EFFECTS OF 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN IN THE NEURONAL DIFFERENTIATION SYSTEM USING HUMAN EMBRYONIC STEM CELLS

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Introduction

It has been known that exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) during the fetal period even by a single dose of 50 ng/kg exerts irreversible reproductive effects in rats (Ohsako et al., 2001). Fetal exposure to TCDD has been shown to affect the advanced brain functions in rodents. Aryl hydrocarbon receptor (Ahr), a TCDD receptor, is present in the brain of mammals and the abnormality of the space learning and central nervous system cells are not found in the *Ahr*-nul mice (Heys et al., 2002, Collins et al., 2008, Powers et al., 2005, Mitsuhashi et al., 2010), indicating that Ahr is a key molecule for developmental neuronal disorder induced by dioxin exposure. Neurological effects resulted from short-term TCDD exposure in the fetal period can be important indices for human health risk assessment and need to be studied.

In this study, to investigate the effects of TCDD exposure during embryonic and fetal stage on human neuronal cell differentiation, we exposed TCDD in the specific short term for the developmental process using neuronal cell differentiation culture system using the human embryonic stem cells (hESCs).

Materials and methods

Human ES cell culture and neuronal differentiation. An hESC line, KhES-1, was provided by Kyoto University, and hESCs were subcultured on mouse embryonic fibroblasts (MEF) as feeder cells in the DMEM/F12 medium, containing 20% KSR, 100 µM NEAA, 2 mM L-glutamine, 100 µM 2-ME, and 5 ng/ml bFGF according to our recent paper (He et al., 2012). After several passages with additional MEFs, the MEFs were eliminated by a brief enzymatic treatment. The hESC colonies left on the dishes were harvested. For the formation of the embryoid body (EB), hESCs (purity > 99%) were seeded at 9×10^3 cells/well of SUMILON PrimeSurface 96 U plate (Sumitomo Bakelite Co., Tokyo, Japan) in the DMEM/F12 medium, containing, 20% KSR, 100 µM NEAA, 2 mM L-glutamine, 100 µM 2-ME, and 10 µM ROCK inhibitor Y-27632 (Day 0) (Fig. 1). The generated EBs were cultured for 9 days followed by growth in the medium without Y-27632 for two days. The growing EBs were cultured for 2 additional days in neuronal induction medium (NIM), DMEM/F12: Neurobasal Medium (1:1), containing, 1X N-2 Supplement, 1X B-27 Supplement, 1x GlutaMAX-I, 1X Penicillin-Streptomycin (P/S) to promote neuronal differentiation. Then, EBs were replated on ornithine/laminin-coated-plates at 20 EBs/well. They were cultured for 7 days in neuronal proliferation medium (NPM), DMEM/F12: Neurobasal Medium (1:1), containing, 2X N-2 Supplement, 2X B-27 Supplement, 1X GlutaMAX-I, P/S, and 20 ng/ml bFGF. NPM medium was exchanged every 3 days until Day 40.

TCDD treatment. TCDD (0.1 nM-10.0 nM) exposure was performed for 24 hours from Day 0, Day 9 and Day 35 (Fig. 1).

Quantitative RT-PCR. Total RNAs from the hESCs (Day1) or hESC derivatives (Day10, Day 28, Day36 and Day 40) were harvested with an RNeasy mini kit (Q□AGEN, Hilden, Germany). The mRNA expressions of *Pou5f1, Mtap2, Cyp1a1, Cyp1b1, Ahr, Actb, Mtap2, Gfap, Nes, Th, Sox17, Foxa2, Pdgfr*, and *Kdr* were measured using a high-throughput real-time thermal cycler Light Cycler 480 system (Roche Diagnostics GmbH, Mannheim, Germany).

Immunocytochemistry. The degree of neuronal differentiation was monitored using MAP2 (dendrite marker) monoclonal mouse antibody and TH (dopamine neuron marker) polyclonal rabbit antibody as the first antibody with Alexa 568- and Alexa 488-labeled second antibodies, respectively. The nucleus was stained with Hoechst 33342.

Results and discussion

Ahr mRNA abundance was detected in hESCs, even though it was lower than that found in EBs and matured neuronal cells (Day 36 cells). The induction of Cyp1a1, a biomarker of dioxin exposure, was found only in EB-stage exposure (Day 9). Without TCDD treatment, its basal level was below the detection limit. No induced levels of Cyp1a1 mRNA in Day 1 sample and the matured neuronal cells (Day 36) were observed. Furthermore, no Cyp1b1 induction was observed in all three stages (Day 1, Day 10, and Day 36, albeit a remarkably high basal level. After being cultured until Day 40, using Mtap2 (neuronal marker) increment as a marker, neuronal differentiation cells was found. During differentiation cultures, it was found that a formation rate of neural rosette, the structure synonymous with the neural tube of embryo, was increased at Day 25 in the Day 9 groups exposed to 1nM and 10 nM TCDD, compared with DMSO control groups. Obtaining RNA from culture at Day 28, the levels of neuronal cell markers and the germ layer markers were measured. Among these markers, we found that the neuronal cell markers Mtap2 and Gfap are increased in the Day 9 group exposed to 1nM and 10 nM TCDD. In contrast, levels of endoderm markers (Sox17 and Foxa2) and the mesoderm markers (Pdgfr and Kdr) were reduced in the TCDD-exposed Day 9 groups.

These results showed that sensitivity of EB to TCDD is higher than other stages, ie., ESCs and matured Day 35 cells. Because of this high sensitivity, it is speculated that endodermal and mesodermal cell differentiations are inhibited, and that a differentiation rate of ectoderm cells is increased.

Fetal exposure to TCDD in rats and mice was shown to induce various effects on brain development. That is, the function of the GABAergic neurons of preoptic area was shown to be damaged in the rat (Heys et al., 2002), and maturation of the granule neuron precursors was reported to be disturbed in the mouse cerebellum (Collins et al., 2008). It has been shown that Ahr is a primary target molecule because the decrease of the spacial learning ability and abnormality of hippocampal nervous system cells were not found in the knockout mouse of the Ahr (Powers et al., 2005). Also, it is known to produce neocorotical dysgenesis through activation of p27Kip1 by TCDD-liganed Ahr in the murine fetal period (Mitushashi et al., 2010).

Human neuronal progenitor cells were reported not to respond to TCDD due to a lack of functional Ahr (Gassmann et al., 2010) whereas growth arrest from G1 to S phase was shown to occur based on the experiment in which p27Kip1 and cyclinD1 were affected by TCDD in human neuronal progenitor cells (Latchney et al., 2011). Although it is still uncertain whether environmental exposures to dioxins affects brain functions in humans, the present study is the first to show that TCDD affects the EB period equivalent to a very early stage of human embryos.

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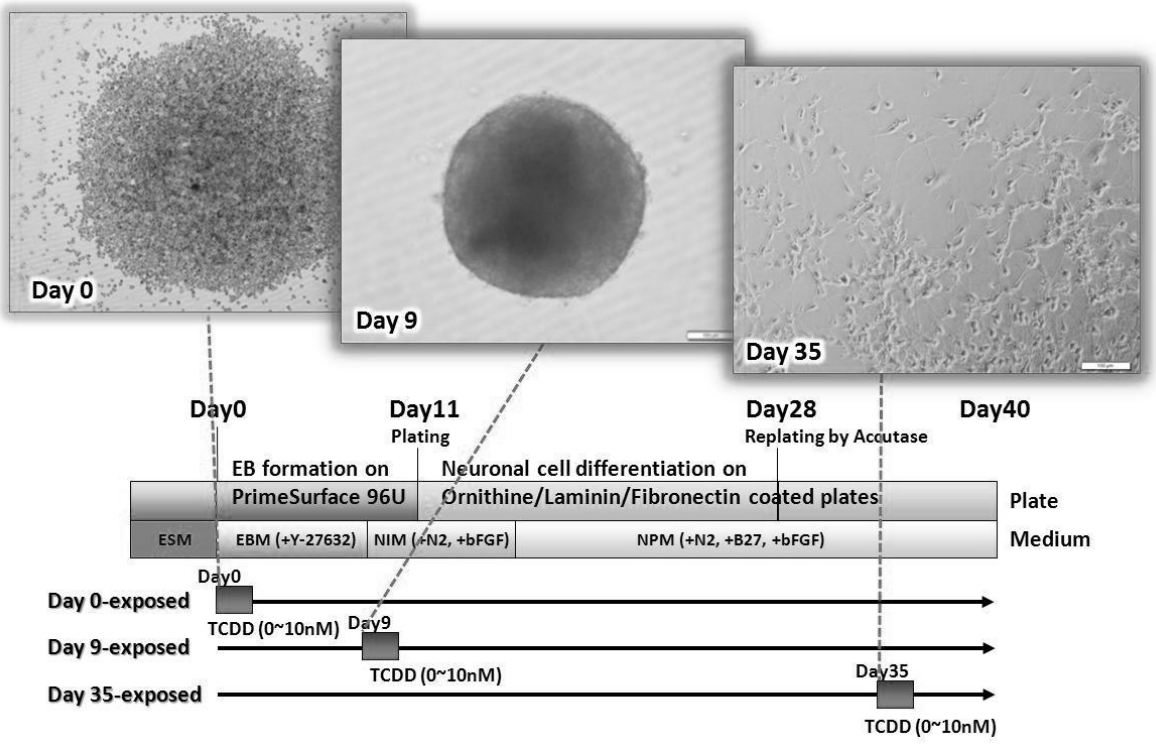

Figure 1.Culture schedule and TCDD exposure.

Human ES cells, KhES-1,were amplified by co-cultivation with MEF (culture medium ES culture media (ESM)). After confirming a sufficient degree of clamp formation, hESCs were separated from MEF and then purified to a single kind of KhES-1cells. On Day 0, the hESCs were plated on SUMILON PrimeSurface 96 U that was an EB formation device. EB culture media was added Rock inhibitor Y-27632 for culture medium to Day 8 and NIM from Day 9. On Day 11 the EBs were disseminated on ornithine/laminin/fibronectin coated plates with NIM. From Day 15 the media was changed to NPM. The maturated neuronal cells were detached with Accutase and then disseminated again on Day 28. Cells were exposed to TCDD (0, 0.1, 1, 10 nM) for 24 hours from Day 0, Day 9, and Day 35.