Toxicology in Vitro 29 (2015) 876–883

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Inhibitory effects on osteoblast differentiation *in vitro* by the polychlorinated biphenyl mixture Aroclor 1254 are mainly associated with the dioxin-like constituents

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ARTICLE INFO

Article history:
Received 7 July 2014
Accepted 1 March 2015
Available online 17 March 2015

Keywords:
Osteoblast
TCDD
Aroclor 1254
Aryl hydrocarbon receptor
Toxic equivalency factors
Mixture toxicity

ABSTRACT

The polychlorinated biphenyl (PCB) mixture Aroclor 1254 alters bone tissue properties. However, the mechanisms responsible for the observed effects have not yet been clarified. This study compared the effect of Aroclor 1254 on the expression of osteoblast differentiation markers in MC3T3-E1 cells with the corresponding effect of the dioxin reference compound 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and two PCB congeners belonging to the category of non-dioxin-like PCBs. The aim of the study was to quantify the relative influence of dioxin-like and non-dioxin-like PCB-components on osteoblast differentiation. Expression of marker genes for AhR activity and osteoblast differentiation were analyzed, and relative potency (REP) values were derived from Benchmark concentration-effect curves. Expression of alkaline phosphatase and osteocalcin were decreased by both Aroclor 1254 and TCDD exposure, while the PCB-congeners PCB19 and PCB52 slightly induced the expression. The relative potency of Aroclor 1254 for inhibitory effects on osteoblast differentiation marker genes was within the expected range as estimated from the chemical composition of Aroclor 1254. These results are consistent with previously observed bone modulations following *in vivo* exposure to Aroclor 1254 and TCDD, and demonstrate that the inhibitory effects of Aroclor 1254 on osteoblast differentiation by the dioxin-like constituents are over-riding the contribution of non-dioxin-like PCBs.

1. Introduction

Experimental studies have demonstrated that exposure to the potent aryl hydrocarbon receptor (AhR) ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) affects bone geometry, densitometry and biomechanical properties (Alvarez-Lloret et al., 2009; Finnila et al., 2010; Herlin et al., 2010, 2013; Hermsen et al., 2008; Jamsa et al., 2001; Lind et al., 1999, 2000, 2004, 2009; Miettinen et al., 2005; Nishimura et al., 2009). TCDD also inhibits the differentiation of osteoblasts (Carpi et al., 2009; Korkalainen et al., 2009; Ryan et al., 2007; Singh et al., 2000) and osteoclasts (Korkalainen et al., 2009) *in vitro*. The AhR is ubiquitously expressed in most organs and cells in the body, including osteoblasts and osteoclasts (Ilvesaro et al., 2005), and data from AhR-knockout mice, which show a partly different bone phenotype compared to wild-type mice (Herlin et al., 2013), suggest that AhR has a role in normal bone development. Further, most bone tissue modulations induced by TCDD in wild-type mice were not observed in AhR-knockout mice (Herlin et al., 2013). Similarly, effects of TCDD on osteoblasts derived from wild-type mice were not seen in osteoblasts from AhR-knockout mice (Korkalainen et al., 2009). These data strongly support that AhR plays a major role in the observed effects TCDD on bone properties.

Bone tissue is continuously undergoing remodeling; a regulated process that is essential for the maintenance of bone size, shape and quality (Hadjidakis and Androulakis, 2006). The balance between bone resorption and formation is dependent on complex interactions between various cell types as well as local and systemic factors. Imbalance in the regulation of bone remodeling

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http://dx.doi.org/10.1016/j.tiv.2015.03.006
0887-2333/

may result in skeletal deformities and impaired bone properties. The complexity of bone remodeling makes it a potential target for insult by chemicals that can interfere with these processes on different levels of regulation.

In addition to altered bone tissue properties and modulated bone cell differentiation by exposure to TCDD alone, also the polychlorinated biphenyl (PCB) mixture Aroclor 1254, containing both dioxin-like and non-dioxin-like congeners, has been shown to alter bone tissue properties similarly to TCDD in terms of decreased bone diameter, cortical thickness and cortical bone mineral density, as well as lower bone stiffness and strength in rats following perinatal exposure (Elabbas et al., 2011). Aroclor 1254 has also been reported to inhibit proliferation, induce apoptosis and suppress the activity of alkaline phosphatase (ALP) in osteoblastic cells (An et al., 2012). Based on the qualitatively similar effects of TCDD and Aroclor 1254 on bone properties following developmental exposure, it has been proposed that the observed alterations are predominantly driven by the dioxin-like congeners in Aroclor 1254 (Elabbas et al., 2011). However, it could not be excluded that also non-dioxin-like components in the mixture contributed to the outcome (Elabbas et al., 2011). Because humans and wildlife are continuously exposed to complex mixtures of chemicals, comparative studies are required in order to establish toxicity profiles for the wide variety of dioxin-like and non-dioxin-like environmental contaminants. Although the main constituents of Aroclor 1254 are non-dioxin-like PCBs, their effect on bone mineralization remains to be studied.

The aim of this study was to elucidate how exposure to Aroclor 1254 and TCDD affects the differentiation of osteoblastic cells, and to quantify the relative influence of dioxin-like and non-dioxin-like PCB-components. The expression of runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP) and osteocalcin (OCN) were used as markers for the various phases of osteoblast differentiation, and time-dependent effects by TCDD were explored. In order to further investigate in a quantitative manner the relative influence of dioxin-like and non-dioxin-like components of the PCB mixture on bone cell differentiation, the dioxin-equivalent potency of Aroclor 1254 for modulating the expression of osteoblast differentiation was estimated and compared with TCDD as well as two highly purified non-dioxin-like PCB congeners alone.

2. Materials and methods

2.1. Cell culture and exposure

Experiments were performed with the murine osteoblastic cell line MC3T3-E1, clone IV, which was obtained from DSMZ (DSMZ no. ACC 210), Germany. The cells were maintained in a basic medium containing αMEM supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin and 100 μg/ml streptomycin. Mineralization medium consisted of basic medium supplemented with 10 mM β-glycerol phosphate (Sigma, USA) and 50 μg/ml ascorbic acid (Wako Chemicals GmbH, Germany). The cells were cultured at 37 °C and 5% CO_2.

Cells were seeded in 6-well plates (45 000 cells/well) and allowed to attach. Next day TCDD (SS Zlotsky, Ufa Oil Institute, Russia), Aroclor 1254 (Supelco Analytical, USA), highly purified PCB19 or PCB52 (purified on a charcoal column mixed with Celite, as described in detail by Danielsson et al. (2008) with only trace levels (<pg/g of dioxin-like compounds (Danielsson et al., 2008) (Chiron, Trondheim, Norway) dissolved in DMSO, or DMSO alone (final concentration of DMSO was 0.1%) was added. No effect of DMSO alone on the cells was observed (Supplementary Fig. 1). Fresh exposure medium was supplied every 3rd day. The first day of exposure was defined as day 0. In one experiment cells were exposed to TCDD either during days 0–6 or during days 11–20. Cells were sampled at day 20, except for the time-course experiment where cells were exposed to 1 nM TCDD and sampled at days 6, 9, 12, 15, 18, 21 and 24. Each exposure was carried out in triplicates. TCDD was tested at concentrations ranging from 0.1 pM to 100 nM, and Aroclor 1254 was tested in the range 0.1 nM–10 μM (= 32.6 ng–3.26 mg/L). PCB52 and PCB19 were tested in the range 100 nM–10 μM.

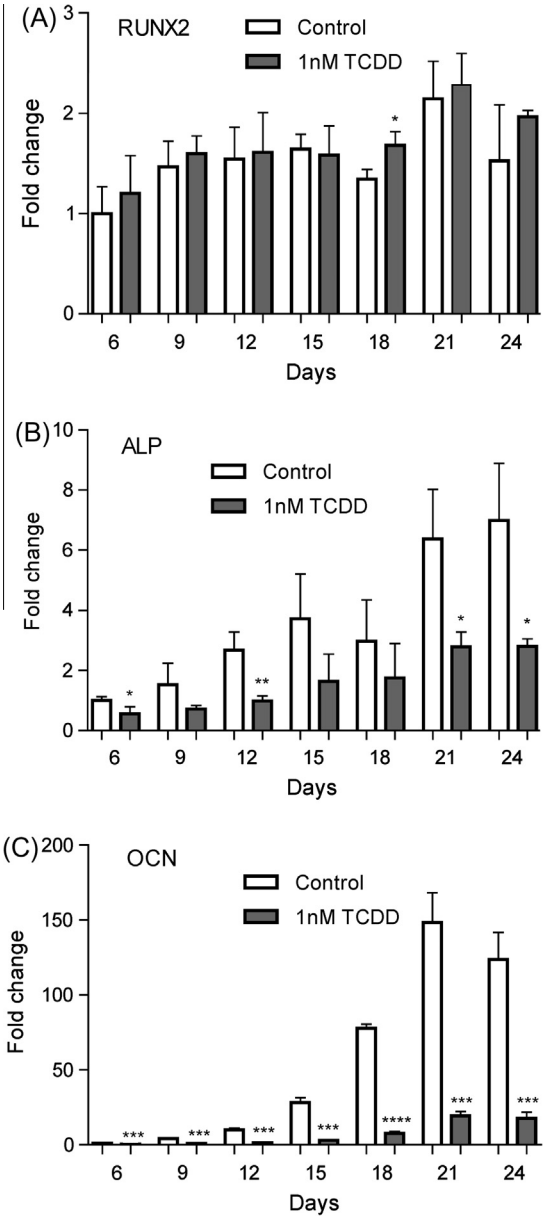

Fig. 1. Relative expression of (A) RUNX2, (B) ALP and (C) OCN at different timepoints with and without exposure to TCDD. All data are expressed as mean ± standard deviation. Pairwise multiple comparisons of means were performed by the analysis of variance (ANOVA) and Dunnet's posthoc test. Significance was considered for values of $p < 0.05$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.00001$.

2.2. RNA isolation and qRT-PCR analysis

RNA was isolated from pelleted cells using E.Z.N.A Total RNA Kit (Omega Biotek, USA) as per the manufacturer's protocol. cDNA was generated using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), and used for quantitative RT-PCR analyses. The expression levels of selected genes were analyzed