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Original Article

Identification of Differentially Expressed Genes by TCDD in Human Bronchial Cells: Toxicogenomic Markers for Dioxin Exposure

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Differentially expressed genes by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) were identified in order to evaluate them as dioxin-sensitive markers and crucial signaling molecules to understand dioxin-induced toxic mechanisms in human bronchial cells. Gene expression profiling was analyzed by cDNA microarray and ten genes were selected for further study. They were cytochrome P450, family 1, subfamily B, polypeptide 1 (CYP1B1), S100 calcium binding protein A8 (calgranulin A), S100 calcium binding protein A9 (calgranulin B), aldehyde dehydrogenase 1 family, member A3 (ALDH6) and peroxiredoxin 5 (PRDX5) in up-regulated group. Among them, CYP1B1 was used as a hallmark for dioxin and sharply increased by TCDD exposure. Down-regulated genes were IK cytokine, interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), nuclease sensitive element binding protein 1 (NSEP1), protein tyrosine phosphatase type VI A, member 1 (PTP4A1), ras oncogene family 32 (RAB32). Although up-regulated 4 genes in microarray were coincided with northern hybridization, down-regulated 5 genes showed U-shaped expression pattern which is sharply decreased at lower doses and gradually increased at higher doses. These results introduce some of TCDD-responsive genes can be sensitive markers against TCDD exposure and used as signaling cues to understand toxicity initiated by TCDD inhalation in pulmonary tissues.

Key Words: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, Human bronchial epithelial cell, cDNA microarray

INTRODUCTION

TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) is one of the most toxic environmental pollutants and the representative compound for polycyclic aromatic hydrocarbons (PAHs). TCDD induces various biological responses, such as induction of xenobiotics metabolizing enzymes, teratogenicity, immunotoxicity, reproductive disorders, hepatotoxicity and cancers (Schwarz et al., 2000; Sweeney and Mocarelli, 2000). Therefore, IARC has upgraded the classification from possible human carcinogen (group 2B) to human carcinogen (group 1) (International Agency for Research on Cancer. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. 1988).

A number of genes altered by TCDD were regulated by a direct receptor-mediated mechanism, involving a basic helix-loop-helix (bHLH) protein known as aryl hydrocarbon receptor (AhR) (Denison and Heath-Pagliuso, 1998; Frueh et al., 2001). AhR is maintained in a ligand-binding state by association with cytosolic proteins. Upon ligand binding, AhR is transferred into the nucleus and forms a heterodimer with another bHLH protein, the aryl hydrocarbon nuclear translocator (ARNT). The AhR/ARNT heterodimer recognize the enhancer DNA element designated xenobiotic responsive element (XRE) sequence located in the promoter region of cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1) and CYP1B1, resulting in enhanced

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expression of both genes (Beedanagari et al., 2010).

Accumulated evidences have shown that the respiratory tract is a main target for TCDD-induced toxicity (Sweeney and Mocarelli, 2000). In addition, oral intake and inhalation are major routes for human exposure to TCDD, which were supported by the findings that chronic exposure of TCDD induced high mortality due to respiratory cancer in a cohort study and oral intake of TCDD increased alveolar-bronchiolar metaplasia in an animal model (Becher et al., 1996; Tritscher et al., 2000). Therefore, elucidation of biological signaling pathways including TCDD-induced toxic mechanisms in bronchial and pulmonary tissues is important. In this study, we tried to identify novel genes which are regulated by TCDD in normal human bronchial epithelial cells and discuss their potential as toxicogenomic markers. To accomplish these goals, gene expression profiling was performed by cDNA microarray and confirmed by northern hybridization in human bronchial epithelial cells.

MATERIALS AND METHODS

Cell culture

HBE4-E6/E7, human bronchial epithelial cell lines (CRL-2078; Rockville, MD, USA), was maintained in keratinocyte-serum-free medium (Gibco-BRL, Gaithersburg, MD, USA) containing 5 μg/L human recombinant epidermal growth factor (EGF), 50 mg/L bovine pituitary extract, and 10 ng/mL cholera toxin, 2 mM L-glutamine and grown in the presence of 5% CO_2 in humidified air at 37℃.

TCDD treatment

Cells were plated on tissue culture dishes and used for chemical exposures when they reached approximately 70% confluence. The cultured cells were treated with either TCDD (0.1, 1, 10, 100 nM) or vehicle (DMSO) for 24 hrs.

cDNA microarray

Total RNA was extracted with Tri-reagent (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. The cDNA microarray analysis was conducted using IntelliGene™ II Human Chip 1 (Takara, Japan). IntelliGene™ II Human Chip 1 is a DNA microarray immobilized with 3,893 cDNA fragments of human identified genes, which included 979 kinds of cancer-related or cytokine-related human cDNA fragments, on a slide glass. The fluorescent intensity of each dye at each spot is determined by the microarray scanner (Affymetrix 428 array scanner, Santa Clara, CA, USA). Red, yellow and green color spots mean up-regulated, not changed and down-regulated in the TCDD-treated cells relative to the non-treated control cells, respectively.

Cloning of PCR product & characterization

The sequences of the PCR primer pairs of 10 selected genes are listed in Table 1. Amplifications were performed using a Gene Amplification System (Applied Biosystems, Carlsbad, CA, USA) and amplified RT-PCR product was used as cloning sources in pGEM®-T Easy Vector System (Promega, Madison, WI, USA). Plasmid was isolated and DNA probe for northern blot analysis was purified by restriction endonuclease digestion.

Northern hybridization

Ten micrograms of total RNA was separated on a 1% formaldehyde-containing agarose gel and transferred to a nylon membrane (Hybond XL, Amersham Bioscience, Little Chalfont, UK). RNA on membrane was immobilized by UV cross-linking (CL-1000 Ultraviolet Crosslinker, UVP, Upland, CA, USA). Membrane was hybridized at 55℃ for 12 hrs with radio-labeled probes (Random Primer DNA Labeling Kit, TaKaRa, Japan). The hybridized membrane was washed twice in washing buffer for 20 min. The membranes were exposed to a phosphoimager screen (Packard, Palo Alto, CA, USA) and developed. The band intensity was quantified by using image analysis software (ImageQuant Version 5.2, Molecular dynamics, Sunnyvale, CA, USA) in Phosphoimager (Typhoon, Amersham Pharmacia, Little Chalfont, UK).

Statistical analysis

The data were expressed as mean ± standard deviations (S.D.). The values were compared to the control using analysis of variance (ANOVA) followed by unpaired

Table 1. Summary of differentially expressed genes induced by TCDD

Fold change	Up-regulated	Down-regulated	Total genes
	Number (%)	Number (%)	Number (%)
>4	2 (0.06)	1 (0.03)	3 (0.1)
3~4	29 (0.9)	4 (0.1)	33 (1.0)
2~3	155 (4.8)	94 (2.9)	249 (7.7)
Total	186 (5.8)	99 (3.1)	285 (8.8)

Percent indicate the proportions of probe number at each category by treatment with 100 nM TCDD for 24 hrs to the total 3,217 significantly signaled probes, which were selected from total 4,800 probes subjected to this analysis by excluding no signaled probes of both position makers and housekeeping genes.

Student's *t*-test. P values less than 0.05 ($P < 0.05$) were considered significant.

RESULTS

Analysis of the cDNA microarray hybridization

To identify TCDD-induced genes, cDNA microarray analysis was applied in HBE4-E6/E7 cells treated with 100 nM TCDD for 24 hrs. Consequently, it was found that 186 and 99 genes were up- and down-regulated by more than 2-fold in the TCDD-treated human bronchial cells. The most prominently responsive genes in the up-regulated group, retinoic acid-, interferon-inducible protein and CYP1B1 were increased 4 times in TCDD treated cells. The other 29 genes fall in the range between 3~4 fold inductions in TCDD treated cells. Predominantly down-regulated gene by 100 nM TCDD is isoleucine-tRNA synthetase with more than 4-fold repression in the TCDD treated cells. The other 4 genes, such as IK cytokine, interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), tousled-like kinase 1, and elastase 2A fall in the range between 3~4 fold repression in the TCDD treated cells. The proportion of total number of the up- and down-regulated genes by more than 2-fold are about 4.8% and 2.9% of total genes analyzed and selected, respectively (Table 1).

Selection of candidate genes

Among 285 differentially expressed genes which showed more than 2-fold induction or repression, 10 candidates exhibited five up-regulated and five down-regulated genes were selected for further study. The criteria of selection were based on their strong induction or reduction ratio and fluorescence strength (exceed 500 arbitrary units), and their suggested functional relevance of the gene to carcinogenesis or toxicity. The selected five up-regulated genes are CYP1B1 known as a hallmark of dioxin-exposure, S100 calcium binding protein A8 (calgranulin A), S100 calcium binding protein A9 (calgranulin B), aldehyde dehydrogenase 1 family, member A3 (ALDH6) and peroxiredoxin 5 (PRDX5). The five selected down-regulated genes are IK cytokine, IFIT1, nuclease sensitive element binding protein 1 (NSEP1), protein tyrosine phosphatase type VI A, member 1 (PTP4A1) and member ras oncogene family 32 (RAB32).

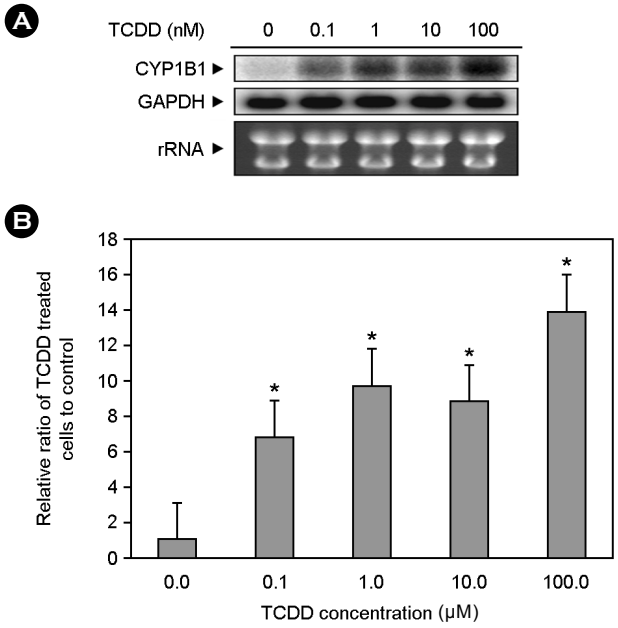

Fig. 1. Northern hybridization of CYP1B1 mRNA expression by TCDD exposure. Human bronchial cells were treated for 24 hrs with serial concentration of TCDD. Northern hybridization of CYP1B1 was used as a hallmark for dioxin. GAPDH and rRNA were used as an internal control. *Significantly different from the no treated control ($P<0.05$).

Northern hybridization

CYP1B1 has been recognized as a hallmark for exposure to dioxin and the expression levels have shown a rough dose-dependent manner with increasing concentrations of TCDD (Fig. 1). The other four up-regulated genes were further studied their dose-dependencies and assessment of induction rates. As shown in Fig. 2, the highest induction

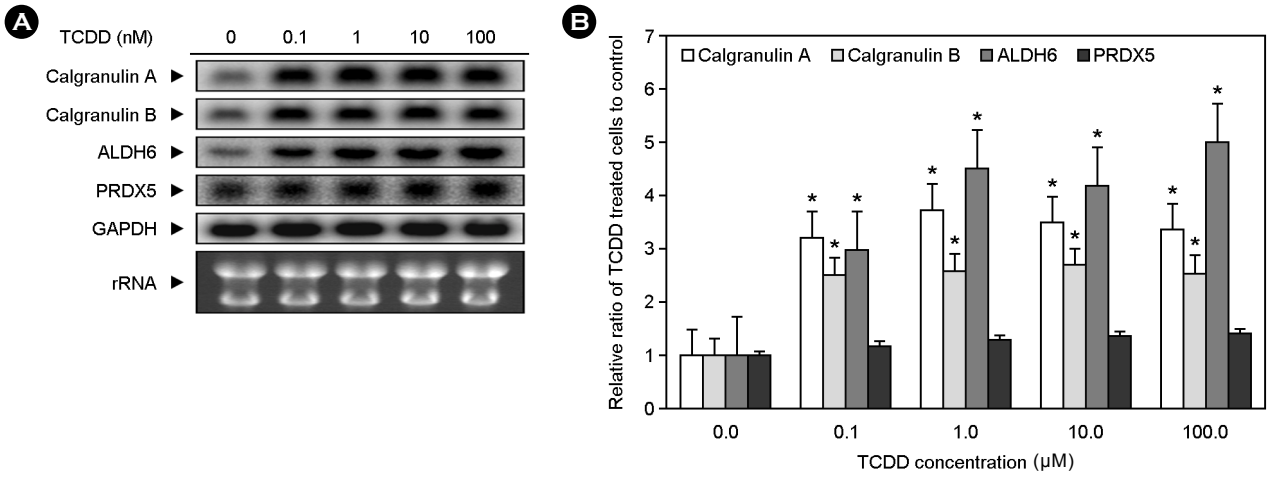

Fig. 2. Northern hybridization of up-regulated genes by exposure to TCDD. Panel A represents northern hybridization results of calgranulin (A, B), ALDH6 and PRDX5. GAPDH and rRNA were used as internal controls. Panel B shows relative band intensities of TCDD-treated samples over the non-treated control. *Significantly different from the non-treated control ($P<0.05$).

Table 2. Summary of expression profiles of the selected genes based on cDNA microarray and northern hybridization analysis

Abbreviation	Gene name	Fold induction	
		cDNA microarray	Northern hybridization
CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1	4.2	13.9
Calgranulin A	S100 calcium binding protein A8	2.9	3.4
Calgranulin B	S100 calcium binding protein A9	2.6	2.6
ALDH6	Aldehyde dehydrogenase 1, family, member A3	2.4	5.0
PRDX5	Peroxiredoxin 5	2.3	1.4
IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	-3.8	-2.7
IK cytokine	IK cytokine, down regulator of HLA II	-3.8	-1.1
NSEP	Nuclease sensitive element binding protein 1	-2.2	-1.1
PTP4A1	Protein tyrosine phosphatase type VIA, member 1	-2.1	-1.5
RAB32	Member Ras oncogene family 32	-2.0	-1.3

rate was obtained with ALDH6 gene which was very similar expression pattern but with lower extent than that of CYP1B1. When detection efficiency between the cDNA microarray and northern blot analysis was compared in 100 nM, the induction rate from northern blot analysis was approximately 2 times higher than that of microarray analysis (Table 2). Up-regulated patterns were similar with each other, and their induction rates seemed saturated during the range between 0.1 and 1.0 nM TCDD. Calgranulin A and B at 100 nM TCDD were induced 3.4 and 2.6 folds compared with those of control, respectively. The induction rates of calgranulins in northern hybridization were similar with the results of microarray analysis, but ALDH6 and PRDX5 showed different patterns than that of calgranulins (Table 2).

Interestingly, five down-regulated genes have shown U-shaped dose dependency, which means dose-dependent decrease at lower doses and increase at higher doses (Fig. 3). The maximum repressions of genes were obtained at 1 nM TCDD except for the PTP4A1 gene (at 10 nM TCDD). The strongest and stable repression was shown in the interferon-induced protein with IFIT1 by overall range of TCDD concentrations. Repression level of IFIT1 was 3.2 fold in 1 nM, which was slowly diminished by increasing TCDD concentration and 2.7-fold repression was obtained at 100 nM. The second strongest repression was observed

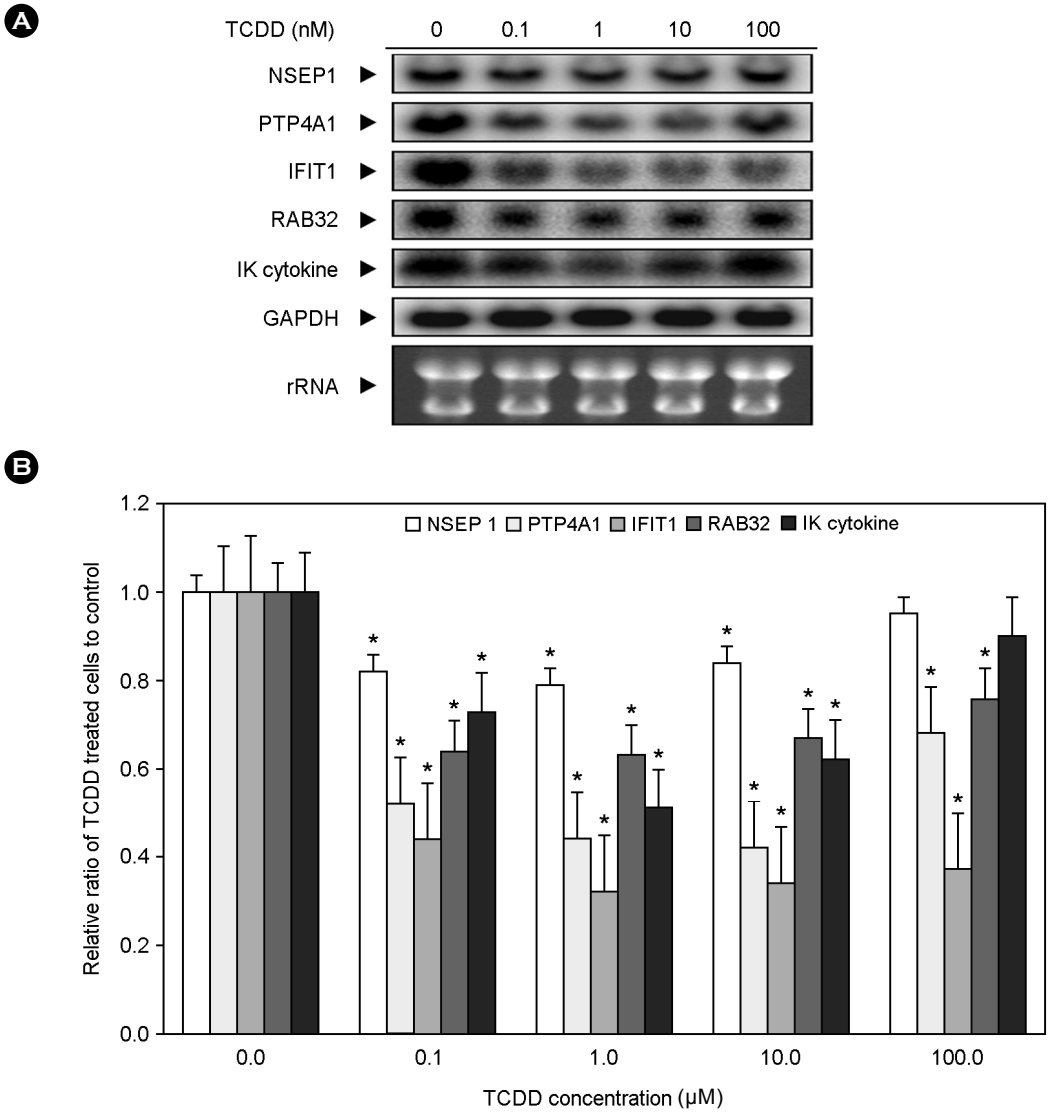

Fig. 3. Northern hybridization of down-regulated genes by exposure to TCDD. Panel (A) represents northern hybridization results of NSEP1, PTP4A1, IFIT1, RAB32 and IK cytokine. GAPDH and rRNA were used as internal controls. Panel (B) shows relative band intensities of TCDD-treated samples over the non-treated control. *Significantly different from the no treated control ($P<0.05$).

in PTP4A1 which has shown 2.3-, 2.4-, and 1.5-fold repressions at 1.0, 10, and 100 nM, respectively. The RAB32 and IK cytokine genes have shown their maximum repressions about 1.6-, 2.0-fold at 1.0 nM and repression levels about 1.3- and 1.1-fold at 100 nM. Although NSEP1 genes have shown a maximum repression about 1.3-fold at 1.0 nM but returned to almost control level (1.05-fold) at 100 nM. When both analysis methods were compared to identify detection efficiencies, expression levels from the northern hybridization at 100 nM were similar with those from microarray except IFIT1 (Table 2).

DISCUSSION

TCDD is known as a potent tumor promoter to enhance tumorigenesis by AhR but its exact mechanisms are far from complete elucidation until now. Moreover, the major routes for human exposure to TCDD are believed through intake of foods and inhalation of air but the biological consequence of the dioxin toxicity in the respiratory system are not well known. In this study, we attempted to identify novel genes induced by TCDD and evaluate them as

toxicogenomic markers for exposure to dioxin. Among analyzed 3,217 genes, 186 genes were up-regulated and 99 genes were down-regulated by over 2 fold. Among the differentially regulated genes, we selected 10 genes and we analyzed their expression patterns by northern hybridization. The up-regulated genes were calgranulin A, calgranulin B, ALDH6 and PRDX5. IK cytokine, IFIT1, NSEP1, PTP4A1 and RAB32 were down-regulated five genes. Their functional properties and possible association with TCDD-induced toxic and carcinogenic mechanisms are discussed below.

Calgranulin A and B are two calcium-binding proteins belonging to the S100 family. These proteins represent about 40% of the total calcium-binding capacity in monocytes, but are not detectable in mature tissue macrophages (Kerkhoff et al., 1998). They are specifically released by monocytes during the course of inflammatory reactions, and serum concentrations have been shown to correlate well with the activity of inflammatory reactions in various diseases (Roth et al., 2001). Calgranulin A and B directly inhibit proliferation of myoblasts and apoptosis of myocytes, which was confirmed by DNA fragmentation analysis and caspase-3 activity assay (Seeliger et al., 2003). But, underlying molecular mechanisms of induced apoptosis through calgranulins' modulation are not clear thus far. In this study, the induction rates of calgranulin A and B at 100 nM TCDD were 3.4 and 2.6 times against control, respectively. This is the first report to show the relationship between TCDD and both genes, and suggested it could be simply related with inflammatory reaction by TCDD-induced toxicity or apoptosis in human bronchial cells.

Retinoids are natural and synthetic analogs of vitamin A which are used to treat oral leukoplakia and reduce second primary hepatocellular or aerodigestive tract cancers (Hong et al., 1990; Muto et al., 1996). All-trans retinoic acid (RA) is the oxidized form of retinol by ALDH6, which is linked to a delayed G_1-S cell cycle transition (Langenfeld et al., 1996). Cyclin D increases in mid-G_1 and produces peak activation near the G_1-S cell cycle transition through forming complex to cyclin-dependent kinase (Cdk) 4 and Cdk6 (Resnitzky et al., 1994). RA directly signals a decline in cyclin D1 in human bronchial cells through induced proteolysis. The ubiquitin-dependent proteasome degradation pathway is implicated in this retinoid effect. RA-signaled cyclin D1 proteolysis is proposed as a mechanism linked to growth suppression in human bronchial cell transformation (Langenfeld et al., 1997). In this study, ALDH6 showed the highest induction rate which contributes to TCDD alterations of vitamin A in human bronchial cells. Thus, an increase in the basic metabolic turnover of vitamin A without concurrent replacement may lead to loss of storage and altered homeostasis, which in turn, cause carcinogenesis of airway epithelial cells.

The lung is continuously exposed to high levels of oxygen and oxidant-generating environmental agents than any other tissues. Thus, levels of reactive oxygen species in lung are further increased by xenobiotic chemicals that lead to DNA damage by free radicals induced mechanism (Ames, 1983). Especially, TCDD exposure could produce a sustained oxidative stress response (Slezak et al., 2000). Human lung expresses several thiol-containing proteins suggested to be major contributors for redox homeostasis (Kinnula et al., 2004). These proteins containing thiol-groups are thioredoxins, thioredoxin reductases, PRDXs and glutaredoxins (Holmgren, 2000). Among them, PRDX constitutes another thiol-containing antioxidant family that has been recently detected from human lung (Kinnula et al., 2002). PRDX appears abundantly in healthy lung and participates in the cellular antioxidant defense to protect cells from undergoing apoptosis (Kang et al., 1998). In this experiment, up-regulated PRDX may have a protective effect in TCDD induced oxidative stress but its induction rate was too low.

NSEP has emerged as key players in cellular metabolism. Diverse biological roles proposed for the NSEP include positive or negative modulation of various genes, redox signaling, and stress response (Kohno et al., 2003). One remarkable research was that the NSEP is a potential negative regulator of the p53 tumor suppressor (Lasham et al., 2003). P53 induces apoptosis that prevents the development of cancer by removing cells with mutated or damaged DNA in order to preserve genome integrity (Lane, 1992). In this study, NSEP has shown a maximum repression 1.3-fold at 1.0 nM but returned to almost non-treated control level (1.05-fold) at 100 nM, which means low concentration

of p53 activation and vice versa. Though the role of NSEP against TCDD exposure can't be suggested due to any report the correlation between them so far, this molecule can be a clue for correlation between dioxin and p53.

The PTP4A1 gene has shown the second strongest repression in this study with about 2.3-, 2.4-, and 1.5-fold repressions at 1, 10, and 100 nM, respectively. When PTP4A1 was stably overexpressed in 3T3 fibroblasts, altered growth characteristics became apparent, including a faster doubling time and growth to a greater saturation density (Kong et al., 2000). In nude mice, overexpressed PTP4A1 in epithelial cells induced tumor formation (Cates et al., 1996). PTP4A1 was down-regulated over 2-fold in low dose TCDD but repressed PTP4A1 was diminished at high concentration. This suggested that there may be a protective mechanism against tumor formation through down-regulation of PTP4A1 mRNA expression. Furthermore, the reduction of decreased PTP4A1 expression suggests to us that this anti-tumorigenic mechanism weakens at high concentration of TCDD.

IFIT1 has shown the strongest and stable repression over the all range of TCDD concentrations tested. In a previous study, mouse IFIT1 promoter contains two functional interferon stimulated response element (ISRE) motifs. ISREs are needed for maximum activity upon IFN-α treatment. Because of its highly similar structure, human IFIT1 was postulated to have an identical mechanism with mouse IFIT1 (Bluyssen et al., 1994). Cellular function of IFIT1 has been revealed that IFI56 binds to the large multimeric complex of eukaryotic initiation factor-3 (eIF-3) via p48 and blocks its function in initiating protein synthesis, which is presumed to mediate inhibition of cell growth and apoptosis (Zhang and Gui, 2004). In this study, decreased IFIT1 indicates to us that TCDD could break the cytokine related immune surveillance. TCDD would weaken the cell defense system by this mechanism that, in turn, normal cells could be transformed into cancer cells by TCDD exposure.

RAB32, a member of the Ras superfamily, interacts with protein kinase A (PKA) and functions as an A-kinase anchoring protein (AKAP). Subcellular fractionation and immunofluorescent approaches indicate that RAB32 and cellular PKA pool are associated with mitochondria. Thus, RAB32 is a dual function protein that participates in both mitochondrial anchoring of PKA and mitochondrial dynamics (Jurchott et al., 2003). However, mitochondria have various functions in apoptosis, the cell stress response, aging and genetically inherited disease. In addition, IK cytokine was secreted by the leukemic cell line K562 and several cancer cells, which inhibit HLA class II antigen induction by interferon (IFN)-γ (Krief et al., 1994). IK cytokine is a pleiotropic cytokine released from activated T cells and natural killer (NK) cells causing growth suppression, immune modulation, and induction of apoptosis. By the way, there is no evidence that IK cytokine and IFN-γ were secreted from epithelial cells. The relevance of RAB32 and IK cytokine to toxicity or carcinogenicity of TCDD should be investigated in a further study.

In this study, differentially expressed genes by TCDD were identified by cDNA microarray in normal human bronchial cells and we confirmed 5 up-regulated and 5 down-regulated genes. Among them, ALDH6 showed outstanding sensitivity and may be a promising toxicogenomic maker for TCDD-exposure in bronchial cells. In addition, IFIT1 gene, one of the down-regulated genes, has also shown characteristic expression pattern but it is doubtful since high concentration and long-term exposure could give a high expression level instead of repression. This question will need to be resolved in future studies. Although their exact functions are still unknown, both candidate genes provided by cDNA microarray may give higher sensitivity and specificity for detecting TCDD exposure.

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