EFFECTS OF TCDD ON THE GENE EXPRESSION IN MOUSE EMBRYONIC STEM CELLS IN CULTURE

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Introduction

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a potent teratogen in several animal species, especially during the period of organogenesis[1]. In addition, maternal TCDD exposure can lead to early pre- and post-implantation embryo loss[1]. Studies indicate that mouse pre-implantation embryos express aryl hydrocarbon receptor (AhR)[2] and TCDD accelerates the differentiation of mouse pre-implantation embryos *in vitro*[3]. Embryonic stem (ES) cells are derived from the inner cell mass (ICM) of the early blastocyst and have the potential to form all embryonic cell lineages[4]. ES cells can differentiate into various cell types within cell aggregated called embryoid bodies (EBs) and this structure consists of ectodermal, mesodermal, and endodermal tissues, which resemble the embryo of egg-cylinder stage[5]. Therefore, it provides an excellent system with which to study the molecular events involved in lineage determination and differentiation. We have utilized this system to study the effects of TCDD on the early-stage embryogenesis. To this end, RT-PCR analysis using molecular markers associated with mouse embryogenesis and c-DNA microarrays analysis, which allows for the study of expression patterns of a large number of genes simultaneously, were conducted.

Methods and Materials

The mouse ES cell lines, E14-TG2a (ATCC) was adopted, because, they can grow in the presence of leukemia-inhibitory factor (LIF) without feeder cells. Undifferentiated ES cells were maintained on gelatinized flasks in Dulbecco's modified Eagle medium supplemented with LIF (1,000 units/ml), 15% fetal calf serum, 1 X non-essential amino acid, 0.8mg/ml adenosine, 0.85mg/ml guanosine, 0.73mg/ml cytidine, 0.73mg/ml uridine, 0.24mg/ml thymidine, 0.1mM beta-mercaptoethanol, 50 units/ml penicillin, 50 μ g/ml streptomycin. For differentiation experiment, ES cells were trypsinized and cultured for 4, 6 and 9 days in suspension condition without LIF. TCDD (10nM) was added into the medium during the suspension culture period. Total RNA for RT-PCR was prepared from the cells with Isogen (Nippon Gene Co. Japan) according to the manufactures recommendations. First strand cDNA was generated by reverse-transcribing total RNA using oligo (dT) as primer. RT-PCR was conducted on mesodermal markers, Brachyury (Forward : TCC AGG TGC TAT ATA TTG CC, Reverse: TGC TGC CTG TGA GTC ATA AC), and FLK-1 (Forward : CAC CTG GCA CTC TCC ACC TTC, Reverse: GAT TTC ATC CCA CTA CCG AAA G), NF-120 (marker of neuron, Forward : ACG GCG CTG AAG GAG ATC, Reverse: GTC CAG GGC CAT CTT GAC) , HNF-4 (marker of visceral endoderm, Forward : CTT CCT TCT TCA TGC CAG, Reverse: ACA CGT CCC CAT CTG AAG), GATA-4 (transcription factor, Forward : TAA CTC CAG CAA TGC CAC TAG C, Reverse: CTG ATT ACG CGG TGA TTA TGT C), G3PDH (house keeping gene, Forward : ACC ACA GTC CAT GCC ATC AC, Reverse: TCC ACC ACC CTG TTG CTG TA, AhR (Forward :

CCT CAC AGT TCT GGT ATC CTG , Reverse: CAA GTT CCT GAA AAC CAA AGT C) , CYP1A1 (Forward : CCA GGA TGC TCA CCA GAC CAG, Reverse: ATG TAG GGT GAA CAG AGG TGC) .

For c-DNA microarray analysis, total RNA (20 micro gram, 4-days-culture) was prepared with RNeasy (QIAGEN K.K. Japan) and was fluorescently labeled with Atlas Glass Fluorescent Labeling Kit (CLONTECH Laboratories, Inc.). The probes were hybridized to glass microarray (Atlas Glass Mouse 1.0 Microarray, CLONTECH Laboratories, Inc.). The fluorescent signals were detected with Gene Pix 4000 microarray scanner (Axon Instruments, Inc.USA).

Results and Discussio

The results of RT-PCR are shown in Fig.1. TCDD increased the both expressions of Brachyury (days 4 and 6) and FLK-1 (day 6). At day 9, the increase was not evident. Brachyury is a transcription factor in T-box genes family and expressed in early mesoderm[6]. Recently, retinoic acid is reported to inhibit the expression of Brachyury in mouse embryonic stem cells[7]. These results suggest that TCDD may be antagonistic to the retinoic acid-signaling in ES cells. FLK-1 is a receptor of VEGF (vascular endothelial growth factor) and expressed in yolk sac hemangioblasts and endothelial cells[8]. Although the species is different, TCDD is known to induce apoptosis in medaka (fish) yolk sac vein[9]. Increase of FLT1, another VEGF receptor, is also reported in TCDD treated human hepatoma cells[10]. In addition, AhR null mouse showed the developmental defect in vasculogenesis[11]. These results suggest that the genes involved in vasculogenesis may be common target for TCDD. Thus, TCDD seems to exert pro-mesodermal activity on mouse embryonic stem cells in culture. Increase of GATA-4 expression was observed at day 6 but not days 4 and 9. Enhanced cardiogenesis was reported in ES cells which overexpressed the GATA-4[12]. In chick, cardiac hypertrophy and septal defects were observed following TCDD exposure[13]. However, GATA-4 is expressed not only heart but also other tissue such as primitive endoderm and gonad[14]. Therefore, *in vivo* study is required to identify the target organ which shows the increase of GATA-4 by TCDD treatment. AhR expression was confirmed in both control and TCDD treated embryoid bodies at day 4. However, these expressions were down-regulated at days 6 and 9. Similarly, Cyp1A1, a down-stream gene of Ahr-Arnt pathway, was also down-regulated at days 6 and 9. At day 4, no clear increase was observed by TCDD treatment. Although expression of AhR protein might be still kept at day 6, the results suggest that the all of observed changes in this experiment at days 6 and 9 would be secondary effects of TCDD. TCDD did not affect the NF120 (neurofilament-120, marker of neuron) and HNF-4 (hepatocyte nuclear factor-4, marker of visceral endoderm) expressions. The results suggest that these tissues might be less sensitive than mesodermal tissue against TCDD treatment.

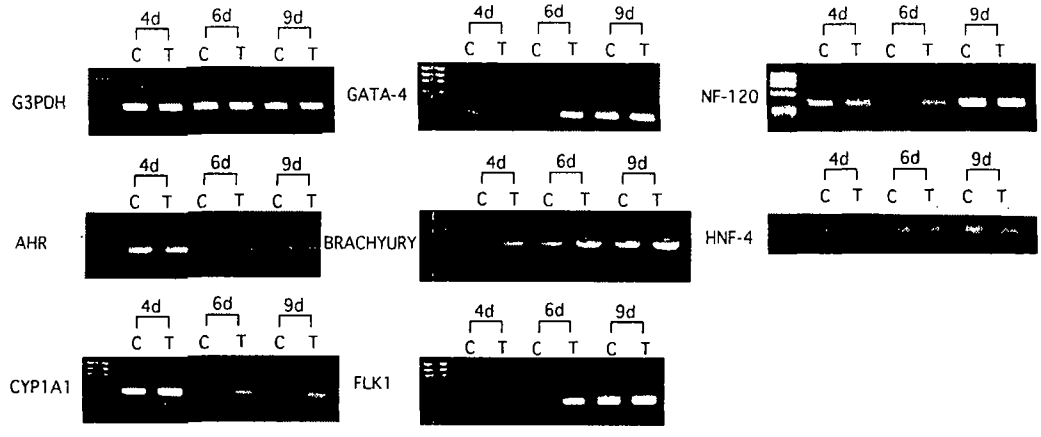

Fig.1 Effects of TCDD on the differentiation of mouse ES cells (RT-PCR)

In c-DNA microarrays analysis, nearly 50% of the genes are expressed in EBs. We find that exposure to 10 nM TCDD for 4days showed that 6 genes are increased and 4 genes are decreased by a factor of 2.0-fold or greater in the 1081 genes. In the changed 10 genes, most strong changes were observed in GATA-4 (2.75 fold up) and Wnt-3a (2.69 fold up) genes. In the genes, GATA-4 is a noticeable, because, we have already shown the increase of GATA-4 by RT-PCR analysis (Fig.1). It has been reported that Wnt-3a encodes a signal that is expressed in the primitive streak of the gastrulating mouse embryo and is required for paraxial mesoderm development and Brachyury is a direct target of Wnt-3a during paraxial mesoderm specification[15]. We have shown that TCDD slightly increased the Brachyury expression (Fig.1). One possible explanation is that TCDD increased Wnt-3a expression and lead to the Brachyury induction. Concerning the other gene, the biological significance is not clear at this time. Further study is required to clarify the biological and toxicological significance of these expression change of genes. And the effort would shed light on the multiple biological effects of TCDD.
Taken together, our findings indicate that ES cells are a suitable *in vitro* model system to study the effect of TCDD on gene expression in early development and differentiation.

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