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Effects of PCB126 and PCB153 on telomerase activity and telomere length in undifferentiated and differentiated HL-60 cells

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Abstract

PCBs are persistent organic pollutants with carcinogenic, immune- and developmental toxicity. This suggests that they may interfere with normal cell maturation. Cancer and stem/progenitor cells have telomerase activity to maintain and protect the chromosome ends, but lose this activity during differentiation. We hypothesized that PCBs interfere with telomerase activity and the telomere complex thereby disturbing cell differentiation and stem/progenitor cell function. HL-60 cells are cancer cells that can differentiate to granulocytes and monocytes. We exposed HL-60 cells to the PCB126 (dioxin-like) and PCB153 (non-dioxin-like) 6 days before and during 3 days of differentiation. The differentiated cells showed G0/G1 phase arrest and very low telomerase activity. hTERT and hTR, two telomerase-related genes, were down-regulated. The telomere shelterins TRF1, TRF2, and POT were upregulated in granulocytes, TRF2 was up- and POT1 downregulated in monocytes. Both PCBs reduced telomerase activity in undifferentiated cells and further reduced telomerase activity in differentiated cells, but had only small effects on the differentiation and telomere-related genes. Long term treatment of undifferentiated HL-60 cells with PCB126 produced a downregulation of telomerase activity and decrease of hTERT, hTR, TRF1, and POT1 gene expression. With PCB153 the effects were less pronounced and some shelterin genes were increased after 30 days exposure. With each PCB no differentiation of cells was observed and cells continued to proliferate despite reduced telomerase activity, resulting in shortened telomeres after 30 days of exposure. These results indicate cell type and PCB congener specific effects on telomeres/telomerase-related genes and, although PCBs do not seem to strongly affect differentiation, they could influence the longevity of stem or progenitor cells through telomere attrition with potential long-term consequences for health.

Keywords

PCB126; PCB153; telomerase; telomeres; HL-60; differentiation

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Conflict of interest

The authors have no conflict of interest.

Introduction

Polychlorinated biphenyls (PCBs) are a class of organic compounds with 1 to 10 chlorine atoms attached to a biphenyl ring (WHO 1993). Commercial PCB products are mixtures of the 209 different PCB congeners to obtain specific levels of chlorination. These products were widely used as dielectric fluid in capacitors and transformers, and to a lesser extent in building materials (Lauby-Secretan et al. 2013). PCBs are persistent organic pollutants which bioaccumulate in plants, animals and environment. Despite the production ban and heavily restricted use since the late 1970s, PCBs can still be found even in the most remote areas like Polar Regions and mountain lakes due to atmospheric transport and precipitation (Fuoco et al. 1996, Grimalt et al. 2004). Humans are exposed to PCBs through contaminated food and indoor/outdoor air (ATSDR 2000). PCBs produce a variety of deleterious health effects, including immunosuppression, neurotoxicity, genotoxicity, endocrine disruption, and cancer (ATSDR 2000, Lauby-Secretan et al. 2013). Among the different types of cancer, Ward et al. showed that PCBs may represent a risk factor for childhood leukemia based on the finding from a population-based case-control study in California (Ward et al. 2009).

Telomeres and telomerase play an important role during hematologic cancer development (Deville et al. 2009, Ohyashiki et al. 2002). Telomeres are 5–10 kb repetitive DNA sequences at the end of chromosomes of most eukaryotic organisms. They are essential to avoid the deterioration of chromosome ends and the fusion with other chromosomes. The conventional DNA replication machinery lacks a mechanism to fill the gap left by removal of the final RNA primer in the most terminal Okazaki fragment; it is therefore unable to fully replicate 3' ends of linear chromosomes (Olovnikov 1971, Watson 1972). This leads to the gradual attrition of telomeric DNA over multiple cell divisions. Telomerase, a ribonucleoprotein complex, is an enzyme that adds the telomeric sequence TTAGGG to the end of DNA strands in the telomere regions in order to hinder the loss of chromosome ends during normal DNA replication (Cerni 2000). The telomerase reverse transcriptase (TERT), is the enzymatic component of the telomerase complex; it uses the intrinsic RNA component (TERC or TR) as a template. In addition, in mammalian cells a telomere shelterin complex composed of TRF1, TRF2, POT1, TIN2, TPP1 and RAP1 is located at the telomeric DNA region and protects the ends of chromosomes from being recognized as double strand breaks and regulates telomerase-dependent telomere elongation (Liu et al. 2004a, Ye et al. 2014).

Telomere shortening and telomerase dysfunction in humans are shown to be highly involved in premature aging syndromes, the development of cancer, and other human diseases like heart disease and diabetes. Studies using knockout mice have demonstrated that the role of telomeres in cancer can both be limiting to tumor growth, as well as promote tumorigenesis, depending on the cell type and genomic context (Chin et al. 1999, Greenberg et al. 1999). Malignant cells become immortalized mostly by activation of telomerase and resulting telomere extension. Shay and Bacchetti found telomerase activation in ~90% of cancers (Shay & Bacchetti 1997), which indicates that telomerase activity and telomere length are highly correlated to tumor development (Bailey & Murnane 2006, Eisenberg 2011, Kim et al. 1994, Stewart et al. 2002, Wu et al. 2003).

Studies from our lab showed that PCBs regulate telomere length and telomerase activity in human keratinocyte (HaCaT) cells (Jacobus et al. 2008, Senthilkumar et al. 2011, Senthilkumar et al. 2012), however, the mechanisms underneath are yet unknown. PCB126 (3,3',4,4',5-pentachlorobiphenyl) and PCB153 (2,2',4,4',5,5'-hexachlorobiphenyl) represent two different groups of PCBs that are involved in different regulatory pathways. PCB126 is a dioxin-like (DL) PCB congener that has a high affinity to the Aryl hydrocarbon receptor (AhR) (Rignall et al. 2013), while PCB153, a non-dioxin-like (NDL) PCB, is an activator of the constitutive androstane and pregnane X receptor system (CAR/PXR) (Kopec et al. 2010, Sakai et al. 2006). AhR and CAR/PXR act as transcription factors and regulate expression of defined sets of target genes, like cytochrome P450 (CYP) 1 for the AhR and CYP 2B and 3A for CAR (Bock 1994, Ueda et al. 2002). PCB126 is the most potent AhR agonist among all PCB congeners and PCB153 is most often the predominated congener in human foods, like fish or mothers' milk, and in human serum and adipose tissue (Robertson & Ludewig 2011).

We hypothesized that PCB126 and PCB153 have distinct, but different effects on telomerase and telomeres in hematopoietic cells, thereby influencing their path towards aging, terminal differentiation, and carcinogenesis. We used human HL-60 cells, myeloblastic/promyelocytic leukemia cells, a model system for studying human myeloid cell differentiation (Birnie 1988, Jasek et al. 2008), to examine the effects of two PCB congeners on telomerase/telomeres before and after induction of differentiation to granulocytes or monocytes/macrophages. We found that both PCB126 and PCB153 decrease telomerase activity and down-regulate hTERT and hTR in HL-60 cells during 30 days of exposure, but they exhibited different effects on the shelterin genes TRF1, TRF2, and POT, and only PCB126 significantly shortened telomere length. The PCBs also slightly enhanced the massive reduction of telomerase activity and hTERT expression caused by differentiation to granulocytes or monocytes. However, PCBs and differentiation had different effects on shelterin genes (TRF1/2, POT1), suggesting different mechanisms and long term consequences of these two pathways of telomere maintenance modification in HL-60 cells.

Materials and methods

Materials

All cell culture media and components, i.e. RPMI 1640 medium, Fetal Bovine Serum (FBS), penicillin-streptomycin (PS), Hanks Balanced Salt Solution (HBSS), and phosphate buffered saline (PBS) were obtained from Invitrogen (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO), resazurin and propidium iodide (PI) were purchased from Sigma (St. Louis, MO, USA). ATRA (all-*trans*-Retinoic acid) was bought from Tocris Bioscience (Bristol, UK), PMA (Phorbol 12-myristate 13-acetate) from Acros Organics (New Jersey, USA), and Hema 3 Staining system from Fisher Scientific (Pittsburgh, PA, USA). PCB126 (3,3',4,4',5-pentachlorobiphenyl) and PCB153 (2,2',4,4',5,5'-hexachlorobiphenyl) were provided by Dr. H.J. Lehmler of the Iowa Superfund Research Program at the University of Iowa. PCR reagents and kits were obtained from Qiagen (Valencia, CA, USA).

Cell culture, PCB exposure, and toxicity determination

The human myeloblastic/promyelocytic leukemia cell line (HL-60) was obtained from the American Tissue Culture Collection (ATCC) (Manassas, VA, USA). Cells were maintained in RPMI 1640 with 10% FBS and 1% penicillin-streptomycin in a humidified incubator at 37° C with 5% CO₂. All experiments were performed using exponentially growing cell cultures at cell densities between 1×10⁵ and 1×10⁶ viable cells/ml.

To identify a non-toxic dose of PCBs in HL-60 cells, 1×10⁴ cells in 1ml medium, were seeded in 24-well tissue culture plates with PCB126 or PCB153 treatment at concentrations of 2–20 µM. Control cultures received solvent (DMSO, 0.05% final concentration) alone. After six days incubation with a change of medium with fresh compounds on the third day, media were removed and cells were incubated with resazurin (5 µM, dissolved in HBSS) for 2 h and the fluorescence intensity of each well at 535 nm excitation and 590 nm emission wavelengths was measured using a microplate reader (GeniosPro from TECAN, Seestrasse, Switzerland). The fluorescence intensity correlates with the number of living cells in the well, since living cells metabolize non-fluorescent blue resazurin (oxidized form) to the red, fluorescing resorufin (reduced form). Data points were measured in triplicate and each experiment was performed twice. Data are given as percent of solvent control.

For the long term exposure experiment, 1×10⁵ HL-60 cells in 10 ml medium were seeded in 10 cm diameter Petri dishes and continuously exposed to PCB126 or PCB153 at a concentration of 5 µM or solvent alone (0.05% DMSO, final concentration) for 30 days. Media with test compounds or solvent were changed every three days. Cells were counted, and re-seeded at low density every sixth day. The remaining cells on days 6 and 30 were used to determine telomerase activity, telomere length, gene expression, cell cycle and cell growth. Cell morphology was monitored throughout the exposure period.

Induction of differentiation

HL-60 cells (2×10⁵ cells/ml) were treated with 5 µM PCB126, or PCB153, or solvent alone (0.05% v/v DMSO) for 6 days, with one subculture to 2×10⁵ cells/ml with medium and compound change on Day 3. On Day 6 cells from each treatment group were transferred into fresh medium without PCBs, divided into 3 groups, and treated with 0.05% (v/v) DMSO (control), 1.25% (v/v) DMSO & 1 µM ATRA (differentiation to granulocyte), or 16 nM PMA (differentiation to monocyte/macrophage). Three days later, cells were collected for cell morphology staining, RNA isolation, and telomerase activity detection.

A modified version of this exposure scheme with continuous exposure to PCBs during the 6 days before and the 3 days during induction of differentiation was also employed.

Cell morphology and cell surface marker analysis

After three days induction of differentiation, a batch of cells was washed with PBS, centrifuged, resuspended in PBS, and transferred onto microscopy slides with a cytospin centrifuge. Cells were stained with the Hema 3 Staining system and inspected at a magnification of 400× using a Zeiss Imager A1 microscope (Carl Zeiss AG, Jena, Germany) connected with a Pixelink Megapixel FireWire Camera (Pixelink, Ottawa, Canada).

For cell surface marker staining about 1×10^6 were washed twice with cold staining buffer with BSA (Cat #554657, BD PharMingen, San Diego, CA), resuspended in 100 μ l staining buffer, and incubated for 20 min with anti-CD11b PE-conjugated antibody (clone ICRF44, BD PharMingen, San Diego, CA) on ice in the dark. After two washings with cold staining buffer, the cell pellet was resuspended in 500 μ l staining buffer and analyzed using an Accuri C6 flow cytometer (BD, San Jose, CA).

Examination of cell growth and cytotoxicity

At different time points of the long term exposure experiment, cell growth was determined by counting the cells using a Neubauer hemocytometer and cytotoxicity was measured by the resazurin assay (described above) by measuring fluorescence of total cells per group after centrifugation, PBS wash, and incubation with resazurin (5 μ M) in HBSS for 2h at 37° C.

Analysis of cell cycle distribution

In the long term exposure experiment, approximately 1×10^6 cell per sample were periodically removed, washed with PBS, fixed with 70% ethanol overnight, washed once with PBS, and treated with RNase (100 μ g/ml) for 30 min and PI (50 μ g/ml) for 1 h. Cell cycle analysis was performed using a Becton Dickinson FACS Calibur flow cytometer with Argon Ion Laser, with 488nm excitation and 585nm band pass filter. Data were collected from 10,000 cells in list mode and the percentage of cells in each phase of the cell cycle was analyzed using MODFIT software.

In the differentiation experiment, 1×10^6 cells per sample were washed with PBS, centrifuged at 250g for 5 minutes, resuspended in 1 mL Vindelov's reagent (Tris buffered Saline, pH=7.6, Ribonuclease A, PI and Nonidet p-40) (Vindelov 1977), and incubated for 1–2 h at 4° C. Cell cycle analysis was performed using a BD Accuri C6 flow cytometer. Data were collected from 10,000 events and the percentage of cells in each phase of the cell cycle was analyzed using the Accuri C6 software.

Measurement of telomerase activity

Telomerase activity was measured following the protocol of Wege et al. (Wege et al. 2003). Briefly, cells were collected by centrifugation, resuspended in cell lysis buffer and incubated on ice for 30 min. Aliquots of cell lysate were centrifuged at 16,000 $\times$ g for 20 min at 4° C and 3/4th of the supernatant was transferred into new micro centrifuge tubes for qPCR. The protein concentration of the supernatant was determined by Bradford method using the Quick Start Bradford Protein Assay Kit from Bio-Rad Laboratories (Hercules, CA, USA). The SYBR Green based qPCR reaction was performed by adding 100 ng (0.2 μ l) of Telomerase Primer (TS), 50 ng (0.1 μ l) of Anchored Return Primer (ACX) (Kim & Wu 1997), 2.5 μ l cell lysate supernatant (40ng/ μ l), 12.5 μ l Real MasterMix Fast SYBR (5 PRIME Inc., Gaithersburg, MD, USA) and 9.7 μ l of DNase- and RNase-free water into the well of a 96-well microplate. Samples were incubated for 20 min at room temperature to allow telomerase to elongate the TS primer by adding TTAGGG repeat sequences, and PCR was performed by incubating the sample at 95 °C for 10 min followed by 35 cycles of 95 °C for 30 s and 60 °C for 90 s using an Eppendorf RealPlex Thermal Cycler (Eppendorf,

Hamburg, Germany). SYBR green fluorescence was determined using an excitation of ~470nm by dedicated light emitting diodes in each well, and emission was measured at 520 nm. The Ct (threshold cycle value) was established from the semi-log amplification plots (log increase in fluorescence versus cycle number) and compared with the standard curves generated from serial dilutions of cell extracts. Each sample was analyzed in duplicate.

Analysis of telomere length

Cellular DNA was isolated using the DNA blood mini kit from Qiagen (Valencia, CA, USA) following the manufacturer's protocol. DNA concentrations were determined by spectrophotometry and PCR reactions were performed following the method of Cawthon (Cawthon 2002). Briefly, 12 ng of DNA from each sample were added to telomere primer 1 (270 nM) and telomere primer 2 (900 nM) for telomere (T) PCR or 36B4u (300 nM) and 36B4d (500 nM) for single copy gene (S) PCR, in 4.25 µl of telomere buffer in separate wells of a 96-well plate. Plates were sealed with a plate sealer (Eppendorf, Hamburg, Germany) and incubated at 95 °C for 10 min followed by 30 cycles of 95 °C for 15 s and 54 °C for 90 s for telomere plates or 35 cycles of 95 °C for 15 s and 58 °C for 1min for single gene plates using an Eppendorf RealPlex thermal cycler. The T/S ratio was calculated for each corresponding well pair. The T/S ratio of the experimental sample to the T/S ratio of the control corresponds to the relative change in telomere length of the experimental sample. Each sample was analyzed in duplicate.

Quantitative real-time PCR

Total RNA was extracted from each group with a RNeasy mini kit from Qiagen (Valencia, CA, USA) following the manufacturer's protocol and quantified using a NanoDrop2000 from Thermo Fisher Scientific (Wilmington, DE, USA). Total RNA was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Foster City, CA, USA). The cDNA was subsequently used for quantitative real-time RT-PCR (qRT-PCR) employing an Eppendorf RealPlex thermal cycler. The applied PCR program started with 95 °C for 10 min followed by 35 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. The primers for the quantification of selected genes are listed in Table 1. GAPDH (glyceraldehyde-3-phosphate-dehydrogenase) served as the house keeping gene for the analysis of data. An amplification threshold in the linear range of each sample was selected to calculate the cycle threshold (C_T) for each sample. The relative mRNA levels were calculated as follows: $C_T(\text{sample}) = C_T(\text{mRNA of interest}) - C_T(\text{GAPDH})$; $C_T = C_T(\text{treatment}) - C_T(\text{control})$; relative expression = 2^{-C_T} .

Statistical analysis

All experiments (except differentiation experiment with continuous exposure to PCBs) were performed at least twice in duplicates or triplicates and every individual qRT-PCR sample was assayed in duplicates. Data from two experiments were combined and analyzed. All data are expressed as the mean $\pm$ SEM of triplicates. The data analysis was performed in SAS software, Version 9.3 of the SAS System for Windows. Differences between groups were examined for statistical significance by analysis of variance (ANOVA) followed by

Tukey's Studentized Range (HSD) multiple comparison *t* test. $p < 0.05$ was considered to be statistically significant.

Results

Cytotoxicity of PCB126 and PCB153 in undifferentiated HL-60 cells

To find a PCB concentration without toxic effects, exponentially growing HL-60 cells were exposed to a concentration range (2–20 μM) of PCB126 and PCB153. PCB126 caused a reduction in viable cells with the two highest concentrations (15 μM and 20 μM ; Supplementary Material, Fig. S1). No cytotoxicity was observed with any concentration of PCB153. Therefore, 5 μM PCB126 or PCB153 were used in the following experiments.

HL-60 cell differentiation to monocytes or granulocytes and effect of pre-exposure to PCBs

PCBs are carcinogenic and have immunotoxic and developmental toxicity, suggesting that they may influence stem/progenitor cell maintenance and/or differentiation. Myeloblastic/promyelocytic HL-60 cells can be induced to differentiate to granulocytes or monocytes. To examine the effects of PCBs on differentiation, HL-60 cells were treated for 6 days with PCBs and then induced to differentiate to granulocytes or monocytes/macrophages by exposure for 3 days to 1.25% (v/v) DMSO & 1 μM ATRA or 16 nM PMA, respectively. PCBs were not added during the differentiation phase.

To confirm the differentiation, the morphologic phenotypes were evaluated. As shown in Fig. 1, control (undifferentiated) HL-60 cells were predominantly myelocytes with round, regular cell margins and large unsegmented nuclei that took up $> 75\%$ of the area of the cell. Treatment with DMSO & ATRA resulted in bilobed and segmented nuclei, comparable to metamyelocytes and banded neutrophils. PMA treated HL-60 cells showed strong adherence to plastic, long cytoplasmic extensions and multiple vesicles in their cytoplasm. PCBs pre-treatment had no significant effects on cell morphology changes in undifferentiated and differentiated cells, but seemed to slightly reduce the number of granulocytes and increase the number of monocytes compared to cultures that were not treated with PCBs (Table S1).

To further confirm differentiation, the cell surface marker CD11b expression was determined by flow cytometry. CD11b is a monocyte- and granulocyte-specific marker, which is only expressed in differentiated cells. Fig. 2 shows the extremely high expression of CD11b in differentiated compared with undifferentiated groups. Pre-treatment with PCB126 had no effect on CD11b expression in differentiated or undifferentiated cells. However, in PMA treated cells, PCB153 significantly decrease CD11b expression compared to the PMA control group, suggesting a possible effect on monocyte/macrophage differentiation.

Cell cycle distribution after differentiation

One mechanism of interference with cell differentiation could be through disruption of the cell cycle. Cells induced to differentiate exhibited a significant decrease in S phase and G2/M phase cells with a corresponding increase of cells in G0/G1 from 68.35% in the

control group to 86.49% and 85.95% after DMSO&ATRA and PMA treatment, respectively (Table 2). PCB126 pretreatment had no influence on cell cycle distribution in differentiated cells. PCB153 significantly increased S phase cells compared to control group from 13.01% to 15.18%, but no significant effect was visible in the differentiated groups. Thus interference with the cell cycle is most likely not a cause for PCB toxicity.

Telomerase activity and telomerase gene expression after differentiation

Progenitor cells express telomerase activity, while differentiated cells usually don't. We examined the effects of pre-exposure to PCBs on this change in telomerase activity after differentiation of HL-60 cells. As expected, telomerase activity was significantly decreased after differentiation to granulocytes or monocytes/macrophages compared with the undifferentiated cells (Fig 3A). Both PCB reduced telomerase activity (non-significantly), but both significantly further decreased telomerase activity in granulocytes.

The most important component of the telomerase complex is TERT, which has the enzymatic activity. TR is the intrinsic RNA component of telomerase serving as a template for TERT. We analyzed the expression levels of hTERT and hTR genes. After differentiation, hTERT mRNA levels were significantly down regulated, an effect that was significantly lessened in DMSO&ATRA cells pretreated with PCB126 (Fig. 3B). Otherwise pre-treatment with PCBs had no effect on hTERT. hTR gene expression was significantly increased after induced to differentiate to granulocytes (DMSO&ATRA), while exposure to PMA or pre-exposure to PCBs produced no significant changes (Fig. 3C).

Gene expression levels of telomere shelterin complex related factors TRF1, TRF2 and POT1 after differentiation

TRF1, TRF2 and POT1, three components of the telomere shelterin complex, directly bind to telomeric DNA. They protect the telomeres and affect the function and activity of telomerase. Therefore gene expression of shelterin components TRF1, TRF2 and POT1 was also determined. TRF1 mRNA level was significantly up regulated after differentiation to granulocytes, and non-significantly elevated in monocytes (Fig 3D). After granulocytic differentiation, PCB126 and 153 both significantly increased TRF1 gene expression in granulocytes while after monocytic differentiation, both PCB congeners significantly down regulated TRF1 gene expression. TRF2 gene expression was significantly increased after monocytic differentiation by PMA treatment while only a non-significant elevation was seen in granulocytes, which was significantly reduced by PCB153 (Fig. 3E). The POT1 mRNA level was non-significantly elevated after differentiation to granulocytes and non-significantly reduced after differentiation to monocytes, an effect that became significant in the PCB153-pretreated group (Fig. 3F).

Telomerase activity and gene expressions after continuous (9 day) exposure to PCBs before and during differentiation

Removal of PCBs during the differentiation induction, the final three days of the experiments, could have alleviated the PCB effect or even caused an overshooting recovery effect. We repeated the same experiments with PCBs present for 6 days before and the 3 days during the differentiation time as a comparison. With 9 days of exposure, PCB126

produced a slight reduction in cell number of all treated cultures, while cell numbers were slightly increased in PCB153-exposed samples (Table S2). A significant effect of PCBs on telomerase activity and hTERT in undifferentiated controls was visible with this exposure regimen (Fig. 4A and 4B). As in the previous experiment, both PCB congeners further reduced telomerase activity in differentiated cells which was significant in the monocytes (Fig. 4A), while no significant effect was seen on hTERT expression (Fig. 4B). The increase in hTR in granulocytes was significantly reduced, an effect that had been non-significant if PCBs were removed during the last 3 days of differentiation. In granulocytes TRF1 and POT1 expressions were reduced by PCB treatment (Fig. 4C, D and F). In monocytes, PCB153 upregulated TRF2 gene expression and PCB126 downregulated POT1 (Fig. 4E and 4F). These results show that short term (6 day) exposure to PCBs and even more the ongoing exposure after induction of differentiation can modulate (enhance or ameliorate) the changes in telomere- and telomerase-related gene expression induced by cell differentiation.

Effect of PCBs on cell growth of undifferentiated HL-60 cells during long term (30 day) exposure

Since relative short term exposure of HL-60 cells before and during induction of differentiation produced changes in telomerase activity and telomere-related genes which could indicate induction or interference with differentiation, we examined the effect of long term (30 days) exposure alone in undifferentiated HL-60 cells. In these experiments medium with compound was changed every third day and the cell number was reduced every sixth day. This continuous exposure to 5 μ M PCB126 or PCB153 did not have any significant effect on the cell numbers or cell cycle distribution of HL-60 cells over a 30 day treatment period as determined by cell counting during subculturing (Supplemental Material Fig. S2, Table S3). Visual inspection of cells during culture and counting did not indicate any obvious change in cell morphology or behavior. This and the constant cell proliferation do not suggest an induction of differentiation by PCBs.

Telomerase activity and telomere length in undifferentiated HL-60 cells after long-term exposure to PCBs

HL-60 cells were treated with 5 μ M PCB126 or PCB153 continuously for 30 days and telomerase activity was determined in cells after 6 and 30 days of exposure. As shown in Fig. 5A, telomerase activity was significantly increased by PCB153 on Day 6, but on Day 30 it was significantly decreased by both PCB congeners.

Measurement of telomere length by qPCR after exposure to PCBs for 30 days revealed that PCB126 significantly shortened telomere length. PCB153 also decreased telomere length but the effect was not statistically significant (Fig. 5B). Telomere length on Day 6 was not determined since our previous experiments showed that measurable telomere length reduction is a late occurring event (Senthilkumar et al. 2011, Senthilkumar et al. 2012)

Gene expression of telomerase (hTERT, hTR) and telomere shelterin (TRF1, TRF2, POT1) genes in HL-60 cells after long-term PCB exposure

hTERT and hTR expression were both down regulated by PCB126 and by PCB153 on Day 6 and Day 30, but the reduction in hTERT expression was no longer significant on Day 30

for PCB153 (Fig.6A and 6B). This is consistent with the observed more pronounced reduction of telomerase activity with PCB126, and a higher sensitivity and earlier occurrence of changes in gene expression than enzyme activity. In undifferentiated HL-60 cells PCB126 down regulated TRF1 and POT1 gene expression on both Day 6 and Day 30. With PCB153 TRF1, TRF2, and POT1 expression was decreased on Day 6, but increased on Day 30 (Fig. 7A–C). These results show that PCBs produce some of the same effects seen by differentiation induction, but to a lesser extent, and they produce different and congener-specific effects on shelterin gene expression (summarized in Table 3).

Discussion

PCBs are ubiquitous contaminants which accumulate in fatty tissues and to a two-to three-fold higher extent in bone marrow (Scheele et al. 1992). This suggests that haemopoietic stem- and progenitor cells may be exposed to unexpectedly high levels of PCBs. Stem cells and progenitor cells express telomerase activity to maintain the length of their telomeres while proliferating, whereas differentiated cells do not express telomerase activity. We previously observed that various PCB congeners downregulate telomerase activity and shorten telomere length in HaCaT cells, human immortal keratinocytes, but the underlying mechanisms are still not understood (Jacobus et al. 2008, Senthilkumar et al. 2011, Senthilkumar et al. 2012). Based on those observations, we hypothesized that PCBs may enhance or even produce cell differentiation or/and may result in premature aging and disturbance of function of stem/progenitor cells. In the present study, we investigated the effect of two PCB congeners, representatives of dioxin-like (PCB126) and non-dioxin like (PCB153) congeners, known to activate very different intracellular receptors and metabolic pathways (Kopeck et al. 2010, Rignall et al. 2013), for their effects on differentiation of HL-60 myeloblastic/promyelocytic progenitor-like cells. HL-60 cells have high expression of hTERT mRNA and active telomerase (Yamada et al. 2000), consistent with the fact that they are cancer cells. HL-60 cells also can be induced to differentiate to granulocytes or monocytes by different inducers (Birnie 1988).

We obtained an efficient induction of differentiation of HL-60 cells to granulocytes or monocytes/macrophages after 3 days exposure to DMSO & ATRA or PMA, respectively, accompanied with a dramatic loss of telomerase activity, a strong downregulation of hTERT gene expression, and an increase in Go/G1 phase cells. This is consistent with previous reports using ATRA with or without TNF or 12-O-tetradecanoyl phorbol-13-acetate (Bartova et al. 2000, Liu et al. 2004b, Meyerson et al. 1997, Ramakrishnan et al. 1998, Wu et al. 2012, Yamada et al. 2000). Telomerase activity was significantly further reduced in granulocytes by pre-exposure to either PCB congener and in monocytes by pre- and continuous exposure during differentiation to this congeners. But the differentiation marker CD11b was not significantly changed by exposure to PCB126 and even reduced in PMA treated cultures by PCB153, suggesting a reduction in differentiation efficiency to monocytes/macrophages.

An analysis of the RNA template in telomerase (hTR) and shelterin genes showed an upregulation of hTR, TRF1, TRF2, and POT1 in granulocytes, which is in agreement with Yamada and coworkers who measured hTR, TRF1 and TRF2 after differentiation (Yamada

et al. 2000). Similarly normal leukocytes express less hTERT but more hTR, TRF1 and TRF2 than malignant hematopoietic cells (Koyanagi et al. 2000). Pre-exposure to PCBs further enhanced the upregulation of TRF1 in granulocytes, which may explain the enhanced reduction of telomerase activity. However, this effect on TRF1 (and telomerase activity) disappeared if PCBs were present also during differentiation while this 9 day exposure caused a downregulation of hTR and POT1 in granulocytes, both of which could possible contribute to a reduction in telomerase activity, although this was not visible in our experiments.

In monocytes we observed only an upregulation of TRF2, no significant changes in hTR and TRF1 expression, and even a down regulation of POT1, revealing that despite the common downregulation of hTERT and telomerase activity, granulocytes and monocytes differ strongly in the expression of the other telomere stabilizing and regulating genes. Also, pre-exposure to PCBs reduced TRF1 expression in monocytes, the opposite effect that they caused in granulocytes, again pointing towards a cell-type specific effect on individual shelterin genes.

It was surprising that most effects seen were similar with both PCB congeners and differed only in magnitude, even though they are known to be toxic by activating very different receptors and genes. One possible exception is TRF2, which was upregulated by continuous exposure to PCB153 before and during differentiation to monocytes, but not changed or even downregulated by PCB126. Whether this is related to the reduced expression of CD11b differentiation marker in PCB153-exposed monocytes and a sign of reduced differentiation of progenitor cells to monocytes *in vivo* remains to be determined.

Overall the presence of PCBs before and during differentiation had only small effects on gene expression and telomerase activity after differentiation. One reason could be that the process of differentiation is so strong and dominating, as the high level of differentiation marker and cells in G0/G1 and strongly reduced telomerase activity and hTERT expression indicate, that smaller effects of PCBs become difficult to distinguish.

The only significant effect of PCBs in differentiated cells that was consistently visible was a further reduction of telomerase activity, indicating an additive effect of PCBs and differentiation and possibly stimulation of differentiation or premature aging of progenitor cells. To explore this possibility, we exposed HL-60 cells for a longer term (30 days), thereby imitating the constant exposure to PCBs in real-life. Our results show that telomerase activity was down-regulated and telomere length significantly shortened after 30 days of exposure to PCB126, but telomere shortening did not reach the level of significance with PCB153. Also, the telomerase component genes hTERT and hTR were both down-regulated by PCB126 and PCB153, but to a lesser extent by PCB153 compared to PCB126. hTERT expression was decreased by about 50% on day 6, which was earlier than the effect on telomerase activity. This is in agreement with our previous findings that significant effects are seen early for hTERT gene expression, followed by a decline in telomerase activity, which is followed a few weeks later by measurable and significant telomere shortening (Kuppusamy 2012). This suggests that PCBs affect telomeres by interference with gene regulation, although the fact that all tested PCB congeners had an effect on

telomerase activity points towards an unconventional mechanism and/or different mechanisms. In agreement with this, differences were seen for effects of PCB126 and PCB153 on the telomere shelterin genes TRF1, TRF2 and POT1: PCB126 down regulated TRF1 and POT1, while PCB153 had a biphasic effect on TRF1 (down after 6 days, up after 30 days exposure) and even caused an upregulation of TRF2 and POT1 after long term exposure. TRF1 is believed to inhibit the action of telomerase at the telomeric region (van Steensel & de Lange 1997). TRF2 was shown to prevent end-to-end chromosome fusions (van Steensel et al. 1998), and POT1, a single-stranded telomeric DNA binding protein, acts as a telomerase-dependent positive regulator of telomere length, facilitating telomere elongation (Colgin et al. 2003). Thus the stronger repression of hTERT, possibly supported by reduction in TRF1 and POT1, seems to be responsible for the lower telomerase activity and for the significant reduction of telomere length with PCB126. On the other hand PCB153's lower reduction of hTERT expression and increased expression of POT1, the telomerase function enhancer, may be the reason for the comparatively lesser efficiency in telomere length reduction with this congener. More experiments are needed to decipher the regulatory pathways that are involved in these effects of these two congeners. It is important to recognize, however, that both congeners reduced telomerase activity without an effect on cell cycle progression or cell proliferation. Continuous proliferation without sufficient telomerase activity is known to shorten telomeres, explaining the shortened telomeres seen here with PCB126 and to a lesser extent with PCB153. If PCBs have the same effects on telomerase and telomeres in stem cells and progenitor cells *in vivo*, then it could result in premature aging of these cells with potentially harmful consequences for the function of the affected organ, like the hematopoietic and immune system.

Conclusion

We have shown that PCB126 down regulated telomerase activity in undifferentiated HL-60 cells, accompanied with decrease of hTERT, hTR, TRF1, and POT1 gene expression. Similar effects with PCB153 on telomerase activity, hTERT, hTR and shelterins were less pronounced except for TRF2 and POT1, where PCB153 may have stronger, upregulating effects, and effects of PCB153 sometimes changing over longer times. Despite the reduction of telomerase activity by both PCB congeners, the cells continued to proliferate which resulted in shortened telomeres after long term exposure. In contrast, induction of differentiation of HL-60 cells to granulocytes or monocytes produced profound G0/G1 phase arrest and almost non-detectable telomerase activity and hTERT mRNA levels, but differences were seen between granulocytes and monocytes regarding hTR, TRF1, TRF2, and POT1 gene expression. Thus all four treatments, PCB126, PCB153, DMSO & ATRA and PMA, caused very different patterns of changes of shelterin and telomerase gene expression (Table 3), suggesting very different underlying mechanisms of action and consequences. Although PCBs had an additive effect on reduction of telomerase activity and hTERT expression during differentiation of HL-60 cells, they may not interfere significantly with differentiation of myelocytic cells, but may have a strong negative effect on telomere maintenance and thereby function and longevity of hematopoietic stem/progenitor-like cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1. Morphology of HL-60 cells after 6 days of exposure to PCBs or solvent (0.05% DMSO) followed by 3 days of exposure to differentiation inducers
The differentiation to granulocytes (DMSO & ATRA) or monocytes/macrophages (PMA) was clearly visible, but no effect of either PCB congeners on differentiation was observed. (Magnification $\times 400$)

Fig 2. Cells induced to differentiate expressed the differentiation marker CD11b; PCB153 pre-treatment reduced CD11b in monocyte cultures

HL-60 cells were treated with 5 μ M PCBs for 6 days before exposure for 3 days to 1.25% (v/v) DMSO & 1 μ M ATRA (granulocyte differentiation) or 16 nM PMA (monocyte differentiation) in the absence of PCBs. * $p < 0.05$, compared to the corresponding control groups (effect of differentiation). # $p < 0.05$, compared to PMA control group (effect of PCB153).

Fig 3. Differentiation reduced telomerase activity and hTERT expression in HL-60 cells and increased TRF1 and TRF2 expression; PCB pre-treatment (without exposure during differentiation) enhanced telomerase reduction and TRF1 increase in granulocytes
 HL-60 cells were pretreated for 6 days with PCBs before exposure to 1.25% (v/v) DMSO & 1 μ M ATRA (granulocyte differentiation) or 16 nM PMA (monocyte differentiation) for 3 days in the absence of PCBs. * p <0.05, compared to the control groups in the same PCB treatment group; # p <0.05, compared to the control groups with the same differentiation phenotype.

Fig 4. Continuous exposure to PCBs before and during differentiation reduced telomerase activity and hTERT in controls, enhanced telomerase reduction in monocytes, but ameliorated hTR, TRF1 and POT1 increase in granulocytes

HL-60 cells were treated with PCBs for 6 days before plus 3 days during differentiation.

* $p < 0.05$, compared to the control groups in the same PCB treatment group; # $p < 0.05$, compared to the control groups with the same differentiation phenotype.

Fig 5. PCB126 significantly reduced telomerase activity and telomere length after 30 days of exposure, PCB153 effect on telomeres was less pronounced

HL-60 cells were exposed continuously to 5 μ M PCB 126 or PCB153 for 30 days with one medium change every 3rd day and subculturing every 6th day. **A.** Telomerase activity in HL-60 cells exposed for 30 days to 5 μ M PCB126 or PCB153 was reduced. **B.** Telomere length was significantly shortened by PCB126 after 30 days of continuous exposure; the similar PCB153 effect did not reach statistical significance. * $p < 0.05$, compared to the control group.

Fig 6. PCB126 and PCB153 reduced the expression of the telomerase components hTERT and hTR after 6 and 30 days
HL-60 cells were continuously exposed to 5 μ M of the PCBs for 30 days with regular medium change and subculturing. * $p < 0.05$, compared to the control group at the individual time point.

Fig 7. PCB126 and PCB153 modified gene expression of the telomere shelterin components TRF1, TRF2, and POT1 in a time- and congener-specific way
 HL-60 cells were continuously exposed to 5 μ M of the PCBs for 30 days with regular medium change and subculturing. * $p < 0.05$, compared to the control groups in individual time point.

Table 1

Primer pairs used for qRT-PCR analysis

Gene	Sequence (5'-3')	Reference
TS	Forward: AATCCGTCGAGCAGAGTT	(Wege et al. 2003)
ACX	Reverse: GCGCGGCTTACCCTTACCCTTACCCTAACC	
Tel1	Forward: GGTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT	(Cawthon 2002)
Tel2	Reverse: TCCC GACTATCCCTATCCCTATCCCTATCCCTATCCCTA	
36B4u	Forward: CAGCAAGTGGGAAGGTGTAATCC	
36B4d	Reverse: CCCATTCTATCATCAACGGGTACAA	
hTERT	Forward: TGTACTTTGTCAAGGTGGATGTGA Reverse: GCTGGAGGTCTGTCAAGGTAGAG	(Gourronc et al. 2010)
hTR	Forward: TCTAACCCTAACTGAGAAGGGCGTAG Reverse: GTTTGCTCTAGAATGAACGGTGGAAG	
TRF1	Forward: GGCAGCGGCAAAAGTAGT Reverse: GTCTTGTTGCTGGGTTCAT	Designed using Primer 3
TRF2	Forward: GTACCCAAAGGCAAGTGGA Reverse: TGACCCACTCGCTTCTTCT	
POT1	Forward: CCTACGTGTTTGGGCATCT Reverse: TTTGTAGCCGATGGATGTGA	
GAPDH	Forward: AAGGTCATCCATGACAACCTTG Reverse: GTAGAGGCAGGATGATGTTCT	

Induction of differentiation causes cell cycle arrests in G0/G1 phase but PCBs have no effect on the cell cycle distribution

Table 2

HL-60 cells were exposed to solvent or PCBs for 6 days, divided into 3 groups, and one sample of each group was treated with solvent, DMSO&ATRA (granulocyte) or PMA (monocyte induction).

Treatment		G0/G1 (%)		S (%)		G2/M (%)	
Pre- (6 days)	Post- (3 days)	Mean	SD	Mean	SD	Mean	SD
Control	Control	68.35	0.76	13.01	2.85	18.64	3.58
	DMSO&ATRA	86.49*	1.36	2.48*	1.01	11.03*	2.34
	PMA	85.95*	7.07	1.24*	0.59	12.82*	6.54
PCB126	Control	67.74	1.55	13.98	2.58	18.28	3.82
	DMSO&ATRA	87.98*	1.52	2.31*	0.70	9.71*	2.21
	PMA	84.81*	8.33	0.85*	0.14	14.34	8.39
PCB153	Control	66.05	4.47	15.18 [#]	1.01	18.78	5.22
	DMSO&ATRA	86.37*	1.42	4.12*	0.59	9.51*	1.45
	PMA	87.00*	5.55	1.13*	0.49	11.87*	6.03

Significance:

* $p < 0.05$ compared with correlating post-treatment Control, indicating effect of differentiation induction.

[#] $p < 0.05$ compared with corresponding group (same shading) in overall Control block, indicating effect of PCB treatment.

Table 3

Comparison of the effects of different treatments on HL-60 cell parameters

	PCB126	PCB153	DMSO & ATRA (granulocyte diff)	PMA (monocyte differentiation)
Telomerase activ.	↓↓	↓	↓↓↓	↓↓↓↓
hTERT	↓↓	↓	↓↓↓	↓↓↓↓
hTR	↓ or O	↓ or O	↑↑	O
Go/G1 increase	no	no	yes	yes
TRF1	↓ (O later)	O or ↓	↑↑	O
TRF2	O	O (↑ later)	↑	↑↑
POT1	↓ or O	O (↑ later)	↑	↓

O = no effect, ↓ = down regulated, ↑ = up regulated