Effects of Dioxin and OH-PCB on Neural Differentiation from Mouse ES cells

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Abstract

Development of the brain in the neonatal period is susceptible to maternal exposure to environmental chemicals, such as dioxins and polychlorinated biphenyls (PCBs), but the toxicity mechanisms are largely unknown. In this study, we investigated effects of these chemicals on neurogenesis by using *in vitro* system for the differentiation from mouse embryonic stem (ES) cell to neural cell line. Neural differentiation form embryoid body (EB) is known to be induced by retinoic acid. During this differentiation period, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 4-hydroxylated-2',3,3',4',5,5'-hexa chlorinated biphenyl (OH-PCB) were added into the cell culture medium. The differentiation process from ES cells to neural lineage cells was monitored by real-time RT-PCR at several differentiating points using respective neural cell markers. The expression of oligodendrocyte-specific markers, olig1 and olig2, decreased by TCDD and OH-PCB, while that of neuron-specific marker MAP2 and astrocyte-specific marker GFAP did not, suggesting that TCDD and OH-PCB suppress oligodendrocyte differentiation form EB. Moreover, high-throughput multi-channel imaging analysis was used to investigate morphological changes in oligodendrocytes and neurons. Among several morphological parameters, the neurite extension of MAP2-positive neuron was suppressed by exposure of TCDD, but no changes were detected in that of oligodendrocyte-specific marker positive cells. These results suggest that TCDD and OH-PCB may perturb differentiation of several neural cells during the embryonic stage.

Introduction

Environmental chemicals, such as TCDD and PCBs, are ubiquitous and persistent environmental contaminants that exert developmental toxicities. Maternal exposure to these compounds affect fetal and early postnatal developing brain via the placenta and breast milk[1], even if the exposure level is too low to induce maternal toxicities[2]. Exposure to PCBs *in utero* was suggested to induce intellectual impairment in children born to mothers who consumed excessive amounts of sporting fish obtained from the Great Lake area in the USA. Disruption of cognitive development among children exposed to TCDD and PCBs has been documented in accidental human exposure, such as in the Yusho and Yuchen incidences, and was proven by experimental animal studies. In addition, exposure to PCBs may alter dendritogenesis in several

brain regions during development[3]. The above-described incidences suggest that effects of TCDD and PCBs on brain development have been mediated not only by arylhydrocarbon receptor-mediated action but also by other signal transduction pathways.

ES cells have the self-renewing capacity to be an unlimited source for the stem cell therapy. Under a specific condition, ES cells can differentiate into certain types of neural cells including neurons, oligodendrocytes and astrocytes. *In vitro* differentiation of ES cells can be carried out in serum-supplemented and LIF-free medium, in which ES cells aggregate into embryoid body (EB). EB-formation enhances the efficiency of ES cell conversion to neural phenotype by adding retinoic acid[4]. The end-products of differentiation by this method are heterogeneous, but this differentiation process is considered to reflect the neural development *in vivo*[5].

To investigate the effects of TCDD and OH-PCB and define a critical window of toxicities during differentiation and development of the neural cells, we employed an *in vitro* system for the differentiation from mouse ES cells to the neural cell lineage. In this study, we harvested differentiated cells at several time points in the differentiation process and investigated changes of gene expression induced by exposure of TCDD and OH-PCB on neural cell differentiation. Moreover, the cellular morphology of differentiated cells was also analyzed with antibodies specific to each type of cells by a multi-channel imaging system.

Materials and Methods

ES cells culture Mouse embryonic stem cells, B6G-2 cells, were cultured with ES medium containing phenolred-free Dulbeco's modified Eagle medium (DMEM; Invitrogen, USA), leukemia inhibitory factor (LIF) (Invitrogen), 15% fetal bovine serum (CHEMICON, USA), non-essential amino acids mixture (Invitrogen) and maintained on gelatinized (0.1%) plates with MEF. Before starting EB formation, the cultures were passaged on feeder-free at least 5 times.

EB formation and differentiation to neural cells The formation of EBs were carried out in the EB medium (DMEM, 15% knockout serum replacement (Invitrogen), non-essential amino acids mixture) with a microsphere array (STEM Biomethod, Japan) that has non-adherent-coated 1020 wells. This microsphere array allowed ES cells to form EBs with a similar diameter (300 μm). ES cells (10^5 cells/array) were applied onto a microsphere array (day 0) and cultured for 8 days, followed by the culture with a fresh medium containing 10^{-8} M all-trance retinoic acid (at-RA) (Sigma-Aldrich, USA). On day 9, the formed EBs were transferred to ornithine/laminin-coated dishes and cultured with differentiation medium (DMEM/F12 (Invitrogen), N2-supplement (Invitorogen), 10ng/ml bFGF (Invitrogen)).

Chemical exposures EBs were exposed to either TCDD (10 nM, Cambridge Isotope Laboratory, USA) or 4-hydroxylated-2',3,3',4',5,5'-hexa chlorinated biphenyl (10 nM, AccuStandard Inc., USA) just after transferring ornithine/laminin-coated dishes and then the exposure was continued until the end of culture.

Real-time RT-PCR Total RNA was extracted from harvested cells and the expression levels of several mRNAs (Nanog, Oct4, Nestin, MAP2, GFAP. Olig1 and Olig2) were analyzed by Light Cycler (Roche Diagnostics Co., Switzerland) using SYBR Green (TAKARA BIO Inc., Japan).

Immunocytochemistry and morphological analysis Cultures on 24-well plates were fixed with 4% paraformardehyde, and/or permeabilization by 0.1% Triton-X 100 solution. Primary antibodies with the appropriate dilutions were applied and incubated overnight. After washing, cells were incubated with ALEXA568-labeled secondary antibody (Invitrogen). Several morphological parameters, e.g., neurite length and neurite branching points, of positively stained cells were analyzed by IN CELL Analyzer 1000 (GE Healthcare Inc., UK).

Results and Discussions

In this study, we used RA-induced neural cell differentiation system using mouse ES cells, which develops into three major cell types, neuron, astrocyte and oligodendrocyte. In general, it is difficult to standardize the differentiation levels and ratio of each cell type because this differentiation system seems to be sensitive for cell numbers and EB size. Therefore, we utilized the microsphere array that can produce EBs with a similar size. To confirm the neural cell differentiation levels, we harvested the differentiating cells at three different stages, (1) just before EB formation (day0), (2) two days after transferring onto ornithine/laminin plate (day10), and (3) the end of culture (day29) (Fig.1). Expression levels of the following marker genes were measured by real-time RT-PCR; Nanog and Oct4 (ES cell markers), nestin (neural stem cell markers), MAP2 (neural cell marker), GFAP (astrocyte marker), and Olig1 (oligodendrocyte marker). During this differentiation process, cells were exposed to TCDD or OH-PCB..

The expression of all the markers were not affected on day 0 and day10 by exposure of TCDD and OH-PCB. On day29, the mRNA levels of olig1 and olig2 that are transcriptional factors for early differentiation and maturation of oligodendrocyte significantly decreased by TCDD or OH-PCB exposure. However, neither of MAP2 and GFAP mRNA levels were not changed during the same period. From these results, we hypothesized that both TCDD and OH-PCB might affect the development of oligodendrocytes from EBs.

Next, we carried out immunocytochemistry and measured several morphological parameters by using the high-throughput multi-channel imaging system. The exposure to TCDD or OH-PCB changed the morphology of MAP2-positive neurons, but not the number of olig1-positive origodendrocytes Especially the neurite length, crossing point of each neurite, and branching point on MAP2-positive cells decreased (Fig. 2). These results suggest that exposure to TCDD or OH-PCB during the neural cell differentiation disrupts their normal development of specific neural cell-type or architecture of the neural cells.

In the present study, we showed that TCDD and OH-PCB affect the expression of transcriptional factors

for oligodendrocyte maturation and suppressed the neurite extension of MAP2-positive neurons. Kimura-Kuroda *et al.* (2005) also reported that OH-PCB suppress dendritogenesis of primary-cultured neural glanuelar cells[3].

Our results suggest that the method of differentiation from ES cells to neural cells could be a new tool to investigate the effects of TCDD and OH-PCBs on neural and brain development.

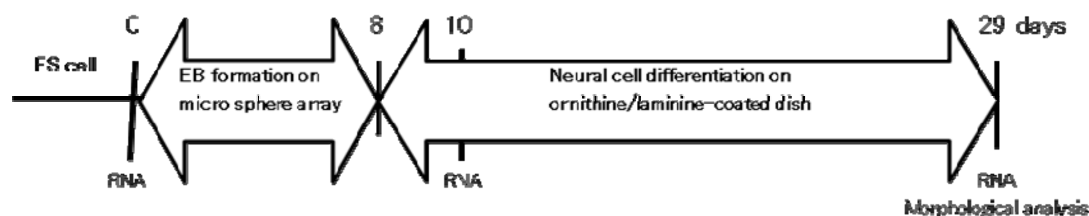

Figure 1. Scheme of sampling during neural differentiation from mouse ES cells

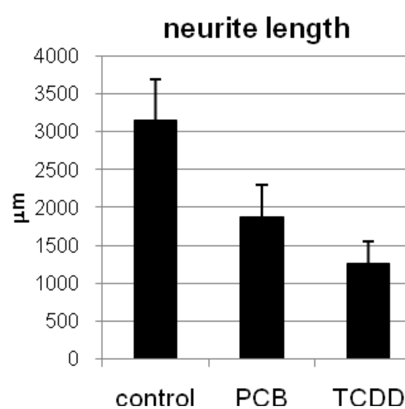

Figure 2. TCDD and OH-PCB suppressed neurite length.

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