INSULIN-LIKE GROWTH FACTOR-BINDING PROTEIN 5: A ESTROGEN-RESPONSIVE GENE NEWLY FOUND TO BE MODIFIED BY TCDD EXPOSURE

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Abstract

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) can induce estrogenic action or inhibit estrogen-induced effects in various tissues because of aryl hydrocarbon receptor (AhR)-estrogen receptor (ER) cross talk. In order to identify the biomarkers of TCDD endocrine disruption, we screened estrogen-responsive genes modified by TCDD exposure using specific cDNA microarrays spotted with estrogen-responsive genes. MCF-7 human breast carcinoma cells and RL95-2 human endometrial carcinoma cells were exposed to TCDD, and an analysis of their gene expression revealed 32 genes exhibiting a significant change. The mRNA expression levels of 27 genes were subsequently verified using real-time RT-PCR. Among these genes, bioinformatic analyses indicated that insulin-like growth factor-binding protein 5 (*IGFBP5*) gene expression might be influenced by estrogen status. In our animal experiments, *IGFBP5* was also shown to be responsive to TCDD exposure in mouse fetuses *in utero*. These results suggest that TCDD affects the expression levels of a series of estrogen-responsive genes, and follow-up fetal studies in mice indicated that *IGFBP5* is useful as a biomarker of TCDD activity.

Introduction

The compound 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) elicits a wide spectrum of toxicities, including carcinogenicity, hepatotoxicity, and reproductive and developmental toxic responses[1,2] Recently, TCDD has attracted a great deal of attention as an endocrine disruptor[3]. Many important studies have demonstrated the existence of inhibitory AhR- estrogen receptor (ER) cross-talk in rodent uteri and mammary glands, as well as in human breast cancer cells. Ohtake et al. (2003)[4]showed that the liganded AhR/ARNT heterodimer directly associated with unliganded ER, with the concomitant activation of the p300 coactivator, resulting in activation of the transcription of estrogen-responsive genes. They have also shown that the activation of AhR by dioxins impaired the expression of estrogen-responsive genes when E2 was present. It was suggested that ligand-activated AhR signaling varies depending on the estrogen-liganded status of the ERs. Based on those observations, it could also be speculated that the interaction between TCDD and ER signaling affects expression of a variety of genes. Although it has now been reported that the interactions between estrogen and TCDD affect the gene expression or protein levels of cathepsin-D, pS2, PR, HSP27 and c-fos, these genes cannot explain the mechanisms of the estrogen-mimicking or inhibitory action of TCDD, and other novel biomarkers may hold the key to the effects of TCDD on estrogen-related development. In order to further shed light on the mechanisms of the interactions between estrogen and TCDD as an endocrine disruptor, a more comprehensive identification of the genes/pathways modulated by TCDD is needed.

In the present study, we identified genes displaying sensitivity to TCDD from a series of estrogen-responsive genes in the customized array using both human breast carcinoma cells (MCF-7) and human endometrial carcinoma cells (RL95-2). Furthermore, the expression levels of genes identified in these microarray screens were investigated in mouse fetuses exposed to TCDD *in utero*.

Materials and methods

Cell culture and treatment

The MCF-7 (human breast carcinoma) and RL95-2 (human endometrial carcinoma) cell lines were obtained from the American Type Culture Collection (Rockville, MD). These cells were grown in phenol red-free DMEM/Ham's F12 medium containing 10% charcoal-stripped FBS, and at 80% confluency the cells were treated with either 0.1, 1 or 10 nM TCDD (99.0% purity; 50 μg/ml DMSO solution, Cambridge Isotope Laboratories, Andover, MA) or with 10 nM estrogen (17-beta estradiol, Sigma-Aldrich, St. Louis, MO) in 0.1%

DMSO for 24 h. The control cell cultures were treated with 0.1% DMSO only.

Animal use and treatment

Pregnant C57BL/6J mice (Clea Japan) in the control and dose groups (three dams in each group) were administered either a single oral dose of corn oil (0; vehicle) or 5 μg TCDD/kg, respectively, on GD12.5. On GD18.5, the dams were sacrificed using diethyl ether anesthesia, and 3 male and 3 female fetuses per dam were removed to enable RNA extraction from the liver, brain and calvaria.

cDNA microarray analysis and Quantitative real-time RT-PCR

The mRNA for the microarray preparations was isolated using the QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech, NJ). For cDNA labeling, mRNA were reverse-transcribed in the presence of Cy5-dUTP and Cy3-dUTP (Amersham Pharmacia Biotech). Microarray analysis of gene expression was carried out using custom cDNA microarrays spotted with estrogen-responsive genes (EstrArray, Infogenes Co., Ltd., Tsukuba, Japan) were used[5]. Total RNA for real-time RT-PCR was isolated using ISOGEN (Wako, Osaka, Japan) and RNeasy (QIAGEN, Valencia, CA). The amplification reaction was performed on an ABI PRISM 7000 Sequence Detector (Applied Biosystems). The gene expression levels were calculated based on the threshold cycle (Ct) and normalized relative to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The specificity of each of the PCR products was confirmed by sequencing with gene-specific primers.

Bioinformatic and statistical analysis

Clustering for gene expression was conducted using GeneSpring 7 (Agilent Technologies, Palo Alto, CA). Gene expression data at 10 nM TCDD verified by quantitative RT-PCR were mapped to biological networks using Ingenuity Pathway Analysis Ver. 4 (Ingenuity Systems, Mountain View, CA), which is a tool for finding pathways based on knowledge information. Information for insulin-like growth factor binding protein 5 (*IGFBP5*) expression in human and mouse tissues was obtained from the public database Gene Expression Omnibus (GEO). In the animal studies, deviations in the expression levels of genes relative to control levels were assessed by Student's *t*-test implemented on StatView 5.0 (SAS Institute Inc., Cary, NC).

Results and discussion

The microarray spots that we utilized in this study consisted of 203 cDNAs corresponding to 165 different estrogen-responsive genes selected as reported previously[5,6]. The gene expression profiles displayed a wide variation within the TCDD treatment range of 0.1 to 10 nM in both cell types. Thirty-two genes were detected to have undergone change due to TCDD treatment in both cell lines. In addition to these 32 genes, the *AHR* and *CYP1A1* genes that were used as positive controls in our screen were also induced by TCDD. Nineteen genes in the MCF-7 cells and 22 genes in the RL95-2 cells, including *AHR* and *CYP1A1*, were significantly altered by TCDD at all concentrations in the examined range. The expression levels of the estrogen-responsive genes *VEGF*, *MAN1A1*, *PCYT1A*, *ENPP2*, *GOS2* and *MAP4K5*, commonly altered in both cell lines, were also found to be altered by TCDD exposure. We anticipated that in different cell types the transcriptional mechanism, including activators and repressors, would differ and result in dissimilar TCDD-induced gene expression profiles.

To quantitatively verify the changes in gene expression that were observed in our cDNA microarray experiments, we determined by quantitative RT-PCR the expression levels of the 27 genes responsive to TCDD. These included *CYP1A1*, the expression of which was used as a positive control. We were able to successfully detect the expression levels of 25 genes in the MCF-7 cells and 15 genes in the RL95-2 cells by real-time RT-PCR. Thirteen genes, including *CYP1A1*, showed altered expression levels following TCDD treatment in both the MCF-7 and the RL95-2 cells. These genes may serve as biomarkers of TCDD exposure, since they responded to TCDD treatment in two different cell types.

The values of the verified expressions of 9 genes (*CDH18*, *CTNND2*, *ERBB2*, CXCL12, *IGF1R*, *IGFBP5*, *TGFA*, *NRIP1* and *CYP1A1*) which were altered by TCDD in the MCF-7 and RL95-2 cells were used for pathway analysis. Figures 1 showed the predictive pathways including these 9 genes, based on pathway analysis. In the MCF-7 cells (Fig. 1), the pathway showed that TCDD influences IGF1R signaling mediated by *AHR* and *MYC*, which in turn are affected by *TGFA* or *NRIP1*. Catenin and cadherin, like *CTNND2* and *CDH18*, also seem to be associated with *IGF1R* signaling. In contrast, the RL95-2 cells did not display the same pathways as the MCF-7 cells due to the lack of change in expression of the genes *ERBB2*, *IGF1R*, CXCL12 and *CDH18*, suggesting the existence of another pathway under conditions of different ER level. The pathway analysis also suggests this battery of genes has functions associated with cancer, cellular growth and proliferation, and the cell

cycle. Therefore, we next focused on the application of *IGFBP5* as an indicator of IGF1R signaling in experimental animals. We analyzed *IGFBP5* gene expression in human and mouse tissues using the public dataset. The data for *IGFBP5* were extracted from the public GEO website. The informatic analysis revealed that the expression of *IGFBP5* in the ovary is much higher than in the brain and liver, in both humans and mice. This analysis suggested that *IGFBP5* could be useful for a marker in experimental animals exposed to TCDD.

IGFBP5 was further investigated in the mouse fetus for responsiveness to TCDD, because cellular growth and proliferation in fetus tissues are very active in comparison to adult tissues. The *IGFBP5* gene expression levels in brain, liver and calvaria of fetuses maternally exposed to TCDD were determined. The brain, liver and calvaria were used since they are known to be ER-rich organs. Ovaries could not be used since in fetuses they are too small to extract RNA for quantitative PCR. The expression of *IGFBP5* was significantly suppressed in the liver of both the male and the female fetuses and in the female fetal calvaria, but was elevated in both the male and the female fetal brain (Fig 2).

IGFBP5 has been suggested to play a significant role in the regulation of organ function, including the development of the central nervous system[7,8], the involution of the mammary glands[9] and bone physiology[10.11]. *IGFBP5* has also been known to be a negative regulator of IGF1-induced proliferation of premalignant cells[12]. Breast cancer tissues showed a positive correlation between ER status and IGF receptor status, and also a negative correlation between ER status and IGFBP3, another member of the IGFBP group[13]. These reports suggest that IGFBP5 could be a good marker for TCDD-induced toxicity in ER-positive tissues. We believe that the *IGFBP5* gene will provide important clues to the TCDD-estrogen interaction, since estrogen associates either directly or indirectly with the pathways of this gene in order to respond to TCDD.

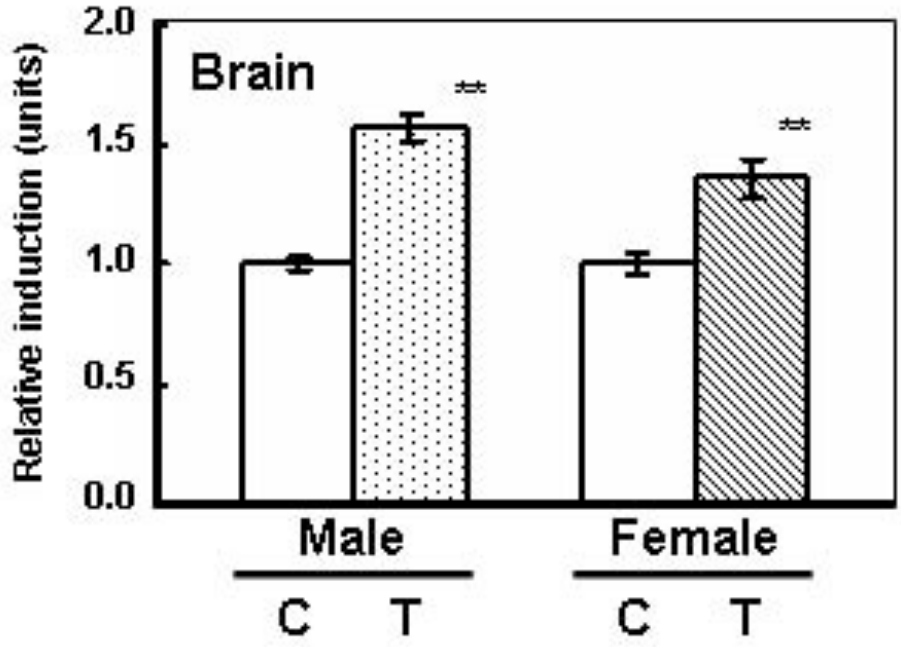

Fig. 1 Application of the identified estrogen-responsive genes induced by TCDD to an animal study model. IGFBP5 is shown here as a typical example. ** at T (TCDD) indicates the difference from the C (control) at $p < 0.01$

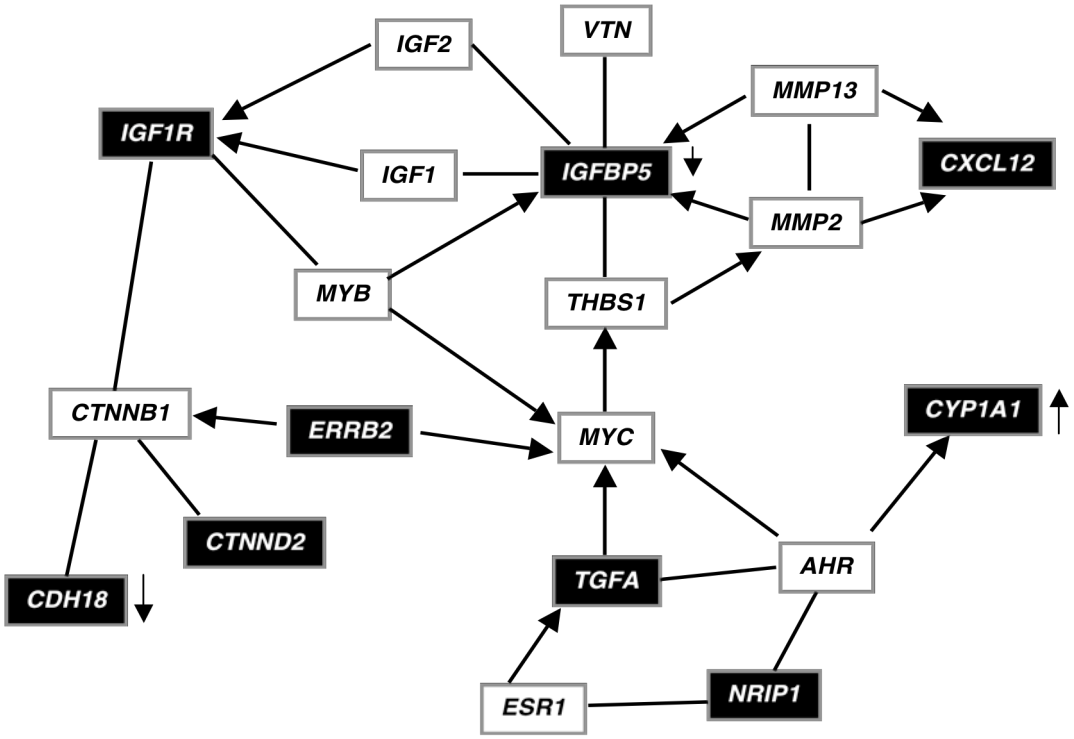

Fig.2 Illustration of the network of the genes verified to be altered by TCDD exposure in MCF-7 cells. The black boxes indicate the verified genes. The plain lines and the line with arrow-heads indicate binding and activating, respectively.

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