DNA microarray expression profiling of leukocyte-expressed genes in the mouse exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin

Yoshihiro Kagami[1], Tetsuo Mitsui[2], Hirotoshi Akane[2], Shuichiro Maeda[2]

[1]Ecogenomics, Inc.
[2]University of Yamanashi

Introduction

Although exposure to dioxins and dioxin-like compounds causes variety of pathological adverse effects to human and wildlife, suitable molecular biomarkers for routine diagnostic analysis or risk assessment have not been discovered yet. As we developed a mouse cDNA microarray containing probes for 508 genes that were selected for their known or potential responsiveness to environmentally toxic compounds (e.g., estrogen-like and dioxin-like compounds), we carried out cDNA microarray gene expression profiling analysis of the C57BL/6 mouse leukocytes. In this analysis, the leukocyte mRNAs obtained from 8-week-old mice (five individuals from each sex), that were exposed to 5µg/kg-body weight of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), were compared with the leukocyte mRNAs from the unexposed 8-week-old mice (five individuals from each sex). Our approach was to discover acute transcriptional gene responses to TCDD in the leukocytes (single dose by gavage/day for three consecutive days, then blood sample was drawn at 72 hours after the last dose) rather than to find immunotoxicological responses from chronic low-dose exposure. In our "short exposure-acute response" study, we identified three TCDD-responsive genes in addition to *cyp1A1* and *cyp1B1*, and those three genes were angiotensin II receptor type 2, matrix metalloproteinase 8, and prostaglandin-endoperoxide synthase 2 (cyclooxygenase-2). Interestingly, these genes did not only show TCDD-responsiveness but also showed sexually dimorphic responses. This result implied a potentially useful feature of the gender-specific biomarkers for the diagnostic measurement and/or risk assessment of the 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 the post-acclimation day one, two and three, 5µg TCDD/kg-body weight was given by single gavage per day to the mice to be exposed (5 mice from each sex), and corn oil (vehicle) was given to the non-exposed control mice (5 mice from each sex). On the post-acclimation day six, 0.5ml of blood was drawn from the posterior vena cava of each mouse. RNeasy Mini Kit (QIAGEN) was used for the extraction and purification of leukocyte total RNA, and 2µg each 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 target samples. The purified aRNA target samples were coupled with amine reactive Cy5 by Amino Allyl MessageAmp aRNA kit (Ambion Inc.). cDNA microarray used for this study contained 508 mouse gene probes (300±25-base-long cDNA fragments), and each probe was spotted six times on the microarray. Each of the labeled target samples were hybridized on one cDNA microarray, thus total of 20 microarrays (5 microarrays per sex per exposure condition) were used in this experiment. Hybridization of the labeled target samples and the gene probes 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 six probe-spots per gene were normalized by the expression of two acidic ribosomal phosphoprotein P0 probes (Arbp100-1 and Arbp100-2) per microarray in each TCDD-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 cDNA microarray containing probes for 508 genes (responsive to the estrogen-like and dioxin-like compounds), and 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 that experienced a “short exposure-acute response” type of exposure test. If some genes 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.

As tables 1 and 2 show, we discovered in our cDNA microarray analysis that the expression of eight genes were significantly affected (FD < 0.5 or 2.0 < FD and $p < 0.05$) in the leukocytes by acute TCDD-exposure. In order to validate such data obtained by cDNA microarray analysis, QRT-PCR analysis was performed on those significantly affected genes. When doing so, we included angiotensin II receptor type 2 (*agtr2*), *cyp1A1*, and *cyp1B1* in the QRT-PCR analysis because of a report showing that *agtr2* mRNA expression was up-regulated by estrogen (TCDD is one of the xeno-estrogenic compounds)[1] and also because of *cyp1A1* and *cyp1B1* having long been investigated as biomarkers for the exposure to dioxin and dioxin-like compounds. This QRT-PCR analysis confirmed that four genes: *agtr2*, *cyp1A1*, *cyp1B1*, and prostaglandin-endoperoxide synthase 2/cyclooxygenase-2 (*ptgs2/cox2*) were up-regulated more than two-fold by TCDD in the leukocytes of female mice, whereas matrix metalloproteinase 8 (*mmp8*) was up-regulated more than two-fold by TCDD in the leukocytes of male mice (tables 1 and 2). Some of the genes, especially *cyp1A1* and *cyp1B1*, showed conflicted FD data between microarray and QRT-PCR, and it was due to their very low level mRNA expression in the leukocytes that generated large variance in the expression data.

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[1]. However, *agtr2* is not known to have an estrogen response element (ERE) in its promoter region, and instead it has an AP-1 site.[2] Thus, in estrogen-overdosed female mice estrogen-ER transcriptional activation of the AP-1 containing *agtr2* might have taken place in kidney tissues[3], 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. 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.[4]

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[5] and in primary human epithelial cells[6], whereas down-regulation in rat thymocytes.[7] Also, AhR-dependent up-regulation of *ptgs2/cox2* was observed in mouse pancreatic β-cells[8], and computational comparative genomic sequence analysis revealed that *ptgs2*/*cox2* had two DREs in its promotor sequence.[9] 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*.

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.[10] Thus, in TCDD-exposed female the expression of MMPs would be induced, and this was the reason for our QRT-PCR FD data of *mmp8* were close to 2.0. 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.

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[11] and *agtr2* mRNA expression as well after renal ablation in rats.[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 (data not shown). 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* (tables 1 and 2). However, the computational comparative genomic sequence analysis revealed no distinct DRE in the ACE promoter region[9], and there has been no report indicating the TCDD-induction of ACE. 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.

Finally, from our gene expression analysis in the leukocytes of mice, sexually dimorphic and significant mRNA expression responses of *agtr2*, *cyp1A1*, *cyp1B1*, *ptgs2/cox2*, and *mmp8* to TCDD-exposure were observed. 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 utilizing a few or all of the above described biomarker candidate genes. Further studies on evaluating their 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. 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.

2. Ichiki T. and Inagami T. (1995) Hypertension. 25: 720-725.

3. Kushner P., Agard D., Greene G., Scanlan T., Shiau A., Uht R., and Webb P. (2000) J Steroid Biochem Mol Biol. 74: 311-317.

4. Nebert D., Puga A., and Vasiliou V. (1993) Ann N Y Acad Sci. 685: 624-640.

5. Vogel C., Schuhmacher U., Degen G., Bolt H., Pineau T., and Abel J. (1998) Arch Biochem Biophys. 351: 265-271.

6. LiuY., Levy N., and Weber W. (1997) Prostaglandins. 53: 1-10.

7. Olnes M., Verma M., and Kurl R. (1996) J Pharmacol Exp Ther. 279: 1566-1573.

8. Yang F. and Bleich D. (2004) J Biol Chem. 279: 35403-35411.

9. Sun Y., Boverhof D., Burgoon L., Fielden M., and Zacharewski T. (2004) Nucleic Acids Res. 32: 4512-4523.

10. Bruner-Tran L., Rier E., Eisenberg E., and Osteen G. (1999) Gynecol Obstet Invest. 48 Suppl 1: 45-56.

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.

Table 1

Data comparison between cDNA microarray and QRT-PCR (Arbp100-1 normalized)

Arbp100-1 Normalized Data					
Microarray		QRT-PCR		Accession	Gene
♀FD	♂FD	♀FD	♂FD		
0.8	0.5	1.5	1.0	BC023851	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1
1.8	1.0	1.7	1.2	NM_027286	angiotensin I converting enzyme 2
2.2	1.4	2.4	1.2	L32840	angiotensin II receptor, type 2
1.2	0.5	0.8	0.7	NM_009696	apolipoprotein E
2.0	1.0	1.6	0.8	NM_009947	copine VI
1.4	0.9	3.0	1.7	NM_009992	cytochrome P450 1A1
1.6	0.6	2.9	1.2	NM_009994	cytochrome P450 1B1
2.4	1.9	1.9	1.2	BC038810	cytochrome P450 7B1
0.8	0.4	1.9	1.0	NM_009995	cytochrome P450 21A1
1.5	2.0	1.9	2.6	BC042742	matrix metalloproteinase 8
1.7	1.8	2.2	1.1	NM_011198	prostaglandin-endoperoxide synthase 2 (cyclooxygenase-2)

FD: fold difference, ratio of TCDD-exposed expression data to unexposed expression data
The highlighted genes were QRT-PCR confirmed and significantly responsive to TCDD-exposure.

Table 2

Data comparison between cDNA microarray and QRT-PCR (Arbp100-2 normalized)

Arbp100-2 Normalized Data					
Microarray		QRT-PCR		Accession	Gene
♀FD	♂FD	♀FD	♂FD		
0.9	0.5	1.5	1.0	BC023851	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1
2.0	1.1	1.8	1.2	NM_027286	angiotensin I converting enzyme 2
2.4	1.7	2.5	1.2	L32840	angiotensin II receptor, type 2
1.4	0.6	0.8	0.7	NM_009696	apolipoprotein E
2.2	1.2	1.7	0.8	NM_009947	copine VI
1.5	1.0	3.0	1.7	NM_009992	cytochrome P450 1A1
1.8	0.8	3.0	1.2	NM_009994	cytochrome P450 1B1
2.7	2.2	1.9	1.2	BC038810	cytochrome P450 7B1
0.8	0.5	1.9	1.0	NM_009995	cytochrome P450 21A1
1.6	2.3	2.0	2.6	BC042742	matrix metalloproteinase 8
1.9	2.1	2.2	1.1	NM_011198	prostaglandin-endoperoxide synthase 2 (cyclooxygenase-2)

FD: fold difference, ratio of TCDD-exposed expression data to unexposed expression data
The highlighted genes were QRT-PCR confirmed and significantly responsive to TCDD-exposure.