GENE EXPRESSION PROFILING IN TCDD-TREATED HUMAN LUNG CELLS

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Introduction

In both rodents and humans, the lung is a TCDD target organ. In rodents, TCDD treatment causes alveolar-bronchiolar metaplasia, hyperplasia, as well as squamous cell carcinoma [1-3]. In humans, TCDD exposure has been linked to lung cancer in males, as well as an increase in chronic obstructive pulmonary disease by cohort analysis [4,5].

We have previously shown that the normal human alveolar type ii pneumocyte cell line (HPL1A) was more responsive to TCDD-induction of Phase I enzyme (CYP1A1 and CYP1B1) expression than a malignant alveolar type ii pneumocyte cell line (A549)[6]. Using toxicogenomic analysis our goal was to examine dose-dependent relationships of other genes that are potential mediators of TCDD toxicity using the NIEHS Human ToxChip v1.0. Different doses of TCDD (0.1, 1, and 10 nM) were used to examine TCDD dose-related changes in outliers from microarray analysis. Lastly, we compared relative fold induction from microarray hybridization values with those measured using real-time fluoresence RT-PCR.

Methods and Materials

Cell Culture: All cell culture reagents were purchased from Life Technologies (Rockville, MA) unless otherwise stated. A549 cells (ATCC No. CCL-185) were purchased from American Type Culture Collection (Manassas, VI) and plated at 4.2 X 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 15 mM HEPES. HPL1A cells were plated at 5.4 X 10^4 cells/ml in media as described previously[7]. A stock TCDD solution was prepared in FBS as reported[8] and diluted with FBS for treatments. Forty-eight hrs after incubation, half of the culture media was removed adding TCDD to achieve final concentrations of 0, 0.1, 1, or 10 nM TCDD. Conditioned media with TCDD was then replaced.

RNA Extraction and cDNA Synthesis: 24 hrs after incubation, cells were washed with PBS, removed by scraping and lysed using TRI Reagent (Sigma, St. Louis, MO). Total RNA was isolated per manufacturer recommendations, quantitated, diluted with DEPC water, and stored at -70 °C. Total RNA (100 ng) was reverse transcribed with random hexamer primers (2.5 mM) and MMLV reverse transcriptase (1.25 U/µl) in 10 µl reactions using a three step cycle: 25°C, 10 mins; 48° C, 30 mins; and 95° C, 5 mins. Reverse transcription reagents were purchased from PE Applied Biosystems (Foster City, CA).

Microarray Experiment Design and Analysis: For toxicogenomic analysis we used the Human v1.0 Tox Chip designed by Emile Nuwaysir, Ph.D at the NIEHS. Probes were obtained from Genbank and filtered using UniGene database for clustering analysis. cDNA clones were obtained from bacteria, cloned into plasmids, purified, PCR amplified and spotted onto poly-L-lysine treated slides using a microarray printer (Beecher Instruments). Triplicate hybridizations were used adding fluor-flips by labeling control with Cy3 in 2/3 hybridizations and Cy5 in 1/3 hybridizations. Calibration to 1 using 84 housekeeping genes was based on the peak of the ratio distribution using Cy3 (red) /Cy5 (green) ratios. Population distribution was used to determine validated outliers at 99 % confidence level (CL). A binomial distribution model was used to detect the chance occurrences in replicate experiments.

Real Time Fluorescence Detection RT-PCR Analysis: Real-time fluorescence detection using Taqman probes was carried with ABI Prism 7700 Sequence Detection System, PCR buffer 1X, $MgCl_2$ (5 mM), dATP, dCTP, dGTP (2.5mM each), dUTP (5mM), Taq Polymerase (0.025 U/µl) (PE Applied Biosystems, Foster City, CA), forward and reverse primers (0.2 µM), (Research Genetics, Huntsville, Al) and cDNA (10µl) in a final PCR reaction volume of 50 µl. PE 7700 amplification parameters were: denaturation at 94°C 10 mins, followed by 40 cycles of: 95°C, 15 sec.; 60° C, 60 sec.

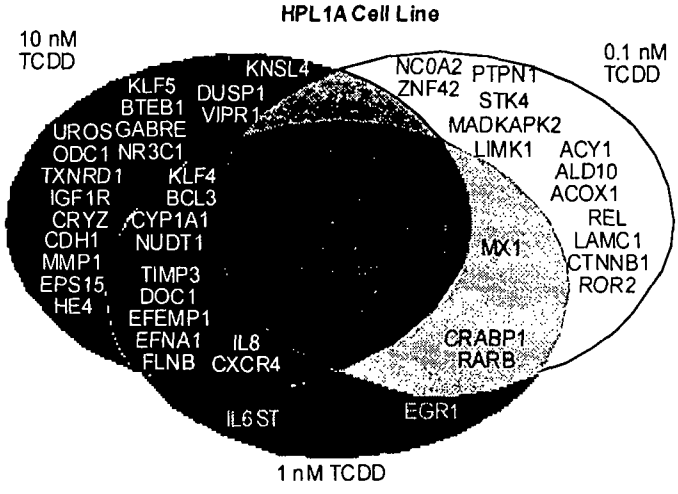
Results and Discussion

CYP1A1 and CYP1B1 were induced by TCDD in a dose-dependent manner in both cell lines and the HPL1A cells showed a greater fold induction than the A549 cells[6]. Thus, we were interested in examining this response in a toxicogenomis analysis. As shown in Figure 1, TCDD induces a pleiotropic set of genes that are classified as: chemokines/cytokines, signal transduction, transcription factors, oncogenes/tumor suppressors, cell proliferation and metabolism. Potential biomarkers are indicated by those genes (DUSP1, EGR1, STK4, UROS, ALD6, ALD3, CYP1B1, CYP1A1, CTNNB1, CDH1, RRAS, F2R) that are induced by TCDD in both cell lines or for those genes that are changed by TCDD at all doses (see Figure 1). The induction or repression patterns vary for individual genes induced by TCDD (Figure 2).

Validation by Sybergreen detection (data not shown) showed that the fold induction by microarray is not directly correlated to copy fold differences as measured by real-time RT-PCR but the trend of whether a gene is induced or repressed was consistent.

In summary, the HPL1A cell line is a more responsive *in vitro* lung cell model with a subset of similar TCDD-induced genes in A549 cells. Differences in some categories of specific subsets of genes affected may reflect the differences in tumorigenicity, as well as the relative sensitivity to TCDD. For example, genes that are involved in differentiation are only altered in the HPL1A cell line, supporting the hypothesis that neoplastic cells are resistant to terminal squamous differentiation. In both cell lines, changes in cell cycle are suggested by the array of TCDD-altered genes that differ, yet appear to have similar functions. Thus, TCDD appears to alter the G1/S phase transition. Finally, the genes such as NCOA2, IRF4, STK4 that are more highly induced at the lowest dose of TCDD [0.1 nM] in HPL1As may reflect a difference in physiological versus pharmacological mechanism of action.

A)



B)

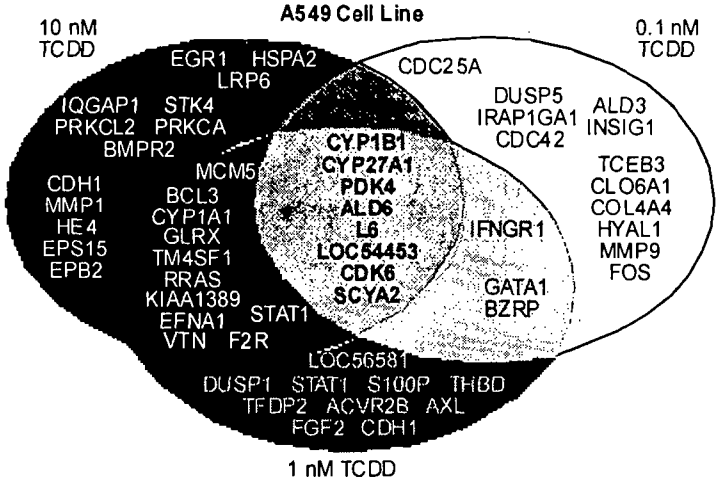


Figure 1. Venn diagram depicting the validated outliers identified by microarray analysis at 99% confidence level and found in two out of three hybridizations in A) HPL1A cell line and B) A549 cell line treated with TCDD for 24 hrs.

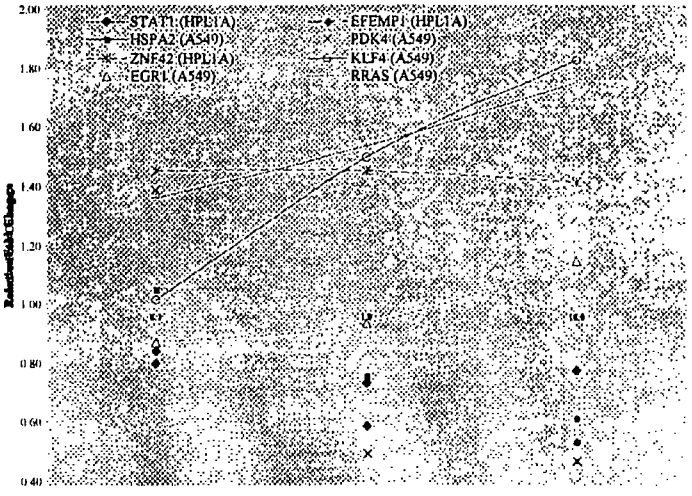


Figure 2. Differential dose-response relationships induced by TCDD were determined using hybridization values from microarray analysis.

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