TCDD AND INDIRUBIN-REGULATED GENES IN HEPG2 CELLS AND HUMAN UMBLICAL VEIN ENDOTHELIAL CELLS

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Introduction

Aryl hydrocarbon receptor (AhR) mediatesa diverse range of toxic and biological effects of dioxins in a variety of species and tissues. Microarray has been used to examine transcriptional signatures of dioxins in their target cells or tissues [1, 2, 3]. In the current study, we examined the gene expression profiles induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and indirubin in human hepatoma HepG2 cells and human umbilical vein endothelial cells (HUVEC). Indirubin, a pink colored pigment synthesized as a by-product of indigo, is a AhR ligand. We previously reported that indirubin was more potent than TCDD in inducing transactivation of a reporter gene in yeast that expressed human AhR and Arnt proteins [4]. In hepG2 cells, indirubin induced CYP1A1 and 1A2 mRNA more potently than TCDD at least under 10 pM exposure (Dioxin2002, Vol.56, p13). Indirubin's bioactivity has been studied because it is also an active component of the Chinese traditional medicine, Danggui Longhui Wan, which is used to cure chronic myelocytic leukaemia (CML). Indirubin is a potent inhibitor of cyclin-dependent kinases and of glycogen synthase kinase-3β (GSK-3β), which may play an important role in development of Alzheimer's disease [5, 6]. Indirubin inhibits inflammation in delayed-type hypersensitivity reactions [7]. Indirubin was also confirmed to be a product of human cytochrome P450-catalyzed metabolism of indole, a product of tryptophan catabolite [8]. We have previously detected indirubin in human urine of healthy individuals and in fetal bovine serum at average concentrations of 0.2 and 0.07 nM, respectively [4]. So we investigated indirubin-induced mRNA expression profile as well as TCDD-induced one and tried to find new target genes induced by indirubin and TCDD.

Methods and Materials

Cell Culture and Treatments

The human hepatocarcinoma HepG2 cell line was obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, Japan. The cells were grown at 37ºC in air supplemented to 5% CO_2. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. $4x10^6$ cells were seeded into 10-cm dishes containing fresh medium. 24 h later (70~80% confluence), cells were exposed to the test chemicals. Chemicals were dissolved in DMSO and added to the medium directly. The final DMSO concentration was 1.0% (v/v). Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics (San Diego, CA). Cultures of HUVEC were maintained in the endothelial growth medium including growth factors (EGM-2 BulletKit™). The cells were cultivated at 37ºC in air supplemented to 5% CO_2, strictly following the protocol provided by Clonetics.

Microarray

Total RNA was extracted from HepG2 cells using RNeasy Mini Kit (QIAGEN, Hilden, Germany). Following the isolation of PolyA (+) RNA from 50 μg of total RNA using Atlas ™ Pure Total RNA Labeling

System (Clontech, Palo Alto, CA), cDNA probes were synthesized with [^{32}P] dATP. Labelled cDNA probes were then hybridised with Atlas ™ Human Toxicology 1.2 Array which consists of 1176 genes. After hybridisation, the array was washed and exposed to Imaging Plate (Fuji, Tokyo, Japan) for one week and scanned with FLA-2000 (Fuji). The captured image was analysed using Atlasimage ™ 2.01.

Total RNA was extracted from HUVEC using RNeasy Mini Kit (QIAGEN, Hilden, Germany). 10 μg of total RNA from control and AhR ligand (TCDD or indirubin) treated cells were reverse transcribed in the presence of Cy3-dCTP and Cy5-dCTP, respectively, using Fluorescent Direct Label Kit (Agilent technologies, Palo Alto, CA). We also synthesized dye-swapped cDNA probe set. Labelled cDNA probe set was then hybridised with Human 1 cDNA Microarray (Agilent technologies) following the provided protocol. After hybridisation, the array was washed and scanned with GenePix4000B (Axon, union City, CA). We analysed the images of hybridised microarrays with GenePix Pro 4.1 (Axon).

Real-time quantitative RT-PCR

To confirm the results generated by microarray analysis, the expression of some genes was also analyzed by RT-PCR. Total RNA was extracted from three independent experiments as described previously. cDNA was synthesized using RNA PCR Kit (AMV) Ver.2.1 (Takara, Shiga, Japan) as the supplier instructed. Synthesized cDNA (2 μl) was amplified in a total volume of 25 μl containing 0.3 mM dNTP, 50 mM KCl, 3.4 mM $MgCl_2$, 1 μl of SYBR Green I (1000X diluted), 1.25 U *TaKaRa Ex Taq*™ R-PCR version (Takara) and 0.2 μM of each primer. Primer sequences are listed in Table 1. PCR was performed using Smart Cycler (Cepheid, Sunnyvale, CA). After 30s denaturation at 94ºC, PCR was carried out for 40 cycles with denaturation at 94ºC for 3s and annealing and extension for 30s at the temperature indicated in Table1. Accurate quantitation was achieved through the generation of standard curves by serially diluting a known amount of cDNA from corresponding synthetic RNA, which was contained in human cytochrome P450 competitive RT-PCR set (Takara). All signals were normalized against actin beta mRNA as a control.

Table 1 RT-PCR primers

gene	sequence	annealing temp. (ºC)	size (bp)
IGFBP1	5'-CCCAGAGAGCACGGAGATAA-3'	60	391
	5'-TATCTGGCAGTTGGGGTCTC-3'		
IGFBP10	5'-CAACCCTTTACAAGGCCAGA-3'	60	449
	5'-TGTAGAAGGGAAACGCTGCT-3'		
CYP19A1	5'-CACTGGCCTTTTTCTCTTGG-3'	60	480
	5'-AGAAGGGTCAACACGTCCAC-3'		
COX-2	5'-CAGGGTTGCTGGTGGTAGGA-3'	60	435
	5'-AAGGGACAGCCCTTCACGTT-3'		
putative lymphocyte	5'-CAAGCATCCACCAAAGGAGT-3'	60	125
G0/G1 switch gene	5'-ATCCTTCCTCCCTAGTGCAA-3'		
actin beta	5'-AGAAAATCTGGCACCACACC-3'	60	435
	5'-CCATCTCTTGCTCGAAGTCC-3'		
CYP1A1	*	60	433
	*		

* CYP1A1 primers are contained in human cytochrome P450 competitive RT-PCR set (Takara).
The sequences of the primers are not available for proprietary reasons.

Results and Discussion

Treatment of HepG2 cells with 10 nM TCDD for 8 h resulted in gene expression changes in 91 genes from a total of 1176 genes. 4 genes were up-regulated and 87 genes were down-regulated. Indirubin changed 23 genes' expression level. 2 genes were up-regulated and 21 genes were down-regulated. 22

indirubin-regulated genes were also regulated by TCDD, suggesting indirubin and TCDD affect the transcriptional level using same pathway via AhR in HepG2 cells. We examined the gene expression level of 4 up-regulated genes by TCDD which were *CYP1A1*, *CYP19A1(aromatase)*, *insulin-like growth factor-binding protein1* (*IGFBP1*) and *insulin-like growth factor-binding protein 10* (*IGFBP10*) by quantitative real-time RT-PCR (Figure 1). Each gene was confirmed to be up-regulated by both TCDD and indirubin. TCDD induced these genes potently than indirubin. We also confirmed that mRNA copies of these genes increased as the dose of indirubin was higher (data not shown). These results clearly show that *CYP1A1*, *CYP19A1*, *IGFBP1* and *IGFBP10* are regulated by TCDD and indirubin in HepG2 cells. *CYP19A1* encodes a member of the cytochrome P450 superfamily of enzymes. This protein localizes to the endoplasmic reticulum and catalyzes the last steps of estrogen biosynthesis, three successive hydroxylations of the A ring of androgens. So up-regulation of this gene may result in increased aromatase activity and affect the estrogen functions both as a sex steroid hormone and in growth or differentiation. Insulin-like growth factor (IGF) peptides are bound in biological fluids with IGFBPs that bind IGF peptides with high affinities and can extend the half-life of IGF and have been shown to modulate the IGF actions [9]. Reproductive abnormalities were observed in humen IGFBP1 overexpressed transgenic mice [10]. In the promoter region of *IGFBP1*, xenobiotic response element (XRE) also exists as well as cAMP response element (CRE), glucocorticoid response element (GRE) and insulin response element (IRE). So, ligand-activated AhR may bind XRE and directly regulate the transcription of IGFBP1 gene.

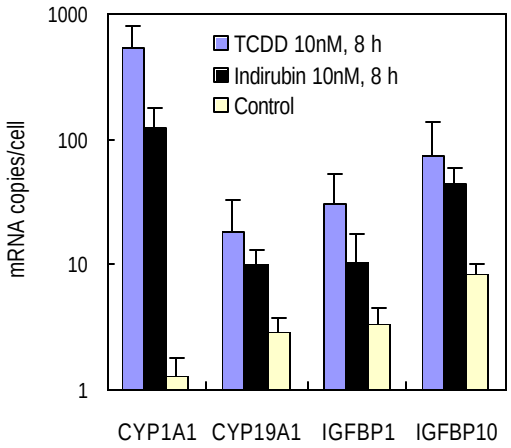

Figure 1. Up-regulated genes by TCDD and indirubin in HepG2 cells.

It is suggested that AhR plays a role in the resolution of vascular structures during development because immature vascular structures were observed in AhR null mice [11]. So, mRNA expression profiles induced by TCDD and indirubin in HUVEC were examined. Using microarray analysis and real-time RT-PCR, we could not confirm the significant change of mRNA expression levels of angiogenesis-related genes. But we found two genes, *prostaglandin-endoperoxide synthase 2* (*COX-2*) and *putative lymphocyte G0/G1 switch gene*, were regulated by TCDD and indirubin as shown in Figure 2. COX-2 is the key enzyme in prostaglandin biosynthesis, and acts both as a dioxygenase and as a peroxidase. COX-2 is expressed in a limited number of cell types and regulated by specific stimulatory events, suggesting that it is responsible for the prostanoid biosynthesis involved in inflammation and mitogenesis. Recent studies have shown that COX-2 is induced by TCDD in mouse fibroblasts [12] and in rat hepatocytes [13]. Putative lymphocyte G0/G1 switch gene (Genebank number; M72885) was identified from blood mononuclear cells [14], but its function is not known well. It is interesting that CYP1A1 mRNA was up-regulated by 10 nM TCDD (18 times for 24 h) and 10 nM indirubin (10 times for 24 h) though AhR mRNA was not confirmed to exist in HUVEC by RT-PCR. It is necessary to confirm the existence of AhR in protein level. If AhR does not exist in HUVEC, this result suggests that there is another AhR-independent pathway to induce CYP1A1 mRNA.

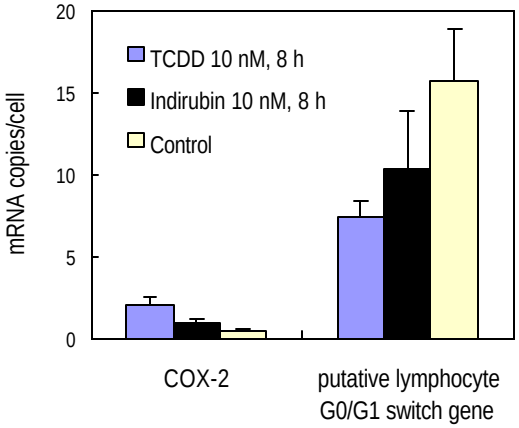

Figure 2. Regulated genes by TCDD and indirubin in HUVEC.

In conclusion, we found 4 genes regulated by TCDD and indirubin in HepG2 cells and 2 genes regulated in HUVEC using microarray and quantitative real-time RT-PCR. We expect our results contribute to bridge the gap between the functions of AhR ligands-regulated genes and the physiological response to exposure.

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References

1. Zeytun A., McKallip R.J., Fisher M., Camacho I., Nagarkatti M. and Nagarkatti P.S. (2002) Toxicology 178, 241
2. Frueh F.W., Hayashibara K.C., Brown P.O. and Whitlock J.P. Jr. (2001) Toxicol Lett. 122, 189
3. Puga A., Maier A. and Medvedovic M. (2000) Biochem. Pharmacol. 60, 1129
4. Adachi J., Mori Y., Matsui S., Takigami H., Fujino J., Kitagawa H., Miller C.A. 3rd, Kato T., Saeki K. and Matsuda T. (2001) J. Biol. Chem. 276, 31475
5. Hoessel R., Leclerc S., Endicott J. A., Nobel M. E. M., Lawrie A., Tunnah P., Leost M., Damiens E., Marie D., Marko D., Niederberger E., Tang W., Eisenbrand G. and Meijer, L. (1999) Nat. Cell. Biol. 1, 60
6. Damiens E., Baratte B., Marie D., Eisenbrand G. and Meijer, L. (2001) Oncogene 20, 3786
7. Kunikata T., Tatefuji T., Aga H., Iwaki K., Ikeda M. and Kurimoto, M. (2000) Eur. J. Pharmacol. 410, 93
8. Gillam E. M. J., Notley L. M., Cai H., De Voss J. J. and Guengerich F. P. (2000) Biochemistry. 39, 13817
9. Mohan S. and Baylink D. J. (2002) J. Endocrinol. 175, 19
10. Froment P., Seurin D., Hembert S., Levine J.E., Pisselet C., Monniaux D., Binoux M. and Monget P.(2002) Endocrinology 143, 1801
11. Lahvis G.P., Lindell S.L., Thomas R.S., McCuskey R.S., Murphy C., Glover E., Bentz M., Southard J. and Bradfield C.A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 10442
12. Wolfle D., Marotzki S., Dartsch D., Schafer W. and Marquardt H. (2000) Carcinogenesis 21, 15
13. Vogel C., Boerboom A.M., Baechle C., El-Bahay C., Kahl R., Degen G.H. and Abel J. (2000) Carcinogenesis 21, 2267
14. Russell L. and Forsdyke D.R. (1991) DNA Cell Biol. 10, 581