GENE EXPRESSION PROFILE IN THE LIVER OF BAIKAL SEALS (*PUSA SIBIRICA*): ASSOCIATION WITH DIOXINS AND RELATED COMPOUNDS

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Abstract
Dioxins and related compounds (DRCs) including polychlorinated dibenzo-*p*-dioxins, dibenzofurans and coplanar polychlorinated biphenyls are highly accumulated in the liver of Baikal seals (*Pusa sibirica*). Our previous study has demonstrated that the expression levels of CYP1A1, 1A2 and 1B1 were positively correlated with DRC concentrations in the liver of individuals of wild Baikal seal population, indicating that these CYP isozymes are induced by these DRCs. To screen DRC-responsive genes other than these CYP isozymes, the present study constructed an oligo array targeting genes expressed in the liver of Baikal seals, and examined the relationships between the hepatic TEQ and gene expression levels. Out of 2374 genes spotted on the oligo array, positive correlations with TEQ were detected for 45 and 26 genes in males and females, respectively. Thirty three and 24 genes were negatively correlated in males and females, respectively. The present study indicated that DRCs may affect the expression of a variety of genes involved in xenobiotic biodegradation and metabolism, immune system and oxidative phosphorylation in the liver of Baikal seals. Although further research is necessary to establish a definite cause-effect relationship, this study provides valuable information on DRC-responsive genes to predict potential effects on wild population of Baikal seal.

Introduction
Polychlorinated dibenzo-*p*-dioxins, dibenzofurans and coplanar polychlorinated biphenyls are ubiquitous contaminants. These compounds are biomagnified in the food web due to their lipophilic and persistent properties, and are notably accumulated in a variety of aquatic mammalian species.[1] The toxic effects of such dioxins and related compounds (DRCs) and the molecular mechanisms in aquatic mammals still remain unclear, but are likely to involve the aryl hydrocarbon receptor signaling pathway.[2]

Our previous study showed that Baikal seal (*Pusa sibirica*) accumulates high levels of DRCs. Total 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxic equivalent (TEQ) levels were in the range of 10-570 pgTEQ/g wet wt in the liver.[3] The hepatic total TEQ levels were positively correlated with expression levels of CYP1A1, 1A2 and 1B1 in the wild population, indicating chronic induction of these CYP isozymes by DRCs.[4]

Since organisms generally react to chemical exposure by altering the expression levels of multiple genes, a wide variety of molecular changes should be monitored to predict potential toxic effects and their mechanisms. Recent advances in microarray technology enabled to evaluate chemical exposure and further toxic effects associated with the gene expression profile. Previous studies have reported expression of genes in few experimental animals such as rats and mice that were treated with DRCs.[5, 6] However, there are only a few microarray studies addressing alteration of gene expression profiles related to DRCs in wild species[7]. To screen DRC-responsive genes and predict potential effects at molecular level in the liver of wild Baikal seal population, the present study constructed an oligo array where genes from the seal liver are targeted, and monitored the gene expression levels associated with DRC levels.

Materials and Methods
Baikal seals were collected from Lake Baikal, Russia, in 1992 and 2005. The liver samples were frozen in liquid nitrogen, and stored at –80 ºC until total RNA extraction. Gene expression levels in the seal livers were measured in 22 samples (6 males and 16 females) collected in 1992 and 10 male samples in 2005. Total TEQ levels were in the range of 11-490 and 7.2-190 pg TEQ/g wet wt in male and female animals, respectively.[3, 8] TEQs were calculated from WHO reevaluated mammalian toxic equivalency factors (TEFs) of individual congeners proposed by Van den Berg *et al* (2006).[9]

A Baikal seal cDNA library was constructed and characterized by sequencing randomly selected 5000 clones. Following BLAST homology search of the cDNA sequences, approximately 4100 cDNA clones whose sequences had high identities with genes deposited in the GenBank database were obtained. Sixty-mer oligonucleotides (7122 probes of 2374 genes; 3 oligonucleotide probes per gene) were designed and spotted onto an 11K format slide glass (Agilent Technologies, Inc., Wilmington, DE).

Total RNA was extracted from the liver tissue. The Agilent Low RNA Input Linear Amplification Kit PLUS, Two-Color (Agilent Technologies) was used to amplify RNA samples following the manufacturer's protocol. RNA samples from the livers of four specimens (2 males and 2 females) that were contaminated by average TEQ levels were pooled to use as a common reference, and labeled with Cy5. Individual RNA samples were labeled with Cy3. After the amplification and labeling, cDNA yields and dye incorporation efficiencies were determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). For hybridization, 500 ng of Cy3-labeled samples and Cy5-labeled references were mixed and incubated on an microarray slide at 65 ºC for 17 h using Gene Expression Hybridization Kit (Agilent Technologies). Following hybridization, the slides were washed and dried using Gene Expression Wash Pack (Agilent Technologies), acetonitrile (Sigma-Aldrich CO., St. Louis, MO), and Stabilization and Drying Solution (Agilent Technologies). The washed slides were then scanned using Fluor-Image Analyzer, FLA-8000 (Fuji Photofilm Co. Ltd, Tokyo, Japan) at 532 nm for Cy3 and at 635 nm for Cy5.

Fluorescent intensities were quantified by ArrayGauge V2.1 (Fuji Photofilm Co. Ltd). The intensity of around each spot was used as background. Expression levels of each spot were represented as Cy3/Cy5 ratios. The ratios were normalized using the Locfit (LOWESS) function in TIGR MIDAS (version 2.19). All samples were analyzed in triplicate on separate arrays, and spots with more than 20 % of coefficient of variation were excluded from subsequent analysis.

To analyze the relationships between gene expression and total TEQ levels, Sperman’s rank correlation test was preliminarily performed for each spot. The spot data that showed significant correlations in Spearman’s rank correlation test were pooled and averaged for each gene, and used for subsequent analysis. Prior to analysis, total

Table 1 List of genes correlated with hepatic total TEQ levels in Baikal seals (n =32, 16 males and 16 females)

Functional classification gene species	male		female	
	positively	negatively	positively	negatively
Xenobiotics biodegradation and metabolism	4	2	5	1
Metabolism of cofactors and vitamins	2	1	-	-
Amino acid metabolism	-	-	4	-
Carbohydrate metabolism	-	-	-	1
Folding, sorting and degradation	3	-	1	1
Biosynthesis of steroids	-	2	-	-
Other enzymes	4	3	3	3
Immune system	3	1	-	1
Receptors	1	-	1	-
Transcription related protein	1	-	-	-
Transcription factor	-	-	1	-
Translation factor	-	1	-	-
Ribosomal proteins	-	12	-	1
Oxidative phosphorylation	-	1	5	-
Menbrane transport	-	1	-	-
Endocrine system	-	-	-	1
Others	27	9	6	15
Total	45	33	26	24

TEQ levels and gene expression levels were logarithmically transformed. The association of concentration of DRCs with gene expression levels was further examined by simple linear regression analysis. Correlation of the microarray data with the real-time RT-PCR data was done by the Spearman's rank correlation test. All statistical analyses were performed using StatView 5.0 (SAS Institute Inc., Cary, NC) and SPSS 12.0J (SPSS Japan, Tokyo, Japan).

Table 2 List of genes correlated with hepatic total TEQ levels in both male and female Baikal seals

gene name	male		female	
Similar to	R^2	r_c [a]	R^2	r_c [a]
cytochrome P450, family 1, subfamily A, polypeptide 1 [*Pusa sibirica*]	0.693	1.057	0.334	1.016
cytochrome P450, family 1, subfamily A, polypeptide 2 [*Pusa sibirica*]	0.501	0.553	0.333	0.516
cytochrome P450, family 1, subfamily B, polypeptide 1 [*Pusa sibirica*]	0.912	4.159	0.480	3.635
phenol sulfotransferase [*Canis familiaris*]	0.306	0.602	0.481	0.982
Alpha-2-HS-glycoprotein precursor (Fetuin-A) [*Sus scrofa*]	0.316	−0.867	0.277	−0.722
brain protein 44 [*Canis familiaris*]	0.314	−0.424	0.283	−0.353
transmembrane protein 4 [*Canis familiaris*]	0.327	−0.345	0.478	−0.279
FK506 binding protein 11 [*Homo sapiens*]	0.317	0.220	0.402	−0.572

[a] r_c represents the regression coefficient

Results and Discussion

We examined the relationships between total TEQ and gene expression levels in the liver of male and female animals. Various classes of genes were significantly correlated with hepatic DRC levels (Table 1). Out of 2374 genes spotted on the oligo array, 45 and 26 genes had positive correlation with TEQs, and 33 and 24 genes showed negatively correlation in males and females, respectively. Seven genes including CYP1A1, 1A2, 1B1, phenol sulfotransferase, alpha-2-HS-glycoprotein precursor (Fetuin-A), brain protein 44 and transmembrane protein 4 exhibited similar responses in both male and female animals (Table 2). These genes may be useful biomarkers of DRCs irrespective of sex. Expression FK506 binding protein 11, which is involved in protein folding,[10] was up-regulated by DRCs in males, but down-regulated in females. Mouse genome database search exhibited that the upstream regions of these 8 genes contain the core sequence of xenobiotic responsive element.

The statistical analysis showed that genes involved in xenobiotic biodegradation and metabolism may be affected by DRC accumulation (Table 1). This gene class includes CYP isozymes, glutathione S-transferase isozymes and hydroxysteroid (17-beta) dehydrogenase 10.

Gene expression levels of 12 ribosomal proteins were negatively correlated with total TEQ levels in male samples. This result showed that the translation machinery might be affected by DRCs in the liver of male seals.

Inflammatory response is one of the effects observed in the liver and other organs of some experimental animals treated with DRCs.[11, 12] The present study indicated that major histocompatibility complex (MHC) II antigen had positive correlation with total TEQ levels in the liver of male Baikal seals (Figure 1). It is known that induction of this gene is associated with hepatic inflammatory response in mouse.[13] Therefore, this suggests that hepatic inflammation may be induced by DRCs in male Baikal seals.

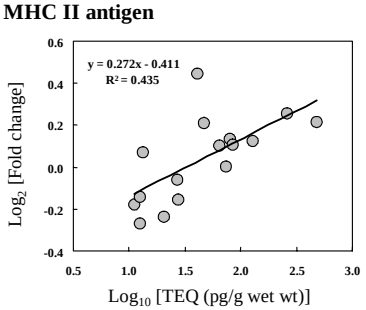

Figure 1. Relationship between hepatic total TEQ and major histocompatibility complex (MHC) II antigen expression levels in male Baikal seals.

In a previous study, we measured CYP1A1, CYP1A2 and CYP1B1 mRNA expression levels by real-time RT-PCR, and indicated the induction of these CYP isozymes by DRCs in the liver of Baikal

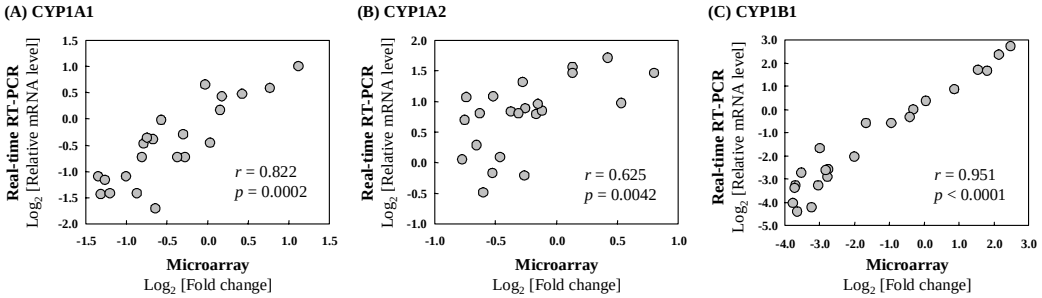

Figure 2. Comparison of gene expression levels quantified by microarray with those by real-time RT-PCR. Data shown here are from expression levels of (A) CYP1A1, (B) CYP1A2 and (C) CYP1B1 in the liver of Baikal seals collected in 1992 (n =22).

seals.[4] To examine whether the alterations in gene expression observed in the previous study are reproducible by the present microarray study, we compared CYP1A1, CYP1A2 and CYP1B1 gene expression levels quantified by microarray and real-time RT-PCR. The results showed significant positive correlations for all CYPs (Figure 2), indicating that our microarray data are quantitatively reliable.

The present study indicated that DRC accumulation affects various classes of genes controlling xenobiotic biodegradation and metabolism, immune system and oxidative phosphorylation in the liver of Baikal seals. Further study is necessary to understand the signaling pathways in which these DRC-responsive genes are involved to link the gene expression profile with the potential toxic effects.

Acknowledgements
The authors wish to thank Professor An. Subramanian, Ehime University, for critical reading of this article. This study was supported by Grants-in-Aid for Scientific Research (A) (Nos. 17208030 and 16201014) and (B) (No. 13480170) from Japan Society for the Promotion of Science. Financial assistance was also provided by ‘‘21st Century COE Program’’ from the Ministry of Education Culture, Sports, Science, and Technology, Japan, and by Feasibility Studies for Basic Research in ExTEND 2005 (Enhanced Tack on Endocrine Disruption) from the Ministry of the Environment, Japan.

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