Analysis of Responsive Genes of Murine Leydig Cells by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

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(Received December 22, 1998; Accepted January 17, 1999)

We examined the effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on changes in gene expression of murine testicular Leydig TM3 cells by a method of differential mRNA display based on a PCR technique. Although no change in viable cell number was observed by TCDD at the concentration of 10 nM, Ah receptor mRNA was down-regulated and CYP1A1 was induced in TM3 cells. One hundred and sixty-one PCR products, differentially expressed in 2% agarose gel electrophoresis after PCR amplification, were detected. Nine cDNAs were confirmed to be differentially expressed in Northern ELISA analysis. Two cDNAs were cloned and sequenced. One was identical to *Homo sapiens* Nedd-4-like ubiquitin-protein ligase WWP2 (WW domain-containing protein), which is associated with the ubiquitin proteolytic process. TCDD decreased expression of WWP2, which might cause the suppression of proteolytic process. The other was identical to the mouse expressed sequence tag. The function of this gene was not known. These genes might provide information for understanding TCDD-induced toxicity or biological responses.

Key words —— TCDD (dioxin), testis, Leydig cell, reproduction, RAP-PCR, WWP2

INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), the most toxic man-made chemical, is released into the environment as a contaminant of herbicides or as a by-product of bleaching paper pulp, and incineration of waste.[1,2] It causes a diverse spectrum of biochemical and toxic responses in many animal species. Some of these responses include carcinogenesis,[3,4] hepatic toxicity,[5-8] diverse endocrine effects[9-11] and reproductive toxicity.[12] TCDD, when given in overtly toxic doses, adversely affects the male reproductive system in laboratory animals. It decreases testis and accessory sex organ weights, alters testicular morphology, decreases spermatogenesis and plasma androgen concentrations, and inhibits testicular steroidogenesis.[8] Exposure to TCDD reduces the total Leydig cell volume in the testis of rats.[13,14] This is explained by the reduced number and size of individual Leydig cells.[13,14]

TCDD induces the so-called Ah gene battery of drug-metabolizing enzymes which includes CYP1A1 and CYP1A2.[15,16] TCDD-induced biological effects are thought to be induced through the Ah receptors.[17,18] However, induction of neither CYP1A1 nor any other TCDD-inducible genes identified to date has been causally linked to a toxic endopoint of TCDD-exposure. Therefore, we attempted to examine the effect of TCDD on the gene expression of testicular cells. A PCR-based technique termed a differential mRNA display is a unique and powerful tool for the identification of genes expressed differently among various tissues or cell lines.[18] Using a differential mRNA display method, we isolated two genes that were down-regulated by TCDD in murine testicular Leydig TM3 cells.

MATERIALS AND METHODS

Cell Culture —— The mouse testis Leydig cell line TM3 was purchased from American Type Culture Collection. The cells were cultured in DMEM/F12 (GIBCO BRL, New York, U.S.A.) supplemented with 5% horse serum (Nacalai, Kyoto, Japan) and 2.5% fetal bovine serum (JRH Biosciences, Kansas, U.S.A.).

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Cells were maintained at 37℃ in 5% CO_2 atmosphere.

TM3 cells in the exponential growth phase were treated with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) containing 0.25% trypsin and 1mM EDTA to detach them from the surface of the tissue culture dishes. They were plated at a concentration of 3×10^5 cells/ml into tissue culture dishes.

Preparation of TCDD —— Cells were treated with 10 nM TCDD (Wellington Lab, Ontario, Canada) dissolved in dimethyl sulfoxide (DMSO), whereas control cultures received solvent only, not exceeding a final concentration of 0.1%.

Measurement of Viable Cell Number and LDH Release —— The cells were seeded at a density of 3×10^5 cells/well in microtiter plates in 0.1 ml of F12/DMEM medium. After 16 h, the medium was replaced with TCDD which contained medium. After incubation for an additional 1, 3, and 48 hours, the viable cell number was measured using the WST-1 kit (Dojin Lab, Japan) at 450 nm by microplate reader (Bio-Rad, Model 550). LDH activity was measured using the LDH release activity kit (Wako Co., Japan) at 595 nm by microplate reader.

Preparation of Cell Homogenates —— TM3 cells in monolayer were rinsed with PBS and were collected by scraping into 10 ml PBS with a rubber policeman. Total RNA was isolated using NP-40 and phenol extraction as described previously.[19]

Arbitrary Primed PCR Fingerprinting of RNA —— Messenger RNA was obtained using Oligotex-dT30 (Super) (Takara Co., Shiga, Japan) according to the instructions, and cDNA was synthesized using M-MLV reverse transcriptase (GIBCO BRL) with hexadeoxyribonucleotide mixture random primer (Takara Co.) according to the instructions. Second-strand cDNA synthesis was also initiated by arbitrary priming using the 9 denominate deoxyoligonucleotides (10-mer, see Table 1) and 2 randomly chosen species were used as sense and antisense primer. Amplification of cDNA was performed by RNA arbitrarily primed polymerase chain reaction (RAP-PCR) using ASTEC (Program Temp Control System PC-700 ; 45 cycles : 95℃ for 25 s, 32℃ for 1 min, and 72℃ for 30 s and an additional 7-min final extension at 72℃) with two deoxyoligonucleotide primers with a defined, but arbitrary sequence (Table 1). After the PCR amplification, the samples were run on a 2% agarose gel in Tris-acetate-EDTA (TAE) buffer at 50 V for approximately 70 min and stained with ethidium bromide. The amplification cDNA bands that were differentially expressed were located and cut, then the cut gel was incubated in 100 μl Tris-ETDA (TE) buffer (pH 8.0) for several minutes at room temperature. An eluted cDNA (2 μl) was reamplified in 50 μl, using the same set of primers and same PCR conditions. The PCR products were then analyzed on a 2% agarose gel electrophoresis.

Table 1. Primer for RAP-PCR

		Sequence	mer	Tm
1	BS-52	5′-CAAGCGAGGT-3′	10	32
2	BS-54	5′-AACGCGCAAC-3′	10	32
3	BS-55	5′-GTGGAAGCGT-3′	10	32
4	BS-57	5′-GGAAGCAGCT-3′	10	32
5	BS-58	5′-CAGTGAGCGT-3′	10	32
6	BS-70	5′-GAGCTATGGC-3′	10	32
7	BS-73	5′-AGCCTGTGTC-3′	10	32
8	BS-76	5′-CTGGTCACAC-3′	10	32
9	BS-78	5′-CACAGTGAGC-3′	10	32

Northern ELISA —— To prepare each Digoxigenin (DIG) probe used in Northern ELISA, the DNA from the gel pieces was isolated by heating the gel at 65℃ for about 5 min and adding 10 μl of TE buffer (pH 8.0). TE solution containing DNA extract was prepared by PCR, using a PCR DIG probe synthesis kit (Boehringer Mannheim GmbH, Mannheim, Germany) with the same primers as for the first PCR amplification. Northern ELISA analysis was performed using the Northern ELISA kit (Boehringer Mannheim GmbH) according to the manufacturers' instructions.

Cloning and DNA Sequencing —— The reamplified cDNA was purified by a Sephaglas BandPrep Kit (Pharmacia Biotech, U.S.A.) from the 2% gel. The product was cloned into pCR2.1 provided by Invitrogen (San Diego, U.S.A.). For each of the RAP-PCR products, several independently isolated clones were sequenced using the Thermo Sequenase fluorescent-labelled primer cycle sequencing kit (Amersham, Buckinghamshire, U.K.).

Northern Blot Analysis —— The cells were treated with 10 nM TCDD for different incubation times and mRNA was isolated. Six μg of mRNA was run on an 1% agarose-formaldehyde gel and transferred onto a nylon membrane (Boehringer Mannheim GmbH). The membranes were prehybridized, then hybridized according to the manufacturer's protocol. To prepare each DIG probe used in Northern blot analysis, the template DNA from the cloning plasmid was prepared by PCR (PCR Thermal Cycler MP TP3000, Takara Co. ; 30 cycles : 95℃ for 10 s, 60℃ for 30 s, and 72℃ for 2 min, from 11 cycle elongation of 20 s for each cycle, and an additional 7 min extension at 72℃), using the PCR DIG probe synthesis kit. After amplification, the products were analyzed by a 2% agarose

gel electrophoresis. Using the PCR product as a Northern ELISA probe, a specific band was visible after ethidium bromide staining. Washed membranes were detected by CSPD® (Boehringer Mannheim GmbH) chemiluminescence for approximately 30 min at room temperature.

RESULTS AND DISCUSSION

Cytotoxicity of TCDD on TM3 Cells

Dose-dependent effects of TCDD on the viable cell number and the LDH release in the medium of murine testicular Leydig TM3 cells were examined. No particular change in viable cell number or LDH release was observed for 48 h up to the concentration of 30 nM that we examined (Fig. 1).

Expression of CYP1A1 and Ah Receptor mRNAs

Although CYP1A1 was not detected in TM3 cells, it was significantly induced from 1 hour after the addition of 10 nM TCDD (Fig. 2). Expression of CYP1A1 mRNA was increased in proportion to the incubation time. As shown in Fig. 2, Ah receptor mRNA, constitutively expressed in TM3 cells, was down-regulated by TCDD. Expression of Ah receptor was decreased and was undetectable at 3 h; however, it appeared again at 48 h.

Gel Analysis of Differentially Displayed mRNAs from TM3 Cells after TCDD Treatment

A typical differential display is shown in Fig. 3 using TM3 cells following treatment with DMSO or 10 nM TCDD for 1, 3 and 48 h. RNA was extracted from the cells and subjected to the first-strand cDNA synthesis and RAP-PCR. TCDD caused a differential display of a number of genes that were displayed using a different set of primers. A total of 36 combinations using two arbitrary primers was analyzed. One hundred and sixty-one PCR products, differentially expressed in 2% agarose gel electrophoresis after PCR amplification, were detected, and the individual bands were cut out.

Northern ELISA Analysis and Northern Blot Analysis

To confirm the expression patterns observed in the differential display profiles, we detected 9

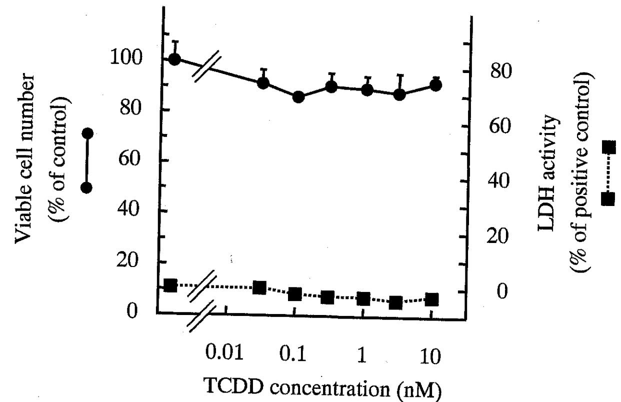

Fig. 1. Dose-dependent Effects of TCDD on Viable Cell Number and LDH Release in TM3 Cells

Cells were cultured with TCDD at the indicated concentrations for 48 h. Viable cell number and LDH release were measured as described in MATERIALS and METHODS. The solid line is the viable cell number and broken line is activity of LDH released in the medium. Each point is the mean±S.D. of three determinations.

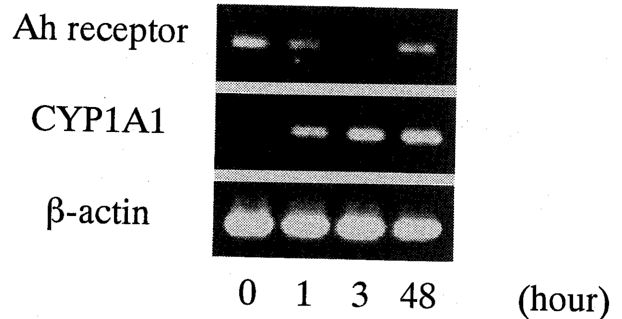

Fig. 2. Expression of Ah Receptor and CYP1A1 mRNAs after Treatment of TCDD in TM3 Cells

mRNA from TM3 cells treated with 10 nM TCDD for the indicated time was extracted for the RT-PCR method. The reaction was run on a 2% agarose gel as described in MATERIALS AND METHODS.

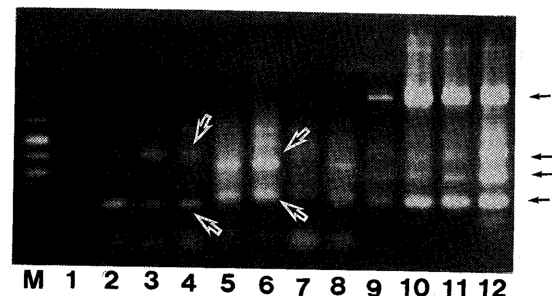

Fig. 3. Gel Analysis of Differentially Displayed mRNA from TM3 Cells after TCDD Treatment

Cells were cultured with 10 nM TCDD for 0, 1, 3 and 48 h, and RNA was extracted. RNA was reverse transcribed using a set of primers. The reaction was run on a 2% gel as described in MATERIALS AND METHODS. The up- and down- primers used in lanes 1—4 were BS52 and BS54, lanes 5—8 were BS52 and BS55, lanes 9—12 were BS52 and BS57. Arrows indicate TCDD-responsive “bands”.

PCR products that were differently expressed in Northern ELISA analysis. These 9 cDNAs were cloned into the plasmid using the TA cloning kit (Invitrogen). To confirm the "correct" expression of mRNA, we examined the expression of the two genes in different TCDD-treatment periods by Northern blot analysis using DIG-PCR probe from cloned cDNA (Fig. 4). Expression of the two cDNA-clones was significantly changed by TCDD treatment. The expression of DL35 mRNA was significantly decreased in 1—48 h, and the expression of DL85 mRNA was also decreased by TCDD treatment.

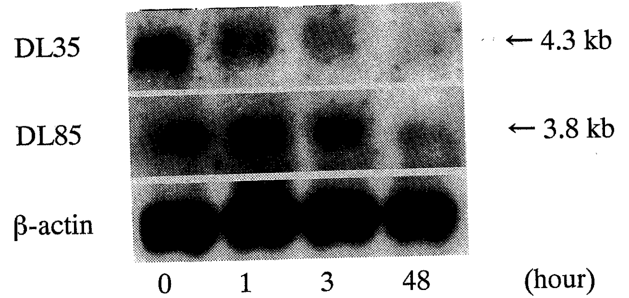

Fig. 4. Time-dependent Northern Blot Analysis of mRNA from TCDD-treated TM3 Cells

The cells were treated with 10 nM TCDD for 1, 3, and 48 h. Messenger RNAs from TM3 cells after TCDD treatment were run on a 1% agarose-formaldehyde gel and transferred to a nylon membrane. The blot was probed with DIG-labelled cDNAs. The calculated size for the DL35 and DL85 was 4.3 and 3.8 kb, respectively.

Cloning and Sequencing of Two cDNAs

These two cDNAs were cloned and the plasmids containing inserts were used for the sequencing. A search among the DNA sequences in the EMBL database (Release 42.0), using the BLAST and FASTA programs, revealed that DL35 was identical to *Homo sapiens* Nedd-4-like ubiquitin-protein ligase WWP2 (WW domain-containing protein) (Fig. 5). WWP2 may be associated with the ubiquitin proteolytic process and/or cell cycle. The WW domain has been reported in a wide variety of proteins of Nedd-4, YAP, Rep5, Dmd, and FE65.[20,21] Although the precise biological function of the WW domain remains undetermined, its presence in diverse proteins involved in signaling, regulatory, and cytoskeletal functions, as well as its rapidly emerging role in signaling mechanisms that underlie several human diseases clearly underscores its importance.[22,23] If the expression of WWP2 were decreased, the proteolytic process of some proteins would be suppressed.

DL85 was identical to the sequence of DNA fragments listed in the mouse expressed sequence tag (EST). DL85 was identical to mouse EST (Fig. 6). A search resulted in the finding that, the sequence of this gene in the protein database, using the Smith-Waterman algorithm DL85, was identical to *Caenorhabditis elegans* cosmid T03F6.[24] *C. elegans* cosmid T03F6 belongs to the DnaJ domain protein superfamily. In this DnaJ superfamily, there are many genes such as mouse CSP,[25] *Homo sapiens* heat shock protein hsp40,[26] and heat shock protein - *Helicobacter pylori*.[27] DL85, which has the DnaJ motif II (Fig. 7), may be a new member of the DnaJ superfamily.

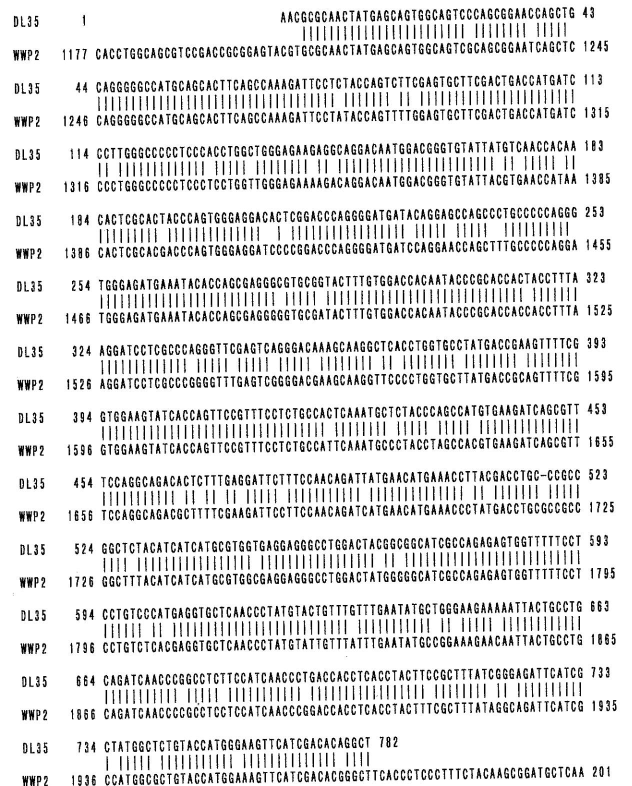

Fig. 5. Alignment of the DL35 and *Homo sapiens* Nedd-4-like Ubiquitin-protein Ligase WWP2 mRNA

Identity is indicated by vertical lines (|). The DL35 shares with WWP2 mRNA a coding nucleotide sequence homology of 90%.

This study shows that the differential mRNA display RAP-PCR method is a powerful tool for the temporal and spatial analysis of differential gene expression, and is able to provide information on the overall patterns of mRNA expression under different exposure conditions. Seven other genes are currently now under sequencing and characterization in our laboratory.

Acknowledgements This work was support-

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DL85  120 AATATCAAGATGACAGCTTAGATCTGCTTCATTATTTCAC 159
                                    ||||||||||
EST     1                           ATTATTTCAC 10

DL85  160 GGTTACCTGTTATTCTGGTTATGGGGACGATGAAAGGGGG 199
          ||||||||||||||||||||||||||||||||||||||||
EST    11 GGTTACCTGTTATTCTGGTTATGGGGACGATGAAAGGGGG 60

DL85  200 TTCTATGCAGTGTATCGTGTTGTCTTTGAGCTGATTGCAA 239
          ||||||||||||||||||||||||||||||||||||||||
EST    61 TTCTATGCAGTGTATCGTGTTGTCTTTGAGCTGATTGCAA 100

DL85  240 AAGAAGAACTGGAGTGTATGTCAGAGGGAGATGTTGAGGA 279
          ||||||||||||||||||||||||||||||||||||||||
EST   101 AAGAAGAACTGGAGTGTATGTCAGAGGGAGATGTTGAGGA 140

DL85  280 TTTTCCAACCTTCGGCGACTCCCAGAGCGACTATGACATG 319
          |||||||||||||||||||||||||||||||||||||| |
EST   141 TTTTCCAACCTTCGGCGACTCCCAGAGCGACTATGACACG 180

DL85  320 GTGGTCCACCCTTTCTACGCTCACTG 347
          ||||||||||||||||||||||||||
EST   181 GTGGTCCACCCTTTCTACGCTCACTGGCAGAGTTTCTGCA 210
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Fig. 6. Alignment of the DL85 and Mouse Expressed Sequence Tag (EST)

Identity is indicated by vertical lines (|). The DL85 shares with EST a coding nucleotide sequence homology of 99%.

ed in part by a Grant-in Aid for Promotion System for Intellectual Infrastructure of Research and Development, Special Coordination Funds for Promoting Science and Technology from Science and Technology Agency.

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Gene/Species	Position	DnaJ motif II Sequence	Accession
T03F6/C.elegans	84	ECTQQ**F**RLLQA**AY**DV**LSD**PRE**R**EF**YD**RHRESI	Z81113
CSP/Mouse	42	EAADK**F**KEINN**A**HAI**LTD**ATK**R**NI**YD**KYGSLG	P54101
DNJ2/Rat	39	NEGEK**F**KQISQ**AY**EV**LAD**SKK**R**EL**YD**KGGEQA	P54102
ZRF1/Mouse	133	GDNDY**F**TCITK**AY**EM**LSD**PVK**R**RAFNSVDPTF	P54103
DnaJ/E.Col.	42	EAEAK**F**KEIKE**AY**EV**LTD**SQK**R**AA**YD**QYGHAA	P08622
DnaJ/Caucr	39	NAAGR**F**KEINE**AY**SV**LSD**SQK**R**AA**YD**RFGHAA	P22305
DNJ1/Human	39	GAEEK**F**KEIAE**AY**DV**LSD**PRK**R**EIF**D**RYGEEG	P25685
CAJ1/Yeast	43	DAQAK**F**QAVGE**AY**QV**LSD**PGL**R**SK**YD**QFGKED	P39101
HSP40-3/Human	40	NAEEK**F**KEIAE**AY**DV**LSD**PKK**R**GL**YD**QYGEEG	AF088982
HSP/H.pylori	41	EAEEK**F**KLINE**AY**GV**LSD**EKK**R**A **YD**RYGKKG	D64686
DL85		EAAEQ**F**KLIQA**AY**DV**LSD**PQE**R**AW**YD**NHREAL	
Consensus		**F** **AY** **L D** **R** **YD**	

Fig. 7. Deduced Amino Acid Sequence and Alignment of DnaJ Motif II

The name and DnaJ motif II of representative DnaJ motif II sequences are shown. Amino acids conserved in >75% of the sequences are shaded. The consensus shows the conserved amino acids.

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