COMPARATIVE GENE EXPRESSION ANALYSIS OF LEUKOCYTE-EXPRESSED GENES IN THE MICE EXPOSED TO 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN AND INDIRUBIN BY CUSTOMIZED DNA MICROARRAY

Kagami Y[1], Kawano K[1], Mitsui T[2], and Maeda S[2]
[1]Ecogenomics, Inc. 1-1 Hyakunenkouen, Kurume, Fukuoka, Japan
[2]Department of Biochemistry, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, 1110 Shimokato, Chuo-shi, Yamanashi, Japan

Abstract

Exposure to dioxins and dioxin-like chemicals adversely affects health and reproduction of human and wildlife animals. However, suitable molecular biomarkers for routine diagnosis or risk assessment of the adverse effects caused by such compounds have not been discovered yet. Since we have developed a mouse DNA microarray which contains 1176 cDNA gene targets of known or potential responsiveness to environmentally toxic chemicals (e.g. POPs and hormone-like chemicals), we carried out microarray gene expression analyses on mouse blood leukocytes that were exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and separately to a natural arylhydrocarbon receptor (AhR) ligand, indirubin. The objectives of this study were to identify and differentiate potential biomarker genes in mammalian leukocytes that were exposed to a pleiotropically toxic and prototypical AhR-ligand, TCDD, and non-toxic AhR-ligand, indirubin. Thus, discovery of TCDD-responsive genes (angiotensin II receptor type 2, matrix metalloproteinase 8, cyclooxygenase-2, and interleukin 1β) in addition to *cyp1A1* and *cyp1B1* was worthwhile, and it was very interesting to note that these genes also showed sexually dimorphic responses. Contrary to our expectations, however, none of the TCDD-responsive genes showed responsiveness to indirubin, yet neuropeptide Y was identified to be a strong indicator of indirubin-exposure in leukocytes, and its transcriptional expression was sexually dimorphic.

Introduction

Upon binding dioxin and dioxin-like chemicals, arylhydrocarbon receptor (AhR) mediates most of toxic and adverse effects of dioxins by activating a series of AhR-transcription factor complex-regulated genes, including cytochromes 1A1, 1A2, and 1B1, glutathione S-transferases, etc. Indirubin, on the other hand, has been known for a long time as an active ingredient of the plant-derived anti-leukemia medicine in China, and it was found to be a natural and non-toxic AhR ligand. Interestingly, indirubin was reported to have higher affinity to AhR than 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in yeast AhR reporter gene assay, however, there is clearly a large toxic difference between TCDD and indirubin. In order to characterize these differences, comparative DNA microarray gene expression analysis using human HepG2 cell culture exposed to TCDD and indirubin was nicely performed by Adachi *et al*, and it was reported that the gene expression profiles for TCDD and indirubin were very similar.[1] Nevertheless, in our study we were interested in *in vivo* comparative DNA microarray gene expression analysis rather than *in vitro* study, focusing upon comparison of the leukocyte transcripts between the adult mice that were exposed to 5μg TCDD/kg-b.w. and the ones that were exposed to 1mg indirubin/kg-b.w. Thus, through this study we were to understand similarity and difference in the gene expression profiles of the leukocyte expressed gene transcripts that could be acutely affected by TCDD and separately by indirubin. This way, if we discover novel biomarker gene candidates, we would be able to apply our findings to diagnostic study and risk analysis of dioxin and dioxin-like chemicals on human health. So far, we have identified four TCDD-responsive mouse leukocyte genes (angiotensin II receptor type 2, matrix metalloproteinase 8, cyclooxygenase-2, and interleukin 1β) in addition to *cyp1A1* and *cyp1B1*. To our surprise, these genes did not only show TCDD-responsiveness but also showed sexually dimorphic responses. Additionally, although the cell type was different (HepG2 cell line vs. mouse leukocyte), we found striking difference in the results of *in vitro* and *in vivo* gene expression studies. None of the TCDD-responsive genes in fact showed responsiveness to indirubin in the mouse leukocytes, but neuropeptide Y was identified to be indirubin-responsive and sexually dimorphic. All in all, these results implied potentially useful features of the gender-specific biomarkers for the diagnostic measurement and/or risk assessment of the dioxin and dioxin-like compounds.

Materials and Methods

7-week-old C57BL/6 mice (SPF grade) were transported to the laboratory and they spent one week for acclimation. On post-acclimation day-one, -two, and -three, 5μg TCDD/kg-b.w. or corn oil (vehicle) was given by single gavage per day to the mice to be exposed (5 mice/sex/chemical), then on post-acclimation day-six 0.35ml of blood was drawn from the orbital sinus of each mouse. In case of indirubin exposure, 1mg indirubin/kg-b.w. or corn oil (vehicle) was given by single gavage to the mice to be exposed (5 mice/sex/chemical) on post-acclimation day-one, then after 8-hour exposure 0.35ml of blood was drawn from the orbital sinus of each mouse. Mouse RiboPure-Blood RNA Isolation Kit (Ambion Inc.) was used for the extraction and purification of leukocyte total RNA, and 2μg of the extracted total RNA was reverse-transcribed with T7-oligo dT primer. Subsequently, ds-cDNA was synthesized and *in vitro* transcribed with amino-allyl UTP to generate amino-allyl labeled aRNA samples. The purified amino-allyl-aRNA samples were coupled with amine reactive Cy5 by Amino Allyl MessageAmp aRNA kit (Ambion Inc.). DNA microarray used for this study contained 1176 mouse target genes (300±25-base-long cDNA fragments), and each target cDNA was spotted three times on the microarray. Each of the labeled aRNA samples were hybridized on one DNA microarray, thus 20 microarrays in total (5 microarrays per sex per exposure condition) were used in this experiment. Hybridization of the labeled samples and the gene targets on the microarray was carried out for 16 hours at 42°C in 50% formamide, 5x SSC, 0.5% SDS solution. For the statistical analysis of the microarray data, the data collected from the three target-spots per gene were normalized by the expression of two acidic ribosomal phosphoprotein P0 targets (Arbp100-1 and Arbp100-2) per microarray in each exposure condition, and they were integrated into ArrayStat z-test (Imaging Research Inc.) with significance determination of $p<0.05$. Quantitative Real Ttime-PCR (qRT-PCR) analysis with SYBR Green Realtime PCR Master Mix (Toyobo) and ABI7300 system (Applied Biosystems) was performed on the selected genes for microarray data validation. For each gene in each sample, three qRT-PCR reactions were set up. In order to relatively quantify qRT-PCR data, two selected primer sequences of the acidic ribosomal phosphoprotein P0 (Arbp100-1 and 100-2) were used.

Results and Discussion

We developed a mouse DNA microarray containing cDNA targets for 1176 genes (selected for their known or potential responsiveness to environmentally toxic chemicals such as POPs and hormone-like chemicals), and firstly we searched for biomarker candidates for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposure by using TCDD-exposed and unexposed mouse leukocytes. This study was to discover TCDD-responsive genes in the leukocytes, particularly the genes showing “acute response”. Therefore, if some genes from our DNA microarray were found to be equivalently or similarly responsive to TCDD-exposure as have been reported *cyp1A1* and *cyp1B1*, then they may become very useful biomarkers in designing diagnostic and risk assessment methods using human and wildlife blood samples for the exposure to TCDD and other dioxin-like compounds. In our DNA microarray and qRT-PCR validation analyses we discovered that the expression of six genes were significantly affected ($FD<0.5$ or $2.0<FD$ and $p<0.05$) in the leukocytes by acute TCDD-exposure. The validation analyses by qRT-PCR on these six genes showed that angiotensin II receptor type 2 (*agtr2*), *cyp1A1*, *cyp1B1*, and prostaglandin-endoperoxide synthase 2/cyclooxygenase-2 (*ptgs2/cox2*) were up-regulated more than two-fold (2.18, 2.34, 2.51, and 2.04, respectively) by TCDD in the leukocytes of female mice only, whereas matrix metalloproteinase 8 (*mmp8*) was 2.77-fold up-regulated in the male leukocytes only, and interleukin 1β (*IL-1b*) were up-regulated more than two-fold (2.35 in male and 2.37 in female) in the leukocytes regardless the gender difference.

The translated product of *agtr2* is associated with blood vessels to regulate renal vascular flow, and it seems to have a role in kidney development as well. There has been a report that female mice expressed renal *agtr2* mRNA substantially higher than that of male mice, and this sexually differentiated renal *agtr2* expression was thought to be due to its estrogen-dependency.[2] However, *agtr2* is not known to have an estrogen response element (ERE) in its promoter region, and instead it has an AP-1 site.[3] Thus, in estrogen-overdosed female mice estrogen-ER transcriptional activation of the AP-1 containing *agtr2* might have taken place in kidney tissues, and similarly in our result xeno-estrogenic TCDD might have acted as overdosed estrogen in the leukocytes of the TCDD-exposed female mice, leading to the up-regulated *agtr2* mRNA expression.[4] Another hypothesis was that TCDD-induction of intracellular Ca^{2+} and accumulation of *fos* and *jun* mRNAs leading to

large increases in AP-1 transcription factor activity might have taken place to up-regulate *agtr2*, and this process could be estrogen-dependent in the leukocytes.[5]

ptgs2/cox2 encodes the enzyme that catalyzes the conversion of arachidonic acid to prostaglandins, and it is induced by tumor promoters, growth factors, cytokines, viruses, and other stimuli such as environmental toxins. There were several reports prior to our report on the inducible mRNA expression and regulation of *ptgs2/cox2* by TCDD, for instance, up-regulation in lung and spleen of C57BL/6 mice and in primary human epithelial cells, whereas down-regulation in rat thymocytes.[6,7,8] Also, AhR-dependent up-regulation of *ptgs2/cox2* was observed in mouse pancreatic β-cells, and computational comparative genomic sequence analysis revealed that *ptgs2*/*cox2* had two DREs in its promotor sequence.[9,10] These species- and cell type-dependent TCDD-response mechanisms involving *ptgs2/cox2* transcriptional regulation might be caused by some unidentified interactions with the other transcription factor complexes that bind to AP-1, AP-2, C/EBP, and CREB sites that are in fact present in the promoter region of *ptgs2/cox2*.

Interestingly, it was recently found that angiotensin II (the ligand of AGTR2) induces *ptgs2*/*cox2* mRNA expression in rat intestinal epithelial cells through small GTPases and *agtr2* mRNA expression as well after renal ablation in rats.[11,12] In our study *ptgs2*/*cox2* and *agtr2* mRNA expressions were both up-regulated in the leukocytes of female mice, and thus it is possible that angiotensin II was involved in the up-regulation of these genes. Also the increase in angiotensin II production was possibly the result of TCDD-regulation at further upstream genes, for example, angiotensin-converting enzyme (ACE, converts angiotensin I to II) mRNA expression might be the target of TCDD-AhR pathway. In fact, our cDNA microarray contained angiotensin I converting enzyme 2 (Ace2, GenBank accession: NM_027286), and the microarray data showed that its FD (induction by TCDD) in male was 1.2, and the FD in female was 1.8. qRT-PCR analysis was also performed on *ace2* mRNA expression, and the FD in male was 1.2, and the FD in female was 1.8. Thus, especially in female it is very likely that TCDD induced the mRNA expression of *ace2*, which increased the concentration of angiotensin II, and then it activated the mRNA expressions of *ptgs2*/*cox2* and *agtr2*. However, the computational comparative genomic sequence analysis revealed no distinct DRE in the ACE promoter region, and there has been no report indicating the TCDD-induction of ACE.[10] Therefore, further investigations will be required for understanding the TCDD-induction of ACE and fine-tuning the use of *ptgs2*/*cox2* and *agtr2*genes as biomarkers for dioxin-like compounds.

The major known function of *mmp8* is to degrade fibrillar collagens imparting strength to the fetal membranes, and *mmp8* is expressed by leukocytes and chorionic cytotrophoblast cells, but there has been no report to show that TCDD-exposure has an influence on *mmp8* mRNA expression. Our finding of more *mmp8* up-regulation by TCDD in the leukocytes of male than that of female was an opposite gender-related response from that of *agtr2*, *cyp1A1*, *cyp1B1*, and *ptgs2/cox2*. It was reported that a physiological role of progesterone was to suppress the expression of matrix metalloproteinases (MMPs), and TCDD interferes with the suppressive role of progesterone on MMPs.[13] Thus, in TCDD-exposed female the expression of MMPs would be induced slightly, and this was the reason for our qRT-PCR FD of *mmp8* expressions was 1.45. In male on the other hand, the physiological role of progesterone should be disregarded and it would be reasonable to consider presence of a different *mmp8* induction mechanism by TCDD-exposure. Although further research will be needed to understand stronger responsiveness in the leukocytes of male, *mmp8* seems to have a potential to become one of the biomarkers for dioxin-like exposure in both sexes.

IL-1b was up-regulated in the leukocytes of both sexes due to acute TCDD-exposure. TCDD is known to promote inflammatory responses, and this effect may result from an up-regulation of the production of inflammatory cytokines such as *IL-1* and tumor necrosis factor. There in fact was a study reporting that TCDD-stimulated AhR-dependent *IL-1b* expression in human keratinocytes was due to post-transcriptional regulation which involved mRNA stabilization.[14] Therefore, perhaps a similar TCDD-response mechanism through AhR is taking place in the leukocytes as well.

Secondly, we were interested in finding the similarity or difference between mouse leukocyte gene expression profiles affected by the toxic and prototypic AhR ligand, TCDD, and natural and non-toxic AhR ligand, indirubin, in order to elucidate mechanisms of AhR-regulated transcription and toxic differences in AhR ligands. In contrast to acute TCDD-exposure, we discovered that none of the six TCDD-responsive genes were significantly affected in their transcriptional expression levels in the leukocytes of the indirubin-exposed mice. Instead, neuropeptide Y (*npy*) was found to be the only gene that was significantly up-regulated (192-fold up) in female and significantly down-regulated (100-fold down) in male. This clearly indicated that the

AhR-mediated response mechanisms to TCDD and to indirubin did not overlap with each other in our *in vivo* (leukocyte) study, even though *in vitro* study by Adachi *et al* showed that TCDD and indirubin were both the ligands of AhR and that they shared their downstream genes that were regulated by the ligand-AhR transcriptional complex.[1] Luckily, the degrees of differentiated transcriptional expressions of *npy* in male and female leukocytes were both quite large (2 orders of magnitude) comparing with the other TCDD-responsive biomarker candidates that we found in this study, and transcriptional expression level of *npy* in the non-exposed leukocytes was high enough to easily monitor its up- or down-regulation. Thus, *npy* appeared to be an excellent biomarker candidate for an overdose of indirubin. Furthermore, referring to an interesting study reporting the body weight loss in TCDD-treated male rats, hypothalamic arcuate nucleus expression of *npy* mRNA was up-regulated and a xenobiotic response element (XRE) was found in *npy* gene.[15] Although *npy* was not the only gene that was involved in the TCDD-induced weight loss in male rats, and although we recognized that the tissues (hypothalamic arcuate nucleus vs. leukocyte) where *npy* might function were totally different, it was still very curious to observe that TCDD never up-regulated *npy* in mouse leukocytes via AhR. Lastly, it was also very interesting to observe sexually differentiated phenotype of the blood samples from indirubin-exposed male and female mice. It appeared that indirubin-exposed female blood samples were much more easily coagulated than the ones of the indirubin-exposed male. Observing that the leukocyte *npy* had shown totally opposite transcriptional response to indirubin-exposure between the genders, it would be worth an investigation to clarify the mechanistic relationship between indirubin-exposure, *npy* and other sexually differentiated gene expressions in leukocyte, and blood coagulation phenotype.

Consequently, from our gene expression analysis in the mouse leukocytes, sexually dimorphic and significant mRNA expression responses of *agtr2*, *cyp1A1*, *cyp1B1*, *ptgs2/cox2*, and *mmp8* to TCDD-exposure were observed, and these genes were not regulated in the same manner by indirubin-exposure. The newly found leukocyte gene that was regulated by indirubin-exposure was *npy*, and its sexually differentiated response to indirubin was observed. As we focused our gene expression analysis and biomarker gene search on TCDD- and TCDD-like compound-exposed blood leukocytes, the next step of our research would be integration and application of these findings into development of novel diagnostic and/or biological risk assessment methods to pragmatically measure human health hazards. However, further studies on evaluation of the identified genes' suitability to be biomarkers, i.e., influences by genetic polymorphisms and inter-individual variation in mRNA induction/repression of these genes, will also be required.

References

1. Adachi J, Mori Y, Matsui S, and Matsuda T. (2004) Toxicological Sciences. 80: 161-169.
2. Armando I, Jezova M, Juorio V, Terron A, Falcon-Neri A, Semino-Mora C, Imboden H, and Saavedra M. (2002) Am J Physiol Renal Physiol. 283: F934-943.
3. Ichiki T and Inagami T. (1995) Hypertension. 25: 720-725.
4. Kushner P, Agard D, Greene G, Scanlan T, Shiau A, Uht R, and Webb P. (2000) J Steroid Biochem Mol Biol. 74: 311-317.
5. Nebert D, Puga A, and Vasiliou V. (1993) Ann N Y Acad Sci. 685: 624-640.
6. Vogel C, Schuhmacher U, Degen G, Bolt H, Pineau T, and Abel J. (1998) Arch Biochem Biophys. 351: 265-271.
7. LiuY, Levy N, and Weber W. (1997) Prostaglandins. 53: 1-10.
8. Olnes M, Verma M, and Kurl R. (1996) J Pharmacol Exp Ther. 279: 1566-1573.
9. Yang F and Bleich D. (2004) J Biol Chem. 279: 35403-35411.
10. Sun Y, Boverhof D, Burgoon L, Fielden M, and Zacharewski T. (2004) Nucleic Acids Res. 32: 4512-4523.
11. Slice W, Chiu T, and Rozengurt E. (2005) J Biol Chem. 280: 1582-1593.
12. Vazquez E, Coronel I, Bautista R, Romo E, Villalon M, Avila-Casado C, Soto V, and Escalante B. (2005) Am J Physiol Renal Physiol. 288: F207-213.
13. Bruner-Tran L, Rier E, Eisenberg E, and Osteen G. (1999) Gynecol Obstet Invest. 48 Suppl 1: 45-56.
14. Henley D, Bellone C, Williams D, and Ruh M. (2004) Arch Biochem Biophys. 424: 112-118.
15. Fetissov S, Huang P, Zhang Q, Mimura J, Fujii-Kuriyama Y, Rannug A, Hokfelt T, and Ceccatelli S. (2004) Regul Pept. 119: 113-124.