagcat	gcattttctgaaccaaagtccagta	- - - - - - - - - - - - -
PTPN1	cacctgctggaaaccacactt	aaggtgaaccgggacagaga	- - - - - - - - - - - - -
RAP1GA1	aaggtgaagctcgagtgcaac	aaatgctccttgccgagaaa	- - - - - - - - - - - - -
RARB	ttcagtgcaagggagatcatgt	gacggactcgcagtgtagaaatc	- - - - - - - - - - - - -
ZNF42	agcccttgctcacgtttcc	ggtttggatgtgccttgctt	- - - - - - - - - - - - -

copy numbers for *CYP1A1* and *CYP1B1* in the MCF-7 RNA standard were determined using competitive RT-PCR assays for *CYP1A1* and *CYP1B1*, as previously described (Spencer *et al.*, 1999). The copy number for the standard curves were; *CYP1B1*, $5.6 \times 10^1 - 5.6 \times 10^6$ copies/μg; *CYP1A1*, $9.6 \times 10^3 - 9.6 \times 10^7$. All standards were analyzed in duplicate, and samples in triplicate.

RESULTS AND DISCUSSION

Quantitation of TCDD-Induced Genes

TCDD induction of cytochrome P450s is a hallmark biochemical effect of TCDD. To compare concentration-dependent induction by TCDD, we quantitated CYP1A1 and CYP1B1 expression using probe-specific Taqman Real Time RT-PCR. EC_{50}s of 0.58 and 0.12 nM for the induction of *CYP1A1* and *CYP1B1* (respectively*)* were determined for the HPL1A cell line In the A549 cell line, *CYP1A1* and *CYP1B1* were induced with EC_{50}s of 1.0 and 0.1 nM (respectively). Studies have shown that the A549 cell line expresses *CYP1A1*, *CYP1B1,* and a number of other cytochrome P450s as well (Foster *et al.,* 1998; Hukkanen *et al.,* 2000). Data in this study indicate that the HPL1A cell line was highly TCDD responsive and is a suitable model for examining the effects of TCDD on nonmalignant human type II cells. Moreover, the lower EC_{50} value for *CYP1A1* indicates that the HPL1A cells may be more sensitive to gene induction by TCDD than the A549 cells.

CYP1A1 was significantly induced by TCDD using specific and absolute quantitation by 5′ nuclease (Taqman) probe-based real time RT-PCR. The effect was dependent on the concentration in both cell lines. In the HPL1A cell line, induction was 2-fold at 0.1 nM, 21-fold at 1 nM, and 29-fold at 10 nM (Fig. 1). In the A549 cell line, *CYP1A1* was induced from 1.1-fold at 0.1 nM, 5-fold at 1 nM, and 13-fold at 10 nM. The fold induction from TCDD exposure for CYP1A1 in the A549 cell line was comparable to that found in the HepG2 cell line that is 12–16-fold (Frueh *et al.,* 2001; Puga *et al.,* 2000b). *CYP1B1* was concentration-dependently induced to similar degrees in both cell lines. In the HPL1A cell line, *CYP1B1* was induced 3.1-fold at 0.1 nM, 4.9-fold at 1 nM, and 5.5-fold at 10 nM. In the A549 cell line, *CYP1B1* was induced 2-fold at 0.1 nM, 3.7-fold at 1 nM, and 3.6-fold at 10 nM. In contrast, expression of actin remained essentially unchanged across the concentration-range (data not shown). These data confirm a preliminary report using intercalating dye-based (Sybergreen) real time RT-PCR detection for the responsiveness of this cell line (Martinez *et al.,* 2000).

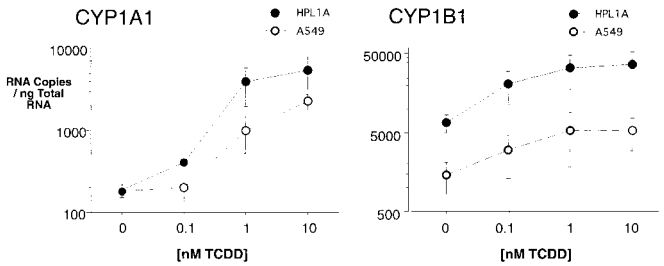


FIG. 1. Concentration-dependent induction of CYP1A1 and CYP1B1 in HPL1A and A549 cell lines treated with TCDD for 24 h. Detection was carried out using real time RT PCR with a Taqman probe and standard curve generated from TCDD-treated MCF-7 cell total RNA serial diluted (5X) and quantitated by competitive RT-PCR. Experiments were repeated three times and samples were analyzed in duplicate. RNA is expressed as copy number per ng total RNA used for analysis.

Toxicogenomic Analysis

Having established that the nonmalignant HPL1A was TCDD-responsive, we next used microarray analysis to compare the concentration-dependent genomic response to TCDD. An aim of this present study was to identify concentration-dependence as well as to compare the breadth of biological complexity associated with TCDD exposure in a nonmalignant versus a malignant lung epithelial cell line. To do this, we used increasing concentrations for a 24-h exposure period. Serum is known to modulate TCDD-mediated effects (Guigal *et al.*, 2001; Morris *et al.*, 1994), so cells were acclimated for 48 h before experiments, and conditioned media was used for all treatments to control for differences in cellular environment. We examined TCDD alterations with 2091 gene clones on the ToxChip for each cell line by paired-microarray analysis comparing treated group to the control group. Genes were identified as altered by treatment if they were statistically different (99% CL) from control in at least two of the three control-treated group hybridizations. Genes that were identified as a significant gene change in only a single microarray hybridization were not classified as altered by TCDD treatment at that concentration.

Based on the above criteria, 121 cDNA clones representing 117 independent genes were identified from the microarray analysis as being significantly altered by TCDD at any concentration and in either cell line. Of these, there were 15 genes altered in both cell lines, in at least one of the three treated (0.1, 1, or 10 nM TCDD) groups. These genes include *AC083883*, *ALDH3*, *ALDH1A3*, *CDH1*, *CTNNB1*, *CYP1A1*, *CYP1B1*, *DKFZp434A1014*, *DUSP1*, *EGR1*, *F2R*, *HSPA4*, *KIAA1389*, *KIAA1007*, and *STK4*. In both cell lines there were 68 genes significantly altered from control at any concentration, and 53 were cell line specific. A Venn diagram of the cell/concentration distribution of the genes that were identified as significantly altered by TCDD is shown in Figure 2. Genes are identified by UniGene symbol/accession number (see http://www.ncbi.nlm.nih.gov/UniGene/Hs.Home.html; where the complete name can be found upon typing in the accession number or the abbreviation). The Venn diagram represents genes that are common (or overlapping) with one concentration, two concentrations, or all three concentrations for each cell line. We found cell line to cell line differences as well as common gene responses to TCDD. This analysis focused only on those genes identified at 99% CL in replicate experiments. The majority (approximately 80%) of gene changes by TCDD in this analysis were cell-line specific. This indicates that caution should be used when extrapolating the specificity of mechanism of action of toxicants between tissue/cell lines and across species.

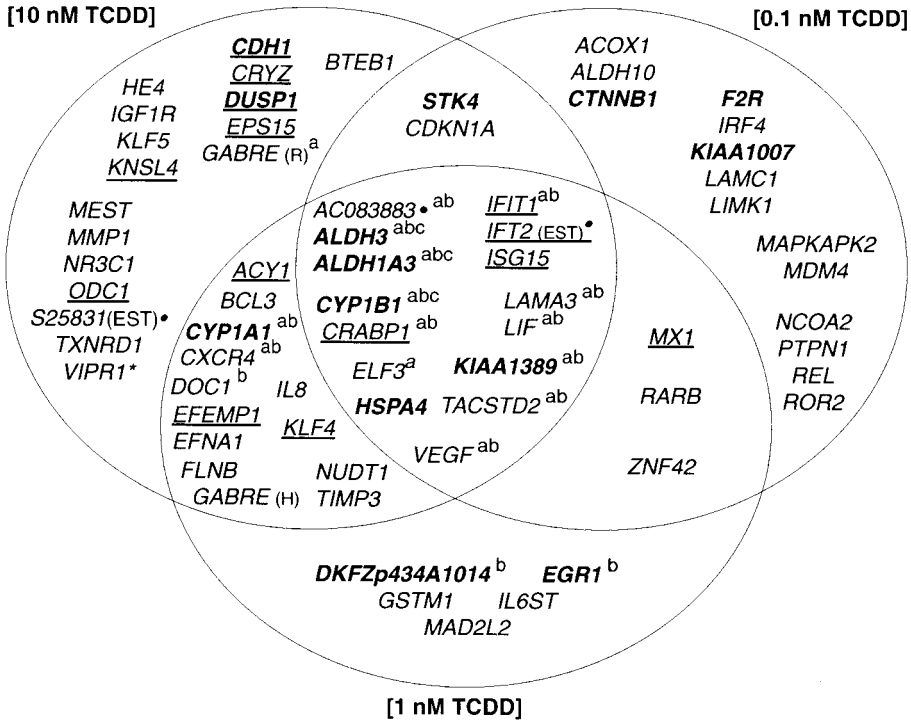
Confirmation of Gene Changes by Real Time RT-PCR

To evaluate the validity of the gene changes identified, we compared the relative fold induction of genes detected at 99% CL by microarray analysis, to those by real time RT-PCR. Table 2 shows this comparison looking at 25 genes that were chosen based on gene function or an unusual concentration response. Dual fluorescence microarray detection is consistently sensitive at the lower end of relative fold changes and the trend of alteration is maintained (Table 2). For example, genes induced including *CDKN1A*, *CTNNB1*, *EGR1*, *IL8*, *IRF4*, *RARB*, and *ZNF42* in the HPL1A cell line had relative fold changes of less than 2-fold by microarray analysis. These inductions confirmed by real time RT-PCR, were indeed less than 2-fold. In the A549 cell line, there were several examples (*CRYZ*, *IL8*, *IRF4*, *RARB*, and *PTPN1*) of genes not identified by the microarray analysis, but were either induced or repressed (*CXCR4*, *NCOA2*, and *ZNF42*) as detected using real time RT-PCR analysis. In the HPL1A cell line, *GLRX*, *HSPA2* are examples of genes that were not identified by microarray analysis, yet were detected by real time RT-PCR. In addition, some genes that were identified only in one cell line, for example *IFNGR1* in the HPL1A or *RARB* in the A549 had fold changes greater than that detected by microarray that fit a concentration-dependent induction. By microarray analysis, the fold change could be muted, perhaps by quenching of fluorescence. The induction of *CYP1A1* is a good example of this effect since it has a very high fold of induction that is not matched by microarray analysis.

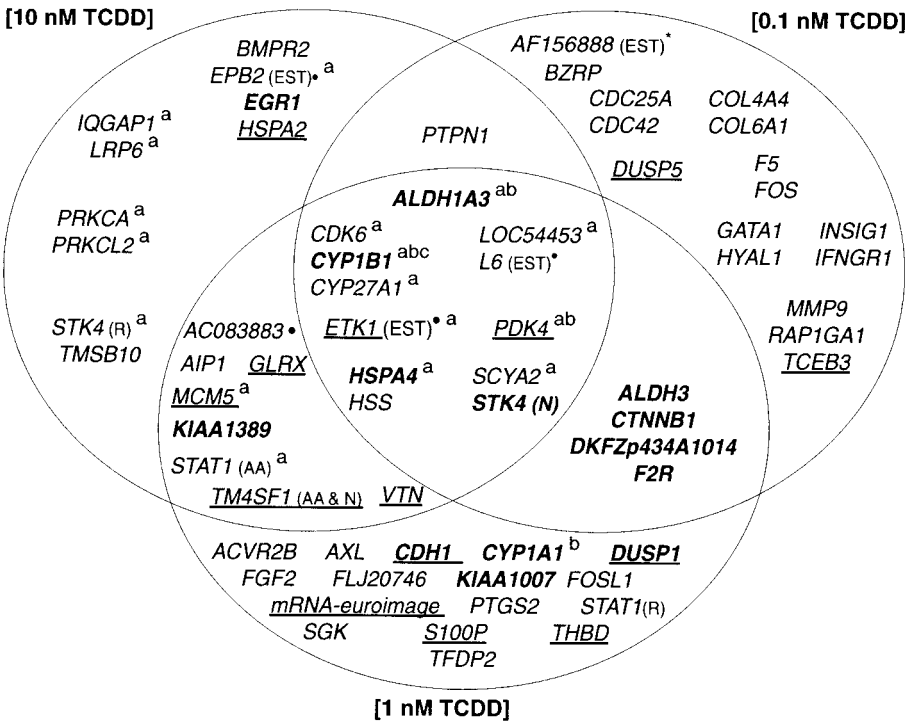
AhR Responsive Genes

The ToxChip is comprised of genes that represent biological pathways activated by a variety of signals or toxicants. This includes genes from various categories including: apoptosis, oxidative stress/redox homeostasis, peroxisome proliferator response, AhR battery, estrogen responsive, housekeeping, oncogenes and tumor suppressor genes, cell cycle control, transcription factors, receptors kinases, phosphatases, heat shock proteins, and cytochrome P450s (Nuwaysir *et al.*, 1999). Consequently its utility is not in gene discovery per se, but in identifying signaling pathways activated following exposure to

HPL1A Cell Line



A549 Cell Line



a given insult. This report confirms and validates the suitability of this chip for characterizing the AhR mediated response and further identifies other pathways altered by TCDD exposure. Of the 12 different clones representing the AhR battery, 8 were detected (99% CL) by microarray analysis. These genes include *CYP1A1*, *CYP1B1*, *CRYZ*, *GSTM1*, *GLRX*, *ALDH3*, *ALDH1A3*, and *ALDH10* (Fig. 2). *CYP1A1* had the highest fold induction in both the A549 and the HPL1A cell line as determined by real time RT-PCR and microarray analysis. AhR battery genes that were not altered include genes like the *AhR* and *ARNT* that are reported as nonresponsive to TCDD in these cell lines (Martinez *et al.,* 2000; Pitt *et al.,* 2001).

The AhR battery of genes altered by TCDD in this study were primarily metabolic enzymes. Due to the overlap of genes involved in many different categories we attempted to categorize based on a biological functional. For example, a transcription factor or receptor that is involved in differentiation was so classified. The 5 major categories discussed are (1) signal transduction, (2) extracellular matrix and basement membrane components, growth factors, and chemokines, (3) genes involved with differentiation, (4) genes involved with cell growth or cell cycle, and (5) others that include stress, glucose regulation and NFκB signaling. Other TCDD toxicogenomic studies have primarily examined TCDD effects in HepG2 cells (Frueh *et al.,* 2001; Puga *et al.,* 2000b) or mouse liver cells (Kurachi *et al.,* 2002; Thomas *et al.,* 2001). Thus tissue specificity may be responsible for the difficulty in making direct gene-to-gene comparisons. Other factors that may contribute to this difficulty are the inherent problems with nomenclature and differences in platforms used. Keeping this in mind, it is not surprising that there were only a few genes found in common with other array studies. Exact matches included *CDN1A*, *CYP1A1*, *ODC1*, *THBD*, *and VEGF*. Genes that matched others within the same family included *ACY1*, *GSTM1*, *HSP70*, *PRKCA*, *STK4*, *S100P*, and *TCEB3* (Frueh *et al.,* 2001; Kurachi *et al.,* 2002; Puga *et al.,* 2000b; Thomas *et al.,* 2001). Hence, our study confirms approximately a dozen genes altered by TCDD in different systems. It extends these studies by identifying genes that are not necessarily involved in the toxic stress mediated by TCDD by using a dose continuum.

Concentration Responses

Examination of the concentration response curves for genes detected by microarray analysis using relative fold changes induced by TCDD is based on the 99% CL. Genes were both induced and repressed by TCDD treatment and a number of different concentration-response patterns were observed (Figs. 3–5). In genes that were repressed, the central tendencies of expression for concentration response were either:

- Equally repressed at all concentrations (*MX1*, HPL1A; Fig. 5),
- Repressed, decreasing with increasing concentrations (*ISG15*, HPL1A; Fig. 5),
- Not repressed at 0.1 nM, then repressed, decreasing with increasing concentrations (*HSPA2*, A549), or
- Induced at 0.1 nM but then repressed at higher concentrations (*PDK4*, A549; Table 2 and data not shown).

For genes whose expression was induced, the trends were similar but in the opposite direction:

- Equally induced at all concentrations (*ZNF42*; HPL1A),
- Not induced at low concentration, then induced in an increasing fashion with increasing concentrations (*KLF4*; A549),

FIG. 2. Venn diagram representation of the concentration dependent changes in HPL1A cell line and A549 cell line. Each circle represents a respective TCDD treatment (0.1, 1, or 10 nM) with genes identified by microarray analysis as validated gene changes compared to control at 99% confidence level and in two out of three hybridizations. Genes are identified by their UniGene symbol or accession number (http://www.ncbi.nlm.nih.gov/UniGene/Hs.Home.html). The complete name can be found upon typing in the accession number (below) or the UniGene symbol. ESTs are identified by bold dots (highly similar to specified gene) or asterisks (weakly similar). If a gene had two clone IDs, the gene is followed by the abbreviation of first letter(s) of the respective accession code. [a]Genes were induced by greater than 2-fold induction at 10 nM; [b]greater than 2-fold induction at 1 nM TCDD treatment; [c]greater than 2-fold induction at the 0.1 nM TCDD treatment. Genes in bold lettering were found in both cell lines. Genes that are underlined were repressed. *ACOX1*-AA040205; *ACVR2B*-R68531; *ACY1*-N35315; *AF15688*-W72271; *AIP1*-H47890; *ALDH10*-H63779; *ALDH3*-AA069024; *ALDH1A3*-AA054748; *AXL*-T91043; *BCL3*-H13606; *BMPR2*-H96519; *BTEB*-W38326; *BZRP*-AA056606; *CDC25A*-H59260; *CDC42*-H23728; *CDH1*-W73312; *CDK6*-H92463; *CDKN1A*-N23941; *COL4A4*-H68555; *COL6A1*-W23873; *CRABP1*-W95693; *CRYZ*-AA035180; *CTNNB1*-N28952; *CXCR4*-R78620; *CYP1A1*-AA418907; *CYP1B1*-AA040872; *CYP27A1*-H05935; *DKFZp434A1014*-AA025987; *DOC1*-T84055; *DUSP1*-W90037; *DUSP5*-W65461; *EFEMP1*-AA040443; *EFNA1*-AA081126; *EGR1*-H27557; *ELF3*-H27939; ESTs, Highly similar to *EPB2* precursor-R39317; *EPS15*-N78949; ESTs, highly similar to *ETK1* precursor-AA070819; *F2R*-N70450; *F5*-H79486; *FGF2*-W44678; *FLJ220746*-H93328; *FLNB*-AA059125; *FOS*-R20750; *FOSL1*-T89996; *GABRE*-H63532; *GABRE*-R07942; *GATA1*-R06446; *GLRX*-R69362; *GSTM1*-H41540; *HE4*-AA025750; *HSPA2*-R12701; *HSPA4*-AA043348; *HSS*-N73677; *HYAL1*-W84634; *IFIT1*-AA052933; *IFNGR1*-T40924; ESTs, Highly similar to *IFT2*-AA143609; *IGF1R*-H13300; *IL6ST*-H85457; *IL8*-W45324; *INSIG1*-AA046719; *IQGAP1*-R37866; *IRF4*-N67844; *ISG15*-AA084813; *KIAA1007*-N29343; *KIAA1389*-AA035484; *KLF4*-H45668; *KLF5*-R25623; *KNSL4*-W95654; ESTs, highly similar to *L6*; *LAMA3*-AA001432; *LAMC1*-AA032191; *LIF*-R50354; *LIMK1*-R41791; *LOC54453*-R83223; *LRP6*-T68228; *MAD2L2*-AA076651; *MAPKAPK2*-W69515; *MCM5*-W80586; *MDM4*-AA456593; *MEST*-N58519; *MMP1*-AA081208; *MMP9*-AA079861; Homo sapiens mRNA full length insert cDNA clone EUROIMAGE *248114*-W86869; *MX1*-AA053117; *NCOA2*-R77770; *NR3C1*-H99414; *NUDT1*-W72569; *ODC1*-AA055467; *PDK4*-AA169469; *PRKCA*-AA029890; *PRKCL2*-AA035285; *PTGS2*-R80217; *PTPN1*-R07707; *RAP1GA1*-N44071; *RARB*-W93713; *REL*-N56815; *ROR2*-T92112; *S100P*-AA053504; *S25831*-AA047276; *SCYA2*-T77817; *SGK*-N77456; *STAT1*-AA076085; *STAT1*-R37747; *STK4*-N23875; *STK4*-R93509; *TACSTD2*-AA115151; *TCEB3*-H83548; *TFDP2*-W46792; *THBD*-AA057068; *TIMP3*-AA043969; *TM4SF1*-AA487893; *TM4SF1*-N47476; *TMSB10*-AA057636; *TXNRD1*-H11561; *VEGF*-W19225; *VIPR1*-W01956; *VTN*-N76949; *ZNF42*-R83364.

TABLE 2

Comparison between Real Time RT-PCR and Microarray Analysis Examining the Relative Fold Increase of Gene Expression in Cells Treated with TCDD

	A549		HPL1A	
Gene ID	RT	M	RT	M
Repressed				
CDH1	0.38	0.78	0.53	0.72*
CRYZ	1.30	1.04	0.53	0.71*
GLRX	0.55	0.67*	1.31	0.87
HSPA2	0.38	0.61*	1.65	1.09
ISG15	1.15	1.01	0.21	0.50*
KLF4	1.00	1.82	0.42	0.70*
MX1	1.79	1.35	0.19	0.63
PDK4	0.88	0.47*	1.15	4.30
RAP1GA1	1.60	1.41	0.84	0.79
Induced				
ALDH1A3	3.56	2.06*	3.42	3.99*
CDKN1A	0.95	1.02	1.34	1.54*
CXCR4	0.70	1.44	9.50	2.39*
CTNNB1	1.36	1.28	1.30	1.12
CYP1A1	12.54	4.48	29.04	12.27*
CYP1B1	3.61	7.15*	5.49	6.36*
EGR1	0.92	1.15*	1.62	1.54
IFNGR1	1.50	1.51	2.40	1.10
IL8	1.64	1.90	1.86	1.76*
IRF4	1.50	1.05	1.14	1.22
NCOA2	0.69	1.01	1.00	1.12
PTPN1	1.93	2.90*	1.47	2.12
RARB	1.31	1.09	1.95	1.41
ZNF42	0.70	1.08	1.15	1.41

Note. RT, real time RT-PCR; M, microarray analysis. RT and M were compared to determine the relative fold increase of gene expression in cells treated for 24 h with TCDD (10 nM), compared to respective control. Genes were identified as induced or repressed from respective control in at least 1 cell line and found in at least 1 TCDD-treatment group (10 nM, 1 nM, or 0.1 nM) using 99% CL. Real time RT-PCR was performed using Sybergreen detection for all genes except CYP1A1 and CYP1B1. Taqman probes were used for CYP1A1 and CYP1B1. Microarray analysis relative fold induction was based on average values of 3 different hybridizations.

*The TCDD treatment at 10 nM was identified as significantly different than control at 99% CL by microarray analysis.

- Induction increased with increasing concentrations (CYP1A1, CYP1B1, *ALDH1A3*; either cell line, Fig. 3), or
- Repressed at low concentration and induced at higher concentrations (*EGR1*; A549).

This study demonstrates that a number of genes altered by TCDD require concentrations that are 10–100-fold lower than those widely used to analyze gene expression in other systems. These data suggest that a physiological response versus a toxic response may be initiated. We show that when one examines global TCDD concentration-response patterns the pattern of trends will vary substantially. Genes that were equally induced or repressed at all concentrations of TCDD may have unusually steep dose-response curves or alternatively, the threshold for effective concentration is lower than that measured in this study.

Signal Transduction Molecules

Many genes altered by TCDD are involved in signaling and lead to changes in cell morphology, motility, cell-cycle progression and the induction of malignant transformation. An important cell surface glycoprotein adhesion molecule is E-cadherin (*CDH1*). *CDH1* was repressed in both cell lines (Table 2), and is implicated in carcinogenesis since it is often lost in human epithelial cancers and appears to be a rate-limiting step in the progression from adenoma to carcinoma. Catenin β 1 (*CTNNB1*), an intracellular partner of *CDH1*, on the other hand was significantly induced in both cell lines (Table 2). *LRP6*, *IQGAP*, and *CDC42* (a Rho family kinase) are other genes involved in Wnt transduction (Fukata *et al.*, 1999, 2001; Tamai *et al.*, 2000) that were altered (99% CL) only in the A549 cells suggesting a cell line specific pathway.

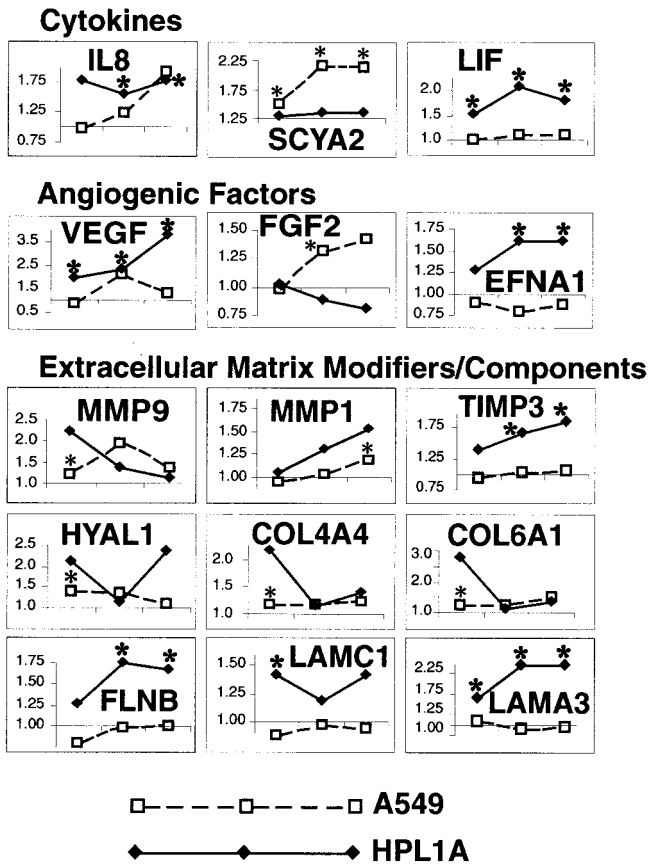


FIG. 3. Hybridization values from genes that are considered downstream products of TCDD action in lung airway epithelial cells, detected by microarray analysis. TCDD concentrations used were 0.1, 1, or 10 nM for 24 h. These data include all average hybridization ratio values for an identified gene, irrespective of whether that treatment value was statistically significantly different based on 99% CL outlier list within a given hybridization. *Treatments identified as significantly altered by microarray analysis.

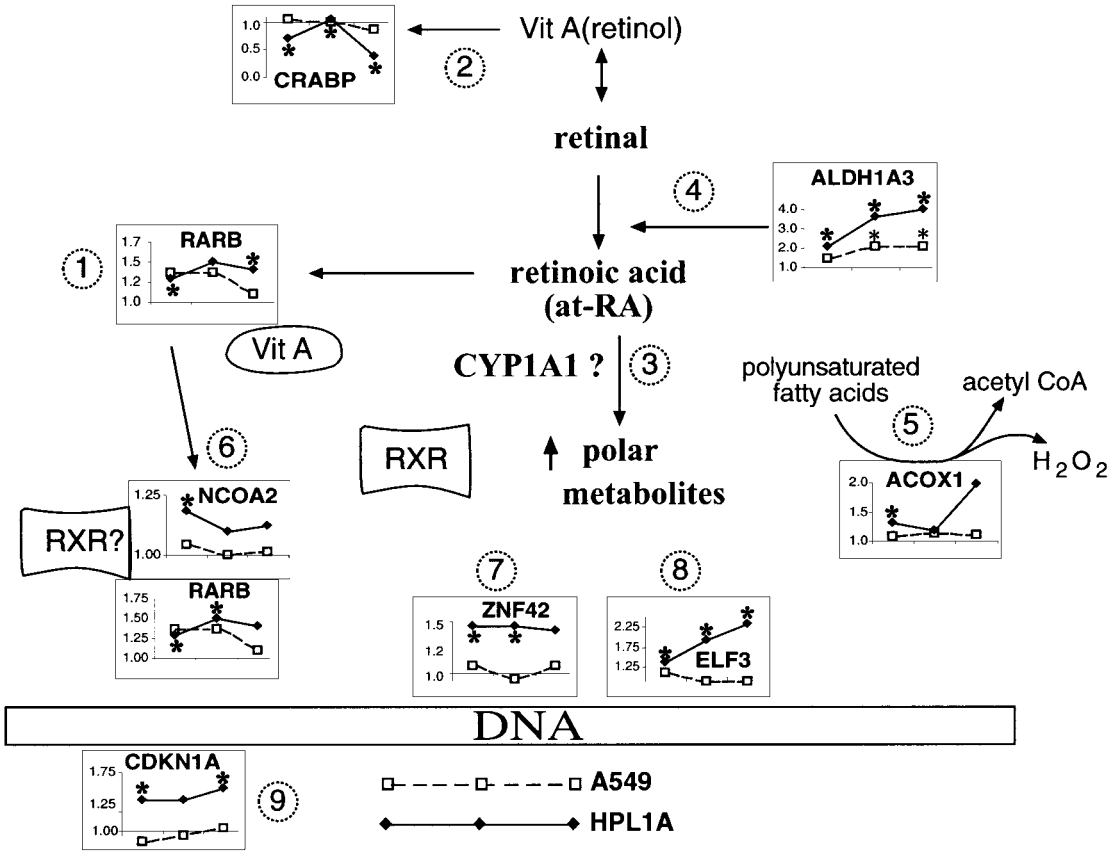


FIG. 4. Schematic representation of retinoid signaling pathway for genes involved in differentiation that were identified as altered by TCDD in lung airway epithelial cells. Detection of genes was based on significant changes at 99% confidence level as determined by microarray analysis. TCDD concentrations used were 0.1, 1, or 10 nM for 24 h. These data include all average hybridization ratio values for an identified gene. Asterisks indicate treatment group was identified as significantly different from control at 99% CL. **(1)** RARB is induced by Vitamin A and has been shown to play a role in cell growth and inhibition (Sun *et al.,* 2000). **(2)** CRABP1 is a specific carrier protein for vitamin A, of which lower levels negatively influence metabolism of retinoic acid and increase the sensitivity to Vit A signaling (Boylan and Gudas, 1992; Ong, 1987). The CRABP1 promoter has been shown to contain an enhancer region through which RA effects inhibition of CRABP1 transcription (Means *et al.,* 2000). **(3)** An inducer of CYP1A1 (β-naphthoflavone) induced the metabolism of all-trans-RA in human intestinal epithelial cells (Lampen *et al.,* 2000). **(4)** ALDH1A3 synthesizes retinoic acid and is a potent inducer of the RAR-dependent signaling pathway (Grun *et al.,* 2000). **(5)** The human peroxisomal acyl-CoA oxidase is the first enzyme of the fatty acid β-oxidation pathway (Varanasi *et al.,* 1994). Fatty acids are known to activate the RXR thereby modulating gene expression (Issemann *et al.,* 1993). **(6)** NCOA2 is also known as GRIP1 and has been shown to interact with the 5 steroid hormone receptor types (Hong *et al.,* 1997; Schmidt *et al.,* 1998). **(7)** ZNF42 (also known as MZF-1) is a putative transcriptional regulator that is induced by retinoic acid in human myeloid cells (Hromas *et al.,* 1991) **(8)** ELF3 is an epithelial specific transcriptional regulator that may play a role in lung carcinogenesis (Tymms *et al.,* 1997). **(9)** CDKN1A is induced by retinoic acid through RARB in human neuroblastoma tumors (Cheung *et al.,* 1998; Liu *et al.,* 1996).

Another similarity between the two different cell lines was the induction of *KIAA1389* (also known as *SPA1*). KIAA1389 belongs to a family of highly diverse, multifunctional signaling proteins that share a conserved 120 amino acid domain (RGS domain) known as regulators of G-protein signaling. KIAA1389 is known to inhibit activation of RAP1-GTP, which is required to maintain cell adhesion (Tsukamoto *et al.,* 1999). *KIAA1389* was altered in the HPL1A cell line at all concentrations of TCDD tested, and in the A549 cell line at 1 and 10 nM in a concentration-dependent manner. Signaling through the Ras cascade is implied by changes in gene levels of downstream mediators through pathways such as Rac, Rap, and Rho. Other evidence for p38 kinase is indicated by repression of *DUSP1* in both cell lines and induction for *MAPKAP2* in the HPL1A cells. It is unclear what membrane receptor may be activating Ras, but potential candidates indicated by microarray analysis include *ROR2*, *IGF1R*, and *F2R* (thrombin receptor). *F2R*, a proteolytically activated cell surface receptor that is involved in cytoskeletal signaling, was found altered in both cell lines. By microarray analysis *F2R* was detected as different from control (99% CL) at the low concentration for HPL1A and the lower two concentrations of A549; however, a concentration-dependent continuum was found for both cell lines. Thus, these data also show that there are several regulators of G protein signaling (*1RAP1GA1*, *IQGAP*, and a SPA-1 homologue *KIAA1389*, and *LOC54453*), as well as multiple signaling networks mediated by TCDD known to be involved with cell adhesion.

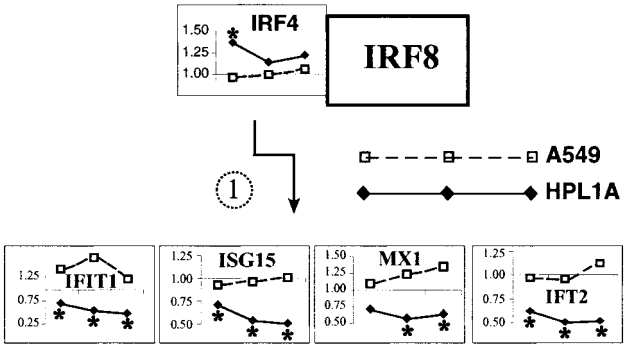


FIG. 5. Repression of interferon stimulated genes by TCDD. TCDD concentrations used were 0.1, 1, or 10 nM. These data include all average hybridization ratio values for an identified gene, irrespective of whether that treatment value was statistically significantly different based on 99% CL within a given hybridization. **(1)** IRF4 forms a complex with IRF8 that leads to repression of ISG15 (Rosenbauer *et al.,* 1999). *Treatment groups that were identified as significantly different from control.

Extracellular Matrix, Basement Membrane Components, Growth Factors, and Chemokines

Effector genes involved in metastasis, cellular transformation, angiogenesis, cell growth, and invasion through the ECM and capillary basement membrane. Theses can be classified as (1) cytokines/chemokines, (2) angiogenic factors, or (3) extracellular matrix components/enzymes. Chemokines themselves regulate angiogenesis, promote cellular transformation, tumor growth, migration, homing, and metastasis (Strieter, 2001). In Figure 3, the concentration-dependent trends for downstream effectors that were detected by microarray analysis are shown. Real time RT-PCR found similar fold induction for *IL8* (also known as *CXCL*8; Table 2). IL8 was induced in A549 cells and is known to be stimulated with tumor necrosis factor and IL1B (Standiford *et al.,* 1990). Chemokines also play a role in the inflammation of airways, and TCDD-induced chemokines expressed detected in this study include the following: *IL8*, *SCYA2*, *LIF* (Fig. 3), and *CXCR4* (Table 2).

By facilitating angiogenesis during lung injury or repair, the highly vascular nature of the lung is critical to its function. Proangiogenic factors detected include *VEGF*, *FGF*, and *ephrin A1* (*EFNA1*). Our data show that these angiogenic factors are differentially induced by TCDD, where induction of *VEGF* and *EFNA1* was found in the "nonmalignant" HPL1A, while *FGF2* was induced in the malignant A549 cell line. Increased vasculature and *VEGF* have been detected in asthma patients suggesting a role for *VEGF* in pulmonary disease, contributing to angiogenesis or chronic inflammation (Hoshino *et al.,* 2001).

Induction of extracellular matrix components such as the hyaluronidase, matrix metalloproteinase 1 and 9 (*MMP1* and *MMP9*), laminins (*LAMC1* and *LAMA3*), and filamin (*FLNB*; Fig. 3) may contribute to tumor invasion and metastasis. The complex interplay of these compounds is observed by induction of *MMP9*, *COL4A4*, and *TIMP3*. MMP9 is gelatinase known to digest basement membrane collagen IV (COL4A4), and TIMP3 is an inhibitor of both MMP1 and MMP9 (Stamenkovic, 2000).

Genes Involved with Cell Growth or Cell Cycle Control

Growth regulation is critical in carcinogenicity, in that uncontrolled growth of aberrant cells leads to tumors. TCDD is a known tumor promoter that modifies the normal cellular proliferation-differentiation process, a process that is linked to altered regulation of gene expression mediated by Ras and Rho GTPases. Many TCDD effects are attributed to its interference with growth regulation, yet the precise mechanism remains unclear. Transcripts detected in this study include previously reported as well as novel TCDD-modified genes. For example, the CDKN1A protein was recently detected by serial analysis of gene expression (SAGE) from the liver of TCDD-treated mice (Kurachi *et al.,* 2002), and TCDD regulates *MAD2L2* by an AhR independent manner (Oikawa *et al.,* 2001). *EGR1*, an immediate early gene that is required to activate transcription of genes involved in negative growth regulation was discovered to be induced by TCDD in HepG2 cells (Puga *et al.,* 2000b). Finally, *TFDP2*, an E2F dimerization partner that plays a role in oncogenesis was increased in a concentration-dependent manner. The E2F complex is an important indirect mediator of TCDD-induced alterations in cell cycle since the AhR is known to interact with the retinoblastoma protein and leads to repression of E2F-dependent transcription and G1 cell cycle progression (Puga *et al.,* 2000a).

There were a number of genes found altered by TCDD that are involved in cellular proliferation. Of these, only *EGR1* was detected in both cell lines. In the HPL1A cell line, cell cycle genes induced by TCDD include *CDKNIA* (also known as *p21*, *Cip1*), *MDM4* (also known as *MDMX*), and *MAD2L2* (Fig. 2). Cell cycle genes altered by TCDD in the A549 cells included *CKD6*, *CDC25A*, *MCM5*, and *TFDP2*. Based on this array of genes, we conclude that TCDD is most likely affecting the G1 phase of the cell cycle, albeit through different effectors. CDKN1A, MDM4, CDK6, and CDC25 are all involved in the G1 phase of the cell cycle and TCDD is known to affect other cells at the G0/G1 progression (Ma and Whitlock, 1996; Weber *et al.,* 1997). Other genes detected by microarray analysis that are not involved directly with cell cycle include negative growth regulators *KNSL4* and *KLF4* in HPL1A cells, *ACVR2B* and *THBD* for the A549 cells.

Genes Involved with Differentiation

A principal component of cancerous growth often depends on deranged patterns and control of differentiation. Thus changes in gene expression that alter the balance of growth and differentiation transcription and growth factors play important roles in pathophysiological changes like hyperplasia, fibrosis, and neoplasia. Cytodifferentiation is the cellular and molecular process that transforms precursor cells into phenotypically

mature cells. The failure of a cell to normally differentiate can be dependent on the neoplastic phenotype (Harris *et al.,* 1985; Nettesheim *et al.,* 1985). In this study, we report distinct differences in genes involved with differentiation altered by TCDD between the A549 and HPL1A cell lines. A hypothetical pathway of how TCDD may be altering differentiation is depicted in Figure 4. Retinoic acid receptor β (*RARB*) is activated by retinoic acid (RA) and causes suppression of tumor cell growth and/or inhibition of keratinization/differentiation (Sun *et al.,* 2000; Zou *et al.,* 1999). RARB is another important potential mediator of TCDD action and it was induced in the HPL1A in a concentration-dependent manner (10−40%; Table 2). These data suggest that the HPL1A cell line is retinoic acid responsive, since several RA responsive genes were detected by microarray analysis and include the following: *RARB*, *NCOA2*, *CRABP1*, *ZNF42*, and *ELF3* (Fig. 4). In contrast, alterations in RA-responsive genes were not detected in the A549 cells, which is consistent with prior observations that report the A549s as a retinoic acid resistant cell line (van der Leede *et al.,* 1993).

A role for TCDD in vitamin A homeostasis is evident by the depleted hepatic stores of vitamin A in chronically TCDD-treated rats (Kelley *et al.,* 2000). Altered mobilization of vitamin A is evidenced by an increase in serum and kidney retinoic acid levels from TCDD treated rats (Nilsson *et al.,* 2000). Cross talk between signal transduction pathways for AhR and the RARs is suggested (Delescluse *et al.,* 2000; Gonzalez and Fernandez-Salguero, 1998). We propose that induction of *CYP1A1,* as well as *ALDH1A3* (also known as ALDH6) contribute to TCDD alterations of vitamin A levels. ALDH1A3 is a retinaldehyde dehydrogenase that can cause the irreversible synthesis of retinal to retinoic acid (Grun *et al.,* 2000; Vasiliou *et al.,* 2000). CYP1A1 has been linked to retinoic acid metabolism (Lampen *et al.,* 2000). Thus, an increase in the basic metabolic turnover of vitamin A without concurrent replacement may lead to loss of storage and altered homeostasis.

Immune Mediated Responses

It is known that alveolar cells play a role in host innate resistance to pulmonary infections by producing proinflammatory cytokines. Suppresion of an immune response will not only leave an organ or tissue susceptible to infections but also to neoplasms as shown by the association of immune defiencies with Kaposi sarcoma, non-Hodgkin's lymphoma and anogenital carcinoma (Ioachim, 1997). In animal models, the immunotoxicity of TCDD is unequivocal (Holsapple *et al.,* 1991), and an extremely sensitive toxic parameter. In both humans and rodents, there are a number of immunotoxic endpoints altered by TCDD, yet the mechanism of immunosuppression is still not clear (Kerkvliet, 2002; Vos and VanLoveren, 1995). Both *in vitro* and *in vivo* data show that immune effects of TCDD may be direct (Sulentic *et al.,* 1998, 2000) or indirect (Shepherd *et al.,* 2000). However, a clear role for the AhR is evidenced by the fact that AhR-deficient mice are resistant to TCDD immunotoxicity (Fernandez-Salguero *et al.,* 1996; Shepherd *et al.,* 2001).

Microarray data from both A549 and HPL1A cell lines suggest that TCDD may signal through the interferon pathway since the *interferon gamma receptor 1* (*IFNGR1*) was upregulated in both cell lines (Fig. 2, Table 1). While *IFNGR1* was induced only in the A549- 0.1 nM TCDD group, we found a concentration-dependent induction based on hybridization values for *IFNGR1* in the HPL1A cell line that was confirmed by real time RT-PCR (Table 2). The mechanism for IFN signaling is through the IFNGR1 association with Jak1, upon activation by INFγ. Transphosphorylation of janus kinases (Jaks) occurs, which phosphorylates IFNGR1, then initiates a cascade of events that leads to altered gene expression (Stark *et al.,* 1998). The data suggest that modulation of the Jak/STAT pathway was mediated through different pathways by the two cell lines since leukemia inhibitory factor (*LIF*), and interleukin 6 signal transducer (*IL6ST,* also known as gp130) were altered in the HPL1A cell line and not in the A549 cell line (Fig. 3). LIF-activated IL6ST/LIFR signaling is mediated through the Jak/Stat pathway, where LIF binds to and activates a heterodimer composed of IL6ST and leukemia inhibitory factor receptor (LIFR; Gearing *et al.,* 1992). A number of genes that are downstream products of Stat activation (Heinrich *et al.,* 1998) were found altered in either the A549 cell line or the HPL1A cell line and include *fos,* interstitial collagenase (*MMP1*), vasoactive intestinal peptide (*VIP*), and *IL6ST* (*gp130*).

One of the most striking and novel immunomodulatory effects of TCDD that was only detected in the HPL1A cell line was the repression of several IFN-stimulated genes. These include interferon-induced protein with tetratricopeptide repeats 1 (*IFIT1*), interferon-stimulated protein 15 (*ISG15*), EST, highly similar to IFT2-human interferon-induced protein with tetratricopeptide repeats 2 (*IFT2),* and myxovirus resistance 1 (homolog of murine interferon-inducible protein p78; *MX1*). These genes appear to be sensitive and robust targets of TCDD since *IFIT1*, *IFT2*, and *ISG15* were repressed at all doses tested, and MX1 was repressed at the two higher doses. The sensitivity for repression for these genes is of interest since immunotoxicity of TCDD occurs at low doses. A potential target activated by TCDD is the interferon regulatory factor 4 (IRF4), which may lead to the repression of these genes (Fig. 5). The mechanism of IRF4 repression of ISG15 results from formation of a complex with interferon consensus sequence binding protein (ICSBP, also known as IRF8; Rosenbauer *et al.,* 1999). Alternatively, IRF4 binding to the immunophilin FKBP52 prevents association of IRF4 with its partner PU.1 (Sharma *et al.,* 2000) as well as transactivation (Mamane *et al.,* 2000). These data strongly suggest indirect immune-modulation by TCDD in lung epithelial cells.

Change in expression of surface antigens is a characteristic often seen in cancerous cells. There were cell line specific alterations of a number of surface antigens induced by TCDD

(Fig. 2). These include epididymis-specific, putative ovarian carcinoma marker (*HE4*); EST, highly similar to tumor associated antigen (*L6*); transmembrane 4 superfamily member 1 (*TM4SF1*); and tumor-associated calcium signal transducer 2 (*TACSTD2*). Both L6 in the A549 cell line and TACSTD2 in the HPL1A cell line were induced at all doses of TCDD, suggesting that they may be potential biomarkers of TCDD exposure.

Other Pathways

Signaling pathways affected also include those involved in glucose homeostasis, stress, and NFκB; a transcription factor for a multitude of genes. Direct interactions of the AhR and NFκB are reported by Tian *et al.* (1999), who propose that the immunosuppressive effects of TCDD are mediated by suppression of NFκB induced cytokines. NFκB interacts with the transcription factor AP-1 and upregulates the IL-8 gene (Shi *et al.,* 1999). Our data suggest a role of NFκB in lung epithelial cells as seen by modulation of IL8, *REL,* and *BCL3*. It is interesting to note that v-Rel causes induction of IRF-4 expression and is linked to transformation of fibroblasts (Hrdlickova *et al.,* 2001). BCL3 is a member of the I kappa B family and binds to the NFκB p50 subunit, thereby allowing activation as a positive regulator of NFκB activity (Franzoso *et al.,* 1997). Evidence of involvement of the AP-1 complex was seen by differential regulation by TCDD in the HPL1A and A549 cells. *FOS* was altered in the HPL1A cells and *FOS*L-1 was altered in the A549 cells (Figs. 2 and 5). In studies using mammary epithelial cell line MCF-10A, TCDD increases the tyrosine phosphorylation of IGF1R (Tannheimer *et al.,* 1998). We have shown in addition to induction of *IGF1R* in both lung cell lines, other genes altered by TCDD that are involved with insulin signaling include *INSIG1*, *PDK4*, and *PTPN1* (Table 2, Fig. 2).

Implications for Evaluating Human Lung Response to TCDD

The classification of TCDD as a known carcinogen and its association with human lung cancer and chronic obstructive pulmonary disease (Bertazzi *et al.,* 2001; Pesatori *et al.,* 1998; Steenland *et al.,* 1999) warrants investigations on its effects in human lung cells. In the lung, there are over 40 different cell types, but tumors are derived mainly from bronchial epithelial cells, Clara cells, and type II pneumocytes. The focus of this study was to investigate TCDD effects using an *in vitro* model of type II pneumocytes comparing a malignant and a nonmalignant cell line. Consistent with the literature, many TCDD-altered gene expression profiles obtained from microarray analysis, included cytochrome P450s, quinone reductase, aldehyde dehydrogenases, fos, ras, protein kinase c, and vascular endothelial growth factor (Lai *et al.,* 1996; Sutter and Greenlee, 1992).

It is becoming increasingly clear that TCDD is a multimechanistic xenobiotic that integrates a variety of signaling networks. This study and others using toxicogenomic approaches reflect the reliability that small changes in gene expression can be detected (Frueh *et al.,* 2001; Kurachi *et al.,* 2002). A majority of the gene expression changes were less than 2-fold and may reflect modulation of signaling pathways, however only a dozen or so genes that had greater than 2-fold alterations are necessary to classify different toxicants (Thomas *et al.,* 2001). These data provide important potential insights into the mechanism of effects of TCDD in the lung; however, it is important to note that these studies have not been extended as yet to a large number of cell lines or *in vivo* conditions. Consequently, the cell line differences observed may simply reflect differences in cell type, growth conditions, or original source. Further validation of these genes affected by TCDD *in vivo* and transpecies comparisons will help to clarify how concordant these responses are with TCDD-induced lung toxicities.

Specific alterations in categories of genes altered from TCDD exposure, seem to depend on whether a cell is malignant or not, thus providing a valuable tool in assessing mechanism of action via toxicogenomic analysis. Gene expression profiles were different between the cell lines, as indicated by the nonmalignant HPL1A cells with altered genes that are immunosuppressive or involved in differentiation. The potential for TCDD-related decrease in immunocompetence or an imbalance in cell growth and differentiation is critical since it could lead to increases in a number of diseases including cancer. Whereas similar genes altered by TCDD in the two cell lines were those that are involved with growth control, cell-cell contact, cell motility, angiogenic factors, and metabolic enzymes.

The implication of these data is that some of these TCDD targets are known important contributors to pulmonary diseases and were only discovered by using nonmalignant cells. TCDD is a known human carcinogen that causes cancer promotion and enhances initiated cellular transformation (Tanaka *et al.,* 1989). The hypothetical pathways presented in this study using genes altered by TCDD provide a thorough overview of the integration of signaling networks as well as added insights into the basic nature of its chemical carcinogenicity.

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