Effects of polychlorinated biphenyls (PCBs) on telomere maintenance in hematopoietic stem cells and progenitor cells

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University of Iowa

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EFFECTS OF POLYCHLORINATED BIPHENYLS (PCBS) ON TELOMERE MAINTENANCE IN HEMATOPOIETIC STEM CELLS AND PROGENITOR CELLS

by

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Human Toxicology in the Graduate College of The University of Iowa

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ABSTRACT

Polychlorinated biphenyls (PCBs) are synthetic persistent organic compounds that are known to be carcinogenic to humans. Changes in telomerase activity and telomere length are hallmarks of aging and carcinogenesis. Retention of telomerase activity and long telomeres are key characteristics of stem cells and progenitor cells. I hypothesize that PCBs modulate telomerase activity and telomeres of hematopoietic stem cells and progenitor cells via interference of gene regulation and potentially disrupt cell differentiation. To investigate this possibility, I used progenitor-like cells, human promyelocytic leukemia cells (HL-60), and stem cells from rat bone marrow. I show that PCB126 and PCB153 display toxic effects on telomerase activity, telomere length and their related gene expression in progenitor-like HL-60 cells, but they did not exert much effect on differentiation. Further, an in vivo/in vitro study using rat bone marrow cells shows that PCB126-induced hematotoxicity, evidenced by reduction in telomerase activity and TERT gene expression, an increase of the differentiation and a change in the differentiation direction towards granulocytes, which indicate an effect on stem cell function. I also show that the most potent dioxin-like congener, PCB126, regulates hTERT gene expression by activation of the AhR pathway. Both AhR and ARNT work together as a repressor of hTERT transcription. This research improves our understanding of mechanisms of PCB126 and PCB153 toxicity on hematopoietic stem cells and progenitor cells, which will ultimately have significant implications for human health.
PUBLIC ABSTRACT

Polychlorinated biphenyls (PCBs) are synthetic organic compounds that resist natural breakdown in the environment. They have various adverse health effects to humans including causing cancers. Telomerase and telomeres are an enzyme complex and DNA structures, respectively, whose functions are highly correlated with aging and cancer. Activated telomerase enzyme and long telomeres are key characteristics of stem cells and progenitor cells. These cells play an important role in repairing our organs and tissues. Aging of stem cells is associated with many adverse health effects including aging and cancer. This dissertation research used two cell models, human progenitor-like cells in culture and rat bone marrow stem cells exposed to PCBs, to investigate the effects of PCBs on stem cells and progenitor cells. My results show that PCBs are toxic to these cells by changing telomerase activity and disturbing telomeres and their maintenance, which can consequently affect the function of these cells. This research improves our understanding of toxicity of PCBs on stem cells and progenitor cells, which may have significant implications for human health.
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AhR: aryl hydrocarbon receptor
α-NF: α-naphthoflavone
ARNT: aryl hydrocarbon receptor nuclear translocator
ATRA: all-trans-Retinoic acid
B(a)P: benzo(a)pyrene
CAR: constitutive androstane receptor
ChIP: chromatin immunoprecipitation
CoCl₂: cobalt chloride
CYP1A1: cytochrome P450 1A1
CYP1B1: cytochrome P450 1B1
DMSO: dimethyl sulfoxide
HaCaT: immortalized human skin keratinocytes
HBSS: hanks balanced salt solution
HSC: hematopoietic stem cell
HL-60: human promyelocytic leukemia cells
HIF-1α: hypoxia-inducible factor-1α
HPRT: hypoxanthine granine phosphoribosyl transferase
HRE: hypoxia response element
hTERT: human telomerase reverse transcriptase
hTR: human telomere RNA
IP injection: intraperitoneal injection
M-CSF: macrophage colony stimulating factor
NHL: non-hodgkins lymphoma

PBS: phosphate buffered saline

PCB: polychlorinated biphenyl

PCB126: 3,3’,4,4’,5-pentachlorobiphenyl

PCB153: 2,2’,4,4’,5,5’-hexachlorobiphenyl

PHD: prolyl hydroxylase domain-containing enzyme

PI: propidium iodide

PMA: Phorbol 12-myristate 13-acetate

POPs: persistent organic pollutants

POT1: protection of telomeres 1

PXR: pregnane X receptor

qRT-PCR: quantitative real-time reverse transcription polymerase chain reaction

RPLP0: ribosomal protein, large, P0

TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin

TRF1: telomere repeat factor 1

TRF2: telomere repeat factor 2

XRE: xenobiotic response element
CHAPTER 1 INTRODUCTION

Polychlorinated Biphenyls (PCBs)

Background

Polychlorinated biphenyls (PCBs) are a group of synthetic organic compounds with 1 to 10 chlorine atoms attached to a biphenyl ring (WHO 1993). The general formula for these compounds is $C_{12}H_{10-n}Cl_n$ shown in Figure 1-1. There are total 209 different PCB congeners based on the different number and position of chlorine atoms on the rings. PCBs are colorless crystals that are easily dissolved in many organic solvents or lipids.

![Chemical structure of PCBs](image)

**Figure 1-1. The chemical structure of PCBs.** The possible positions of chlorine atoms on the benzene rings are denoted by numbers assigned to the carbon atoms.

PCB products were commercially manufactured for industrial purpose, and were mixtures of many different PCB congeners to obtain a specific level of chlorination. These products were widely used as dielectric fluids in capacitors and transformers, plasticizers in paint and inks, and to a lesser extent in building materials such as caulking in school buildings (Lauby-Secretan et al. 2013). An estimated 2 million tons of PCBs products were manufactured and used from 1929 to early 1980s in many countries.
including the United States, Germany, France, Spain and Japan, of which about 0.2 million tons still remain in the environment (WHO 1993).

Despite the production ban and heavily restricted use since late 1980s, 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). In the environment, lower chlorinated PCBs can be degraded by photolysis and biodegradation (Tiedje et al. 1993), while higher chlorinated PCBs are difficult to degrade but are subject to reductive dechlorination by microbes under anaerobic conditions. In higher organisms, PCBs can be metabolized via hydroxylation by cytochrome P450 (CYP) enzymes and phase II enzymes and finally eliminated in bile, urine or feces. Higher chlorinated PCBs are predominantly found at the top of the food chain since among PCB congeners the possibility of metabolism decreases as the chlorination of the biphenyl rings increases, resulting in bioaccumulation in fatty tissues of animals and biomagnification in the food chain. Therefore, humans are still exposed and will continue to be exposed to these persistent environmental pollutants.

Individual PCB congeners

The toxicity among the different PCB congeners varies based on their differences in chlorination and chemical structure. PCBs can be divided into two groups based on their structure: dioxin-like and non-dioxin-like PCBs. The congeners that are substituted at both para and at least two meta positions, but not in any of the ortho positions, may form a more coplanar configuration, as seen in 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and bind strongly to the aryl hydrocarbon receptor (AhR) (Safe 1994). The toxicity of
individual dioxin-like PCBs is evaluated by toxic equivalency factors (TEFs), which are the ratio of the toxicity of a given PCB congener compared to TCDD (TEF = 1).

3,3’,4,4’,5-pentachlorobiphenyl (PCB126) is a coplanar PCB, which has been shown to affect transcription via the AhR (Fukuzawa et al. 2003; Hassoun et al. 2001). It is the most potent AhR agonist in the PCB family with the highest TEF of 0.1 (Safe 1990). 40-60% of the total toxic potency of all dioxin-like PCBs is due to PCB126 (NTP 2006).

2,2’,4,4’,5,5’-hexachlorobiphenyl (PCB153) is most often the predominant congener in human foods, like fish or mothers’ milk, and in human serum and adipose tissue (Robertson and Ludewig 2011). Its half-life may be over 100 years in marine sediments (Jonsson et al. 2003), which means that PCB153 is one of the most persistent compounds in the environment. PCB153 is a non-dioxin-like PCB congener and it is an activator of the constitutive androstane and pregnane X receptor systems (CAR/PXR) based on its structural similarity to phenobarbital and regulation effect on gene expression (Kopec et al. 2010; Sakai et al. 2006).

Human health effects of PCBs

Since PCBs are persistent in the environment, humans are exposed to PCBs through food, indoor and outdoor air and other pathways. Studies have shown that PCBs are immunotoxic (Iwanowicz et al. 2009; Sormo et al. 2009; Tryphonas 1994) and correlate to childhood leukemia (Ward et al. 2009) and Non-Hodgkin lymphoma (NHL) (Bertrand et al. 2010; Hardell et al. 1996). Dioxin and dioxin-like PCBs are carcinogenic, possibly due to their ability to inappropriately activate the AhR and consequently stimulate the clonal expansion of preneoplastic cells or inhibit apoptosis of these cells.
TCDD exposure affects the ability of hematopoietic stem cells (HSCs) to proliferate and differentiate into mature lineage cells by inappropriate AhR activation (Singh et al. 2009). Some studies demonstrated that non-dioxin-like PCBs suppress the antibody production and immune response of phagocytosis (Levin et al. 2005; Selgrade 2007), and also non-dioxin-like PCBs exposed infants or adult humans show a decreased resistance to infection (Dallaire et al. 2006; Luebke et al. 2004). Ward et al. reported 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). In addition, epidemiologic studies have shown the PCB levels in peripheral blood or adipose tissue are related to increased risk of NHL. Engel et al. (Engel et al. 2007) demonstrated that concentrations of certain PCBs (PCB118, 138, and 153) in blood are associated with increased risk of NHL based on three cohorts. A case control study nested within the Physicians’ Health Study also showed a positive association between PCB exposure and development of NHL in men (Bertrand et al. 2010).

Moreover, PCBs are carcinogenic in other organs and genotoxic. PCB126 has been found to cause hepatocellular carcinoma, cholangioma and biliary duct cancer in rats (NTP 2006), and be involved in the development of diabetes through inhibition of adipogenesis of human preadipocytes (Gadupudi et al. 2015). Sandal et al. showed that PCBs 52 and 77 cause DNA damage in cultured human peripheral lymphocytes (Sandal et al. 2008). A study using a fish cell line (RTG-2) demonstrates that PCBs 153, 138, 101, 118 cause evident genotoxic damage, probably as a consequence of oxidative stress (Marabini et al. 2011). However, the mechanisms of their adverse effects are not yet fully understood.
A clear conclusion is that exposure to PCBs results in a variety of deleterious health effects including immunosuppression, neurotoxicity, genotoxicity, endocrine effects, and they are classified as human carcinogens due to the strong evidence of their initiating and promoting activity (ATSDR 2000; Lauby-Secretan et al. 2013).

**Telomeres, telomerase, stem cells and cancer**

Telomeres, telomerase and cancer

A telomere is a 5-10 kb repetitive DNA sequence that caps the end of chromosomes of most eukaryotic organisms to avoid their deterioration or fusion with other chromosomes. Eukaryotic nuclear genomes are organized into a variable number of chromosomes each consisting of a single linear DNA molecule. In contrast, the genomes of all archaebacteria and the majority of bacteria are retained in circular DNA replicons. This difference represents an important evolutionary innovation and consequently advances nuclear expansion and increases complexity of eukaryotic genomes (Fulcher et al. 2013). Chromosome linearity, however, poses a major challenge to the internal cellular machinery that requires a unique solution. The conventional DNA replication machinery lacks a mechanism to fill in 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 requirement of the gradual attrition of telomeric DNA over multiple cell divisions.

A ribonucleoprotein complex, telomerase is an enzyme that adds the telomeric sequence (TTAGGG in mammals) to the end of DNA strands in the telomere regions in order to prevent the loss of important DNA from chromosome ends during normal DNA
replication (Cerni 2000). The telomerase reverse transcriptase (TERT, in humans called hTERT), is the enzymatic component of telomerase, which plays its role by using an intrinsic RNA component (TR, hTR in human) as a template. TEP1 (telomerase-associated protein 1), hsp90 (heat shock protein 90), p23, and dyskerin are other important components of the telomerase complex (Figure 1-2.) (Wojtyla et al. 2011).

![Human telomerase complex](image)

**Figure 1-2. Human telomerase complex.** NHP2, NOP10, GAR1, members of the H/ACA snoRNPs (small nucleolar ribonucleoproteins) (Wojtyla et al. 2011).

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 (Figure 1-3) and protects the ends of chromosomes from being recognized as double strand breaks and regulates telomerase-dependent telomere elongation (D Liu et al. 2004; Ye et al. 2014). Abnormal telomere shortening can result from toxic effects of chemicals and toxicants (Lansdorp 2005).

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1 Springer and Molecular Biology Reports, 38, 2011, 3339-3349, Human telomerase activity regulation, Aneta Wojtyla, Marta Gladych, Blazej Rubis, Fig. 1, © The Author(s) 2010, is given to the publication in which the material was originally published, with kind permission from Springer Science and Business Media.
Most somatic cells do not have telomerase activity and thus telomeres are progressively shortened during cell division. This eventually leads to aging and cell senescence (Allsopp et al. 1995). Such a mechanism is thought to prevent genomic instability and development of cancer in human aged cells by limiting the number of cell divisions. However, shortened telomeres impair immune function, which might also increase cancer susceptibility (Eisenberg 2011). Studies using knockout mice have demonstrated that the role of telomeres in cancer can both limit tumor growth, as well as promote tumorigenesis, depending on the cell type and genomic context (Chin et al. 1999; Greenberg et al. 1999). On the other hand, malignant cells become immortalized with stabilized telomeres due mostly to the activation of telomerase. Shay and Bacchetti have shown that telomerase activation is observed in ~90% of cancers (Shay and Bacchetti 1997). These indicate that telomeres and telomerase activity are highly correlated to tumor development (Bailey and Murnane 2006; Kim et al. 1994; Stewart et al. 2002; Wu et al. 2003).
Stem cells and cancer

Stem cells are functionally defined by two important characteristics: the capacity of self-renewal and differentiation into mature tissue. Stem cells may divide either asymmetrically or symmetrically, producing a novel stem cell, progenitor cells, transit cells, transit amplifying cells, or terminally differentiated cells (Zhang and Ju 2010). There are two types of stem cells in mammals: embryonic stem cells isolated from the inner cell mass of blastocysts, and adult stem cells found in various tissues, including bone marrow, liver, and skin. In a developing embryo, stem cells are pluripotent cells that can differentiate into all the specialized cells, while in adult organisms, stem cells and progenitor cells act as a repair system for the body, replenishing adult tissues.

Many diseases are highly correlated to stem cell dysfunction, such as loss of immune function, accelerated aging and cancer (Symonds et al. 2009; von Figura and Rudolph 2009). One of the molecular characteristics of stem cells is the retention of telomerase activity. However, telomerase activity is not sufficient to maintain stable telomeres in aging stem cells in humans, and telomere shortening may contribute to the accumulation of DNA damage in adult stem cells during aging (Vaziri et al. 1994). Telomere attrition and telomerase dysfunction are reported to be involved in stem cell dysfunction (failure to self-renew and differentiate) (Zhang and Ju 2010) and immune system damage (Eisenberg 2011).

Hematopoietic stem cells (HSCs) are important for the immune function. However, telomerase expression in HSCs is lower compared to the germline cells or other stem cells, which means that it cannot fully compensate for the shortening of telomeres during cell division. Similar to most other cancer cells, in malignant hematopoietic disorders,
telomerase activity is much higher with the highest level found in acute leukemias and NHLs (Norrback and Roos 1997). Dyskeratosis congenita (DKC) is one of the diseases that relate to telomerase dysfunction. DKC patients have mutations in six genes that encode components of both the telomerase complex and telomere shelterin complex, which results in reduced telomerase activity, premature telomere shortening, and bone marrow failure or malignancy prior to age 50 (Greenwood and Lansdorp 2003; Panichareon et al. 2015). Gradual loss of telomeres due to aging and rapid proliferation of HSCs might account for immunosenescence, exhausted hematopoiesis, and increased risk of malignant transformation (Ohyashiki et al. 2002). In chronic myeloid leukemia, telomere length is significantly shorter in granulocytes compared to T lymphocytes from the same individuals (Brummendorf et al. 2000). Therefore, telomerase and telomeres in HSCs are highly correlated to HSC aging and bone marrow failure and malignancies.

PCBs effects on telomeres and telomerase

Although adverse effects of PCBs have already been a focus of research for several decades, very few studies have been conducted to investigate the effects of PCBs on telomerase and telomeres. The only studies about this are from our laboratory using mammalian cell lines. Jacobus et al. demonstrated that 2-(4’-chlorophenyl)-1,4-benzoquinone (PCB3pQ), one of the metabolites of 4-chlorobiphenyl (PCB3), significantly decreased telomere length in HaCaT cells (Jacobus et al. 2008). Senthilkumar et al. showed that PCB28, 52, 153 and the Chicago Airborne Mixture (CAM) of PCBs reduce telomerase activity and shorten telomere length after different exposure times in HaCaT cells (Senthilkumar et al. 2011;
Senthilkumar et al. 2012). However, the mechanisms of PCBs toxicity on telomerase and telomeres are not yet well known. Since telomeres and telomerase play an important role in the development of cancer, stem cell dysfunction, and aging, more studies were needed to investigate how PCBs regulate telomerase activity and telomere length, especially in stem cells and progenitor cells, which might provide valuable information in these areas.

PCBs effects on stem cells

PCBs have been reported to be toxic to different types of stem cells and progenitor cells in humans and animals, including human cord blood progenitor cells, adipose-derived stem cells, and neural stem and progenitor cells. Van Den Heuvel et al. demonstrated that environmental compounds, such as benzo[a]pyrene (BaP), PCB126 and lead are hematotoxic to human progenitor cells derived from cord blood and to murine bone marrow cells, with human cells more sensitive than murine cells (Van Den Heuvel et al. 2001). A study using human multipotent adipose-derived stem cells exposed to TCDD, PCB126 and PCB153 showed that genes that are involved in the inflammatory/immune response are the most significantly regulated genes by these compounds and the precursor cells are more sensitive than adipocytes (Kim et al. 2012). This study also showed that the AhR ligands TCDD and PCB126 have more adverse effects than non-dioxin-like PCB153. But PCB153 is more neurotoxic as reported in Kang’s study (Kang et al. 2001). They exposed rat brain neural stem cells to PCB153 and endosulfan, and the results suggest that these compounds can inhibit differentiation and proliferation of neural stem cells. However, no study has investigated the toxic effects of PCBs on telomerase and telomeres in hematopoietic stem cells and progenitor cells.
**Regulation of telomerase activity and the TERT gene**

Telomerase is only expressed in stem cells, not in normal somatic cells, and it is also reactivated in 90% of cancer cells (Shay and Bacchetti 1997). Telomerase dysfunction is highly correlated to many other diseases besides cancer, such as premature aging, heart disease, and diabetes (Demerath et al. 2004; Qi Nan et al. 2015). Therefore, the regulation of telomerase activity and expression seems to be one of the most crucial factors to improve therapies and prevention for cancer and disease. Since the telomerase complex has multiple subunits, telomerase can be regulated at various levels, including transcription, alternative splicing, chaperone-mediated folding, phosphorylation, nuclear translocation of each subunit, assembly of the telomerase complex, and its accessibility to telomeres (Chan and Chang 2010; Cong et al. 2002; Gladych et al. 2011; Wojtyla et al. 2011). However, telomerase activity is usually correlated with the expression of TERT gene, which is generally repressed in normal cells and upregulated in immortal cells (Cong et al. 2002). Therefore, the transcriptional regulation of TERT gene, which encodes the enzymatic subunit of telomerase, seems very important to the regulation of telomerase.

**hTERT gene regulation**

Studies have found that activation of human TERT (hTERT) gene transcription is the dominant, rate-limiting step in telomerase activation. Several proto-oncogenes and tumor suppressor genes have been implicated in the regulation of hTERT gene expression including c-myc, a proto-oncogene product that activates telomerase by direct binding to the E-boxes of the TERT promoter (Wang et al. 1998; Wu et al. 1999). Repression of
TERT transcription may be accomplished by inhibiting c-myc via TGFβ/Smad3 signaling pathway both in human and rat (Hu et al. 2006; Lacerte et al. 2008). TCDD has been shown to regulate TERT and telomerase activity through c-myc signaling (Fiorito et al. 2013; Sarkar et al. 2006). AhR is reported to influence the expression of c-myc at both mRNA and protein levels (Yang et al. 2005), which indicates that it could be involved in the regulation of TERT. There are many other factors that regulate TERT expression as shown in Wojtyla’s review (Wojtyla et al. 2011). However, no study has reported that the AhR signaling pathway can play a role in transcription of TERT. When one searches for the transcription factor binding sites on the Qiagen website, it is found that there is one AhR/ARNT binding site shown in the promotor region of hTERT gene (as shown in Figure 1-4) 


**Figure 1-4.** Transcription factors and binding sites in the promoter of hTERT gene. (https://www.sabiosciences.com/chipqpcrsearch.php?factor=Over+200+TF&species_id=0 &ninfo=n&ngene=n&nfactor=y&gene=TERT)
The aryl hydrocarbon receptor signaling pathway

AhR is a transcription factor known to regulate transcription of drug-metabolizing enzymes [cytochrome P450 1A1 (CYP1A1), CYP1A2, CYP1B1, glutathione S-transferase (GST), and UDP-glucuronosyltransferase (UGT)] and the expression of genes involved in cell growth control (e.g., transforming growth factor-α and -β, B-Jun, and Jun-D). Upon ligand binding, AhR translocates into the nucleus, dissociates from several cytoplasmic chaperone proteins including heat shock protein 90 (HSP90) and XAP-2 (Carver and Bradfield 1997; Lees and Whitelaw 1999; Perdew 1988), heterodimerizes with the aryl hydrocarbon nuclear translocator (ARNT) protein (Lees and Whitelaw 1999) and then binds to DNA response elements known as the xenobiotic/dioxin response element (XRE/DRE) on the target genes mentioned above (Kewley et al. 2004; Lees and Whitelaw 1999). The XRE core sequence has been identified as 5’-TNGCGTG-3’ (Whitlock et al. 1996). AhR can be activated by exposure to various endogenous and exogenous ligands. Bilirubin or various tryptophan metabolites are considered as endogenous ligands for AhR. Exogenous chemicals that serve as ligands of AhR include TCDD, B(a)P, 3-methylcholanthrene (3MC), β-naphthoflavone (β-NF) and dioxin-like PCBs (Denison and Nagy 2003).

Studies in mice expressing a constitutively active AhR indicate that the AhR plays a pivotal role in tumor promotion during liver carcinogenesis. Stevens et al. reported that inappropriate activation of AhR resulted in immunosuppression in different animal models (Stevens et al. 2009). AhR has also been shown to be involved in the endogenous development during hematopoietic stem-cell maintenance and differentiation (Casado et al. 2011). TCDD and related dioxins, which can activate the AhR, were correlated with
increased risk for certain hematological diseases such as NHL, chronic lymphocytic leukemia and multiple myeloma in humans (Casado et al. 2011; Frumkin 2003). However, the molecular mechanisms of AhR-dependent tumor promotion and regulation of telomerase in stem cells are still poorly understood, especially under the sustained activation status by exposure to PCB126.

The hypoxia pathway

The hypoxia pathway is an important sensor of changes in oxygen level that can happen during various physiological and pathological conditions. Hypoxia-inducible factor-1α (HIF-1α) is the key factor that regulates the cellular responses to hypoxia. Similar to AhR pathway, when hypoxia occurs, HIF-1α also binds with ARNT to serve as a transcriptional regulator of hypoxia-inducible genes (Kewley et al. 2004). In normal oxygen conditions, prolyl hydroxylase domain-containing enzymes (PHDs) continuously hydroxylate HIF-1α to degrade it. During hypoxia, HIF-1α escapes from hydroxylation, translocates into the nucleus, dimerizes with ARNT and binds to the hypoxia response element (HRE) of target genes to regulate their expression (Majmundar et al. 2010).

In cancer, hypoxia-inducible factors (HIFs) are disrupted and exhibit increased levels (Talks et al. 2000; Zhong et al. 1999). Alteration of HIF activity in tumors results from the hypoxic environment within solid tumors and also from various genetic changes. Target genes of HIF play crucial roles in cancer biology, such as cell survival, metabolism, invasion and metastasis (Pouyssegur et al. 2006; Semenza 2010). HIFs are also correlated to many diseases in the circulatory system, like ischemic disease (Hashimoto and Shibasaki 2015). The hypoxia pathway has clinical significance because
the hypoxic response correlates with tumor progression and resistance to therapy (Vaupel and Mayer 2007).

Connection between the AhR pathway and the hypoxia pathway

Both AhR and hypoxia pathways are important for the response to changes in the environment. The AhR pathway is involved in the responses to exposure to environmental chemicals whereas the hypoxia pathway mediates responses to low oxygen conditions. To perform their functions, both AhR and HIF-1α need to heterodimerize with ARNT (Kewley et al. 2004), which has been shown to bind to the nucleotide sequence 5’-GTG-3’ present in both the XRE and HRE core sequence (Bacsi et al. 1995). Since ARNT is a key factor for these two pathways, there is a possible crosstalk between them. Activation of the hypoxia pathway may cause HIF-1α to bind with ARNT and consequently inhibits the activation of AhR in response to a PCB ligand (Vorrink et al. 2014b). On the contrary, AhR activation by PCB exposure may inhibit HIF-1α mediated responses in low oxygen conditions (Vorrink et al. 2014a).

Many studies have investigated the competition of ARNT between AhR and hypoxia pathways. To clarify the role of ARNT in the two pathways, Schults et al. showed in a study using human A549 cells (human epithelial lung carcinoma cells) that the formation of AhR/ARNT was reduced in CoCl₂-induced hypoxia-like conditions and conversely B(a)P inhibited the formation of HIF-1α/ARNT heterodimers (Schults et al. 2010). Another study by Gradin et al. demonstrated that human HeLa cells with overexpression of HIF-1α also showed decrease of AhR/ARNT heterodimer after exposure to TCDD, whereas HIF-1α/ARNT formation was not affected by TCDD.
exposure, which indicates that HIF-1α has a higher affinity for ARNT (Gradin et al. 1996). Similar results were also reported by Khan et al. (Khan et al. 2007), where it was shown that hypoxia decreased CYP1A1 mRNA and protein levels after TCDD exposure in human cells, but overexpression of ARNT did not attenuate this effect. These data suggest that HIF-1α dominates over AhR when competing for ARNT.

Though numerous studies regarding the competition of ARNT between the two pathways have already been reported, most used TCDD as a ligand for AhR activation. Only a few studies used PCB126 as a test compound. And no study has ever investigated how the hypoxia pathway affects the regulation of hTERT gene by PCB126.

**Hypothesis**

PCBs have been shown to have diverse adverse health effects, including reproductive problems, neurobehavioral problems, immunotoxicity and increased risk of cancer. PCBs are known to affect telomere length and telomerase activity in HaCat cells, although the mechanism is not yet understood. Telomerase is activated in stem cells, proliferating tissues and most cancer cells. Many diseases are highly correlated to stem cell dysfunction, such as failure of organ renewal, aging and cancer. Telomere attrition and telomerase dysregulation are reported to be involved in stem cell dysfunction (failure to self-renew and differentiate) and aging. Understanding the mechanism of how PCBs regulate telomere length and telomerase activity and affect differentiation in stem cells and progenitor cells may provide important insight into the onset and development of many diseases. The studies described in this dissertation are designed to address the hypothesis that PCBs interfere with the maintenance of telomeres and telomerase in
stem cells and progenitor cells and disrupt their differentiation and proliferation.
The long term goal is to understand the possible toxic effects of PCBs on stem cells and progenitor cells and the underlying mechanisms.

Specific Aim 1: Analyze *in vitro* effects of PCBs on the telomere complex and differentiation of progenitor cells

Specific Aim 1 was designed to test the effects of PCB126 and 153 on telomere maintenance and differentiation of human myeloblastic/premyelocytic leukemia cell line (HL-60). This aim was divided into two sub aims: 1) to investigate how PCBs affect the differentiation of hematopoietic HL-60 cells; 2) to assess the effects of long term exposure to PCBs on telomerase activity, telomere length and their related gene expression in undifferentiated HL-60 cells.

The data presented in Chapter 2 show that PCB126 has a more pronounced effect of inhibiting telomerase activity and telomere length than PCB153 in undifferentiated HL-60 cells. But the two congeners produced different effects on telomerase and telomere related gene expression. 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 into granulocytes or monocytes produced profound G0/G1 phase arrest and almost nondetectable telomerase activity and hTERT mRNA levels, but differences were seen between granulocytes and monocytes regarding hTR, TRF1, TRF2, and POT1 gene expression. 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. They may have a strong negative effect on telomere maintenance and thereby function and longevity of hematopoietic stem/progenitor-like cells.

Specific Aim 2: Examine in vivo effects of PCB126 on the differentiation and telomerase in rat bone marrow cells

Closely related to the previous aim, I asked whether treatment of rats with PCB126 could reduce telomerase activity and affect the differentiation in stem cells. Specific Aim 2 was designed to elucidate the effects of PCB126 on the telomerase activity and differentiation of rat bone marrow cells.

In Chapter 3 I show that PCB126 reduced telomerase activity in bone marrow cells, which is likely due to the decrease of TERT mRNA and protein expression. I also observed a promotional effect of PCB126 on the myelocytic differentiation of the bone marrow stem cells, accompanied with the decreased number of stem cells and the increased number of downstream differentiated cells, which is consistent with the reduced telomerase activity and decreased TERT expression in stem cells. These indicate that PCB126 could interfere with the proliferation of stem cells by downregulation of telomerase activity and TERT gene expression, and promote the differentiation of stem cells, which results in premature aging of these cells with potentially harmful consequences for the function of the affected organ, like the hematopoietic and immune system.
Specific Aim 3: Elucidate the mechanism of PCB126 regulation of the hTERT gene expression

Specific Aim 3 was designed to determine the mechanism of PCB126 regulation of the hTERT gene through the AhR pathway and whether this effect could be disturbed by hypoxia pathway activation.

The results in Chapter 4 show that the AhR pathway activation by PCB126 decreased hTERT gene expression which indicates that AhR/ARNT could be the key factor for the regulation of hTERT. This is verified by the employment of the AhR antagonists that reversed the decrease of hTERT gene expression by PCB126. CoCl\(_2\)-induced hypoxia-like condition enhanced the reduction of PCB126 on hTERT gene expression when combining the treatment, which indicated there might be a competition with ARNT between the two pathways. siRNA knock down AhR and/or ARNT suggested that both of them are playing an important negative role in the regulation of hTERT expression. However, there is no AhR or ARNT binding site on the analyzed promoter region of hTERT using the ChIP assay technology, which suggested that PCB126 indirectly regulated hTERT gene expression through AhR pathway, probably by regulating other transcription factors of hTERT through AhR.

Summary

The successful completion of the above described Specific Aims will provide substantial insights into the interrelationships and involvement of telomerase, telomere and AhR pathway on the proliferation and differentiation of hematopoietic stem and
progenitor cells and may provide new avenues and possibilities for further studies that will aid our understanding of the deleterious biological effects of PCB exposure.
CHAPTER 2 ANALYZE IN VITRO EFFECTS OF PCBS ON THE TELOMERE COMPLEX AND DIFFERENTIATION OF PROGENITOR CELLS²

Abstract

PCBs are persistent organic pollutants that are carcinogenic, immunotoxic and have 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 be differentiated into 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 POT1 were upregulated in granulocytes, TRF2 was up- and POT1 down regulated in monocytes. Both PCBs further reduced telomerase activity in differentiated cells, but had only small effects on the differentiation and telomere-related genes. Treatment of undifferentiated HL-60 cells for 30 days 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

² With permission of Springer, this chapter was reproduced from accepted manuscript: Xin X, Senthilkumar PK, Schnoor JL, Ludewig G. 2015. Effects of PCB126 and PCB153 on telomerase activity and telomere length in undifferentiated and differentiated HL-60 cells. Environmental science and pollution research international. DOI: 10.1007/s11356-015-5187-y. Copyright: © Springer-Verlag Berlin Heidelberg 2015. My role in this work was to conduct all the experiments, analyze data and prepare the manuscript.
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 telomere/telomerase-related genes. Although PCBs do not seem to strongly affect differentiation, they may influence stem or progenitor cells through telomere attrition with potential long-term consequences for health.

**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-Secrétan et al. 2013). PCBs are persistent organic pollutants which bioaccumulate in 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-Secrétan 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 prevent 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 5’ ends of the DNA leading strands (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 (D Liu et al. 2004; 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 and Bacchetti 1997), which indicates that telomerase activity and telomere length are highly correlated to tumor development (Bailey and 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 immortal human skin 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 congener, 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 predominant congener in human foods, like fish or mothers’ milk, and in human serum and adipose tissue (Robertson and 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/premyelocytic 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 into 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 POT1, and only PCB126 significantly shortened telomere length. The PCBs also slightly enhanced the massive reduction of telomerase activity and hTERT expression caused by differentiation into 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 Toecris 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 and PCB153 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 and PCB exposure, and toxicity determination

The HL-60 cell line 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% v/v DMSO, final concentration) for 30 days. Media with test compounds or solvent were changed every three days. Cells were counted, and reseeded 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 (2×10^5 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 2×10^5 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 and 1 µM ATRA (differentiation into granulocyte), or 16 nM PMA (differentiation into 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 6 days before and 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 cytopsin 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 $1 \times 10^6$ were washed twice with cold staining buffer with BSA (Cat #554657, BD PharMingen, San Diego, CA), resuspended in 100 µl 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 µl 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 µM) in HBSS for 2h at 37°C.

Analysis of cell cycle distribution

In the long-term exposure experiment, approximately $1 \times 10^6$ cells per sample were periodically removed, washed with PBS, fixed with 70% ethanol overnight, washed once with PBS, and treated with RNAse (100µg/ml) for 30 min and PI (50µg/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 250×g for 5 min, 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 4ºC. 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. The cell lysate were centrifuged at 16,000×g for 20 min at 4 ºC and three fourths of the supernatant was transferred into new microcentrifuge 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). After adding 100 ng (0.2 µl) of Telomerase Primer (TS), 50 ng (0.1 µl) of Anchored Return Primer (ACX) (Kim and Wu 1997), 2.5 µl cell lysate supernatant (40ng/µl), 12.5 µl Real MasterMix Fast SYBR (5 PRIME Inc., Gaithersburg, MD, USA) and 9.7 µl 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. qPCR was performed by incubating the sample at 95ºC for 10 min followed by 35 cycles of 95 ºC
for 30 s and 60 °C for 90 s using an Eppendorf RealPlex Thermal Cycler (Eppendorf, Hamburg, Germany). The Ct (threshold cycle value) was established from the semilog 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 separate wells of a 96-well plate with 4.25 μl of telomere buffer and 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. Plates were 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 1 min 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 an RNaseasy 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 using the high capacity cDNA reverse transcription kit from Applied Biocystems (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 2-1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 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: \[ \Delta C_T \text{(sample)} = C_T \text{(mRNA of interest)} - C_T \text{(GAPDH)} \]; \[ \Delta\Delta C_T = \Delta C_T \text{(treatment)} - \Delta C_T \text{(control)} \]; relative expression = \(2^{-\Delta\Delta C_T}\).

Statistical analysis

All experiments (except differentiation experiment with continuous exposure to PCBs) were performed at least twice in duplicate or triplicate, and every individual qRT-PCR sample was assayed in duplicate. Data from two experiments were combined and analyzed. All data are expressed as the mean ± 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.

**Table 2-1. Primer pairs used for qRT-PCR analysis**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS</td>
<td>Forward: AATCCGTCGAGCAGAGTT</td>
<td>(Wege et al. 2003)</td>
</tr>
<tr>
<td>ACX</td>
<td>Reverse: GCGCGGCTTACCCTTACCCCTACCTAACC</td>
<td></td>
</tr>
<tr>
<td>Tel1</td>
<td>Forward: GGTITTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT</td>
<td></td>
</tr>
<tr>
<td>Tel2</td>
<td>Reverse: TCCCAGCTATCCCTATCCCTATCCCTATCCCTATCCCTA</td>
<td>(Cawthon 2002)</td>
</tr>
<tr>
<td>36B4u</td>
<td>Forward: CAGCAAGTGGAAGGTGAATCC</td>
<td></td>
</tr>
<tr>
<td>36B4d</td>
<td>Reverse: CCCATTCTATCATCAACGCGGTACAA</td>
<td></td>
</tr>
<tr>
<td>hTERT</td>
<td>Forward: TGTACTTTTGCAAGGGTGATGTGA</td>
<td>(Gourronc et al. 2010)</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCTGGAGGTCTGTCAAGGGTGAG</td>
<td></td>
</tr>
<tr>
<td>hTR</td>
<td>Forward: TCTAACCTAACTGAGAAGGCGGTAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: GTTTGCTCTAGAATGAACGGGTTGAGA</td>
<td></td>
</tr>
<tr>
<td>TRF1</td>
<td>Forward: GGCAGCGGCAAAAGTAGT</td>
<td>Designed using Primer 3</td>
</tr>
<tr>
<td></td>
<td>Reverse: GTCTTGTGGCTGGGTTCCAT</td>
<td></td>
</tr>
<tr>
<td>TRF2</td>
<td>Forward: GTACCCAAAGGCAAGTGGGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: TGACCCACTCAGTCTTTCTTC</td>
<td></td>
</tr>
<tr>
<td>POT1</td>
<td>Forward: CCTTACGTGTTTGGCGCATC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: TTTGAGGCGATGGATGTA</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: AAGGTCATCCGACAAACTTTT</td>
<td>(Gourronc et al. 2010)</td>
</tr>
<tr>
<td></td>
<td>Reverse: GTAGAGGGAGGGATGATTCT</td>
<td></td>
</tr>
</tbody>
</table>
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 and 20 µM, Figure 2-1). No cytotoxicity was observed with any concentration of PCB153. Therefore, 5 µM PCB126 or PCB153 were used in the following experiments.

Figure 2-1. PCB126 reduces viable cells at concentrations above 10 µM; PCB153 has no effect on cell number. HL-60 cells were exposed to 2-20 µM of the PCBs for 6 days with one medium and compound change on day 3. Cytotoxicity was measured using the resazurin reduction assay.

HL-60 cell differentiation into monocytes or granulocytes and the effect of preexposure to PCBs

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

![Figure 2-2](image.png)

**Figure 2-2. 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 into granulocytes (DMSO and ATRA) or monocytes/macrophages (PMA) was clearly visible, but no effect of either PCB congeners on differentiation was observed. (Magnification x400)

To confirm the differentiation, the morphologic phenotypes were evaluated. As shown in Figure 2-2, 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 and 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 pretreatment had no significant effects on cell morphological 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 2-2).

### Table 2-2. Cell numbers after differentiation with PCBs pretreatment for 6 days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell number (×10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1 - 6</td>
</tr>
<tr>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>DMSO and ATRA</td>
</tr>
<tr>
<td></td>
<td>PMA</td>
</tr>
<tr>
<td>PCB 126</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>DMSO and ATRA</td>
</tr>
<tr>
<td></td>
<td>PMA</td>
</tr>
<tr>
<td>PCB 153</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>DMSO and ATRA</td>
</tr>
<tr>
<td></td>
<td>PMA</td>
</tr>
</tbody>
</table>

HL-60 cells were pretreated for 6 days with PCBs or solvent alone, after which each sample was divided into 3 groups and treated for 3 days with solvent, DMSO and ATRA, or PMA before cell number per sample was determined by cell count. # p<0.05 compared with corresponding group (same shading) in overall Control block, indicating effect of PCB.

To further confirm the 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. Figure 2-3 shows the extremely high expression of CD11b in differentiated compared with undifferentiated groups. Pretreatment with PCB126 had no effect on CD11b 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.

Figure 2-3. Cells induced to differentiate expressed the differentiation marker CD11b; PCB153 pretreatment reduced CD11b in monocyte cultures. HL-60 cells were treated with 5 µM PCBs for 6 days before exposure for 3 days to 1.25% (v/v) DMSO and 1 µM 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 the PMA control group (effect of PCB153).

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 and ATRA and PMA
treatment, respectively (Table 2-3). PCB126 pretreatment had no influence on cell cycle distribution in differentiated cells. PCB153 significantly increased S phase cells compared to the 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.

Table 2-3. Induction of differentiation causes cell cycle arrests in G0/G1 phase but PCBs have no effect on the cell cycle distribution

<table>
<thead>
<tr>
<th>Treatment</th>
<th>G0/G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td>Pre- (6 days)</td>
<td>Post- (3 days)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>68.35</td>
<td>0.76</td>
<td>13.01</td>
</tr>
<tr>
<td>DMSO and ATRA</td>
<td>86.49*</td>
<td>1.36</td>
<td>2.48*</td>
</tr>
<tr>
<td>PMA</td>
<td>85.95*</td>
<td>7.07</td>
<td>1.24*</td>
</tr>
<tr>
<td>PCB126</td>
<td>Control</td>
<td>67.74</td>
<td>1.55</td>
</tr>
<tr>
<td>DMSO and ATRA</td>
<td>87.98*</td>
<td>1.52</td>
<td>2.31*</td>
</tr>
<tr>
<td>PMA</td>
<td>84.81*</td>
<td>8.33</td>
<td>0.85*</td>
</tr>
<tr>
<td>PCB153</td>
<td>Control</td>
<td>66.05</td>
<td>4.47</td>
</tr>
<tr>
<td>DMSO and ATRA</td>
<td>86.37*</td>
<td>1.42</td>
<td>4.12*</td>
</tr>
<tr>
<td>PMA</td>
<td>87.00*</td>
<td>5.55</td>
<td>1.13*</td>
</tr>
</tbody>
</table>

HL-60 cells were exposed to solvent or PCBs for 6 days and divided into three groups, and one sample of each group was treated with solvent, DMSO and ATRA (granulocyte), or PMA (monocyte induction). * 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.
Telomerase activity and telomerase gene expression after differentiation

Progenitor cells express telomerase activity, while differentiated cells usually don’t. We examined the effects of preexposure to PCBs on this change in telomerase activity after differentiation of HL-60 cells. As expected, telomerase activity was significantly decreased after differentiation into granulocytes or monocytes/macrophages compared with the undifferentiated cells (Figure 2-4A). Both PCBs reduced telomerase activity (nonsignificantly), 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 and ATRA cells pretreated with PCB126 (Figure 2-4B). Otherwise, pretreatment with PCBs had no effect on hTERT. hTR gene expression was significantly increased after induced to differentiate into granulocytes (DMSO and ATRA), while exposure to PMA or preexposure to PCBs produced no significant changes (Figure 2-4C).

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 POT was also determined. TRF1 mRNA level was significantly upregulated after differentiation into granulocytes and nonsignificantly elevated in monocytes (Figure 2-4D). After granulocytic differentiation, PCB126 and 153 both significantly increased TRF1 gene expression in granulocytes while after monocytic differentiation, both PCB congeners significantly downregulated TRF1 gene expression. TRF2 gene expression was significantly increased after monocytic differentiation by PMA treatment, while only a nonsignificant elevation was seen in granulocytes, which was significantly reduced by PCB153 (Figure 2-4E). The POT1 mRNA level was nonsignificantly elevated after differentiation into granulocytes and nonsignificantly reduced after differentiation into monocytes, an effect that became significant in the PCB153-pretreated group (Figure 2-4F).

Figure 2-4. Differentiation reduced telomerase activity and hTERT expression in HL-60 cells and increased TRF1 and TRF2 expression; PCB pretreatment (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 and 1 μM 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.
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 3 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 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 2-4). A significant effect of PCBs on telomerase activity and hTERT in undifferentiated controls was visible with this exposure regimen (Figure 2-5A, B). As in the previous experiment, both PCBs congeners further reduced telomerase activity in differentiated cells which was significant in the monocytes (Figure 2-5A), while no significant effect was seen on hTERT expression (Figure 2-5B). The increase in hTR in granulocytes was significantly reduced, an effect that had been nonsignificant if PCBs were removed during the last 3 days of differentiation. In granulocytes, TRF1 and POT1 expressions were reduced by PCB treatment (Figure 2-5C, D and F). In monocytes, PCB153 upregulated TRF2 gene expression and PCB126 downregulated POT 1 (Figure 2-5E, F). 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.
Table 2-4. Cell numbers after differentiation with PCBs treatment before and during differentiation for 9 days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell number (x 10^6)</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1 - 9</td>
<td>Day 7 - 9</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Control</td>
<td>17.28 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>DMSO and ATRA</td>
<td><strong>6.14 ± 0.08</strong></td>
</tr>
<tr>
<td></td>
<td>PMA</td>
<td><strong>2.29 ± 0.03</strong></td>
</tr>
<tr>
<td>PCB 126</td>
<td>Control</td>
<td>15.82* ± 0.07</td>
</tr>
<tr>
<td></td>
<td>DMSO and ATRA</td>
<td><em><em>5.57</em> ± 0.05</em>*</td>
</tr>
<tr>
<td></td>
<td>PMA</td>
<td><em><em>2.12</em> ± 0.03</em>*</td>
</tr>
<tr>
<td>PCB 153</td>
<td>Control</td>
<td>17.71* ± 0.26</td>
</tr>
<tr>
<td></td>
<td>DMSO and ATRA</td>
<td><em><em>7.05</em> ± 0.07</em>*</td>
</tr>
<tr>
<td></td>
<td>PMA</td>
<td><em><em>2.47</em> ± 0.02</em>*</td>
</tr>
</tbody>
</table>

HL-60 cells were pretreated for 9 days with PCBs or solvent alone. During the last 3 days solvent, DMSO and ATRA, or PMA was added to induce cell differentiation. Cell number per sample was determined by cell count at the end of the differentiation phase. *p<0.05 compared with corresponding group (same shading) in overall Control block, indicating effect of PCBs.
Figure 2-5. 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 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.

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 μM 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 (Figure 2-6, Table 2-5). 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.

Figure 2-6. Continuous exposure to 5 µM PCB126 or PCB153 for 30 days did not have a significant effect on cell numbers. HL-60 cells were continuously exposed to 5 µM PCBs for 30 days with regular medium plus compound change every third day and cell count determination with subculturing every sixth day.

Table 2-5. PCB126 or PCB153 at 5 µM concentration has no influence on cell cycle distribution

<table>
<thead>
<tr>
<th>Time</th>
<th>Treatment</th>
<th>G0/G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td>Day 6</td>
<td>Control</td>
<td>39.57</td>
<td>0.07</td>
<td>51.70</td>
</tr>
<tr>
<td></td>
<td>PCB 126</td>
<td>40.33</td>
<td>0.16</td>
<td>51.09</td>
</tr>
<tr>
<td></td>
<td>PCB 153</td>
<td>37.90</td>
<td>1.79</td>
<td>51.89</td>
</tr>
<tr>
<td>Day 12</td>
<td>Control</td>
<td>40.30</td>
<td>0.54</td>
<td>53.24</td>
</tr>
<tr>
<td></td>
<td>PCB 126</td>
<td>42.32</td>
<td>1.18</td>
<td>50.95</td>
</tr>
<tr>
<td></td>
<td>PCB 153</td>
<td>40.14</td>
<td>1.09</td>
<td>52.73</td>
</tr>
<tr>
<td>Day 30</td>
<td>Control</td>
<td>37.88</td>
<td>0.97</td>
<td>55.02</td>
</tr>
<tr>
<td></td>
<td>PCB 126</td>
<td>39.04</td>
<td>1.91</td>
<td>53.24</td>
</tr>
<tr>
<td></td>
<td>PCB 153</td>
<td>39.08</td>
<td>2.31</td>
<td>53.63</td>
</tr>
</tbody>
</table>

HL-60 cells were continuously exposed to 5 µM of the PCBs for 30 days with regular medium-plus-compound change every third day and subculturing every sixth day.
Telomerase activity and telomere length in undifferentiated HL-60 cells after long-term exposure to PCBs

HL-60 cells were treated with 5 µM PCB126 or PCB153 continuously for 30 days, and telomerase activity was determined in cells after 6 and 30 days of exposure. As shown in Figure 2-7A, 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 (Figure 2-7B). 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).

![Graphs showing telomerase activity and telomere length](image)

Figure 2-7. PCB126 significantly reduced telomerase activity and telomere length after 30 days of exposure, and the effect of PCB153 on telomeres was less pronounced. HL-60 cells were exposed continuously to 5µM PCB126 or PCB153 for 30 days with one medium change every third day and subsulculturering every sixth day. A. Telomerase activity in HL-60 cells exposed for 30 days to 5µM PCB126 or PCB153 was reduced. B. Telomere length was significantly shortened by PCB126 after 30 days of continuous exposure; a similar PCB153 effect did not reach statistical significance. *p<0.05, compared to the control group.
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 downregulated 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 (Figure 2-8A,B). 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 downregulated 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 (Figure 2-9A-C). These results showed 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 2-6).

Figure 2-8. PCB126 and PCB153 reduced the expression of the telomerase components hTERT (A) and hTR (B) after 6 and 30 days. HL-60 cells were continuously exposed to 5 µM 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.
Figure 2-9. PCB126 and PCB153 modified gene expression of the telomere shelterin components TRF1 (A), TRF2 (B), and POT1 (C) in a time- and congener-specific way. HL-60 cells were continuously exposed with 5 μM 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 2-6. Comparison of the effects of different treatments on HL-60 cell parameters

<table>
<thead>
<tr>
<th></th>
<th>PCB126</th>
<th>PCB153</th>
<th>DMSO and ATRA (granulocyte differentiation)</th>
<th>PMA (monocyte differentiation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Telomerase activity</td>
<td>↓↓</td>
<td>↓</td>
<td>↓↓↓</td>
<td>↓↓↓↓</td>
</tr>
<tr>
<td>hTERT</td>
<td>↓↓</td>
<td>↓</td>
<td>↓↓↓</td>
<td>↓↓↓↓</td>
</tr>
<tr>
<td>hTR</td>
<td>↓ or O</td>
<td>↓ or O</td>
<td>↑↑</td>
<td>O</td>
</tr>
<tr>
<td>Go/G1 increase</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>TRF1</td>
<td>↓ (O later)</td>
<td>O or ↓</td>
<td>↑↑</td>
<td>O</td>
</tr>
<tr>
<td>TRF2</td>
<td>O</td>
<td>O (↑ later)</td>
<td>↑</td>
<td>↑↑</td>
</tr>
<tr>
<td>POT1</td>
<td>↓ or O</td>
<td>O (↑ later)</td>
<td>↑</td>
<td>↓</td>
</tr>
</tbody>
</table>

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

Discussion

PCBs are ubiquitous contaminants which bioaccumulate in organisms in fatty tissue 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, 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 (Kopec 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 and progenitor cells. HL-60 cells can also be induced to differentiate into granulocytes and monocytes by different inducers (Birnie 1988).

We obtained an efficient induction of differentiation of HL-60 cells into granulocytes or monocytes/macrophages after 3 days of exposure to DMSO and ATRA or PMA, respectively, accompanied with a dramatic loss of telomerase activity, a strong downregulation of hTERT gene expression, and an increase in G0/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; L Liu et al. 2004; 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 preexposure to either PCB congener and in monocytes by preexposure and continuous exposure during differentiation to these 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 had less hTERT but more hTR, TRF1 and TRF2 than malignant hematopoietic cells (Koyanagi et al. 2000). Preexposure 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 downregulation 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, preexposure 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 down regulated by PCB126. Whether this is related to the reduced expression of CD11b differentiation marker in PCB153-exposed monocytes or this is a
sign of reduced differentiation of progenitor cells into 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 downregulated 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 downregulated 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 downregulated 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 and 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. 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 into granulocytes or monocytes produced profound G0/G1 phase arrest and almost nondetectable 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 and 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.

Acknowledgments

We thank Dr. Hans Joachim Lehmler, The University of Iowa, for providing the PCB126 and PCB153, and the staff of the Flow Cytometry Core Facility of the
University of Iowa for their help in cell cycle analysis. This work was supported by the National Institute of Environmental Health Sciences Superfund Research Program, grant number P42 ES013661. X. Xin was supported from the Training Core of this grant.
Abstract

PCBs are persistent chemicals which are immunotoxic and correlated to childhood leukemia, Non-Hodgkin lymphoma and hematotoxicity. Dioxin-like PCBs, an exceptionally potent class of PCB, are carcinogenic possibly due to their ability to inappropriately activate the AhR and consequently stimulate the clonal expansion or inhibit the apoptosis of preneoplastic cells. The AhR is reported to be active in the control of hematopoietic stem cells (HSCs) and/or progenitor cells (HPCs) by regulating the balance between quiescence and proliferation. Similarly, telomerase malfunction and telomere attrition are involved in stem cell failure to self-renew or differentiate and immune system damage. Our hypothesis was that PCB126, a dioxin-like PCB, is hematotoxic by affecting telomerase activity and thereby proliferation and differentiation of HSCs and HPCs. Male Sprague-Dawley rats were treated with a single i.p. injection of a low or high dose (1 or 5 μmol/kg body weight) of PCB126. Two weeks later, bone marrow nucleated cells were isolated to determine the number of viable and functional progenitor cells, cell surface marker expression, telomerase activity and gene and protein expression. We observed that PCB126 significantly decreased telomerase activity and TERT gene expression and protein levels. Bone marrow HSC differentiation into the granulocytic direction was promoted, as indicated by increased numbers of CFU-G (colony forming unit – granulocytes) and CFU-GM (colony forming unit – granulocytes and macrophages), and increased number of differentiated granulocytes (CD11b positive...
cells). PCB126 did not influence the differentiation of bone marrow cells into macrophages. These results suggest that PCB126 exhibits hematotoxic effects through decreased proliferation of HSCs by reduction of telomerase activity and through modification of the differentiation pattern of HSCs.

**Introduction**

PCBs are lipophilic and persistent chemicals that are hard to degrade and which bioaccumulate in the environment and biomagnify in the food chain (Safe 1994). Despite the cessation of PCBs production over 30 years ago in most countries, PCBs still remain as ubiquitous contaminants. They are reported to be immunotoxic (Iwanowicz et al. 2009; Sormo et al. 2009; Tryphonas 1994) and associated with childhood leukemia (Ward et al. 2009) and Non-Hodgkin lymphoma (Bertrand et al. 2010; Hardell et al. 1996). Dioxin and dioxin-like PCBs are carcinogenic possibly due to their ability to inappropriately activate the AhR and consequently stimulate clonal expansion ofpreneoplastic cells or inhibit apoptosis of these cells (Singh et al. 2009). It is known that the AhR is involved in the regulation of cell cycle and proliferation *in vitro* and *in vivo*, and endogenous development functions during hematopoietic stem-cell maintenance and differentiation (Bock and Kohle 2005; Vogel et al. 2007). TCDD treatment significantly increased the number of HSC-enriched Lin⁻/Sca-1⁻/cKit⁺ (LSK) cells due to the presence of the AhR in hematopoietic cells (Murante and Gasiewicz 2000; Sakai et al. 2003). This indicates that inappropriate AhR activation by xenobiotic ligands may affect the ability of HSCs to proliferate and differentiate into mature lineage cells (Singh et al. 2009). However, the molecular mechanisms of AhR-dependent HSCs changes are still poorly
understood, especially under the sustained activation status by exposure to PCB126, which is the most potent dioxin-like PCB congener (Fukuzawa et al. 2003; Hassoun et al. 2001). Although many studies have investigated the mechanisms of its adverse effects, hardly any studies have focused on the toxicity of PCB126 in HSCs.

HSCs, which are located in the bone marrow and important for immune function, are one type of stem cells. Stem cell dysfunction is highly associated with many diseases, such as defective immune function, accelerated aging and cancer (Symonds et al. 2009; von Figura and Rudolph 2009). One of the molecular characteristics of stem cells is the retention of telomerase activity. However, telomerase activity is not sufficient to maintain stable telomeres in aging stem cells in humans, and telomere shortening may contribute to the accumulation of DNA damage in adult stem cells during aging (Vaziri et al. 1994). Telomere attrition and telomerase dysfunction, which are reported to be involved in stem cell dysfunction (failure to self-renew and differentiate) (Zhang and Ju 2010) and immune function injury (Eisenberg 2011), can also be induced by PCBs in HaCaT cells (Jacobus et al. 2008; Senthilkumar et al. 2011; Senthilkumar et al. 2012) and HL-60 cells (Xin et al. 2015). However, no study so far has investigated if PCB126 affects telomeres and telomerase of stem cells and impacts their differentiation capacity.

In this study, we investigated the effects of PCB126 on telomerase in rat bone marrow stem cells and progenitor cells, and the proliferation and differentiation capacity of these cells after exposure in vivo or/and in vitro. Male Sprague-Dawley rats received a single i.p. injection of PCB126 and two weeks later, bone marrow nucleated cells were isolated for different assays. Together, the data reported here indicate that PCB126 exhibits hematotoxic effects through decreased proliferation of HSCs, by reduction of
telomerase activity and downregulation of TERT expression, and through modification of the differentiation pattern of HSCs.

Materials and methods

Materials

PCB126 was prepared by an improved Suzuki-coupling method with 3,4,5-trichlorobromobenzene and 3.4-dichlorophenyl boronic acid utilizing a palladium-catalyzed cross-coupling reaction (Luthe et al. 2009). All cell culture media and components, i.e. Dulbecco’s Modified Eagle Medium (DMEM), Iscove’s Modified Dulbecco’s Medium (IMDM), FBS, PS, 0.25% trypsin with EDTA, and PBS were obtained from Invitrogen (Carlsbad, CA, USA). DMSO was purchased from Sigma-Aldrich (St. Louis, MO, USA). PCR reagents and kits were obtained from Qiagen (Valencia, CA, USA).

Animals

The animal experiment was conducted with approval from the Institutional Animal Care and Use Committee of the University of Iowa. Male Sprague-Dawley rats, weighing 75-100 g and 4-5 weeks old, were purchased from Harlan Sprague-Dawley (Indianapolis, IN). The animals were housed in individual wire hanging cages in a controlled environment maintained at 22 ºC with a 12 h light-dark cycle and water ad libitum. Animals were randomly divided into three groups, and were fed ad libitum a modified AIN-93G diet purchased from Harland Teklad (Madison, WI). The modified diet used egg white solid in place of casein. After three weeks of acclimatization, the animals were
given a single i.p. injection of vehicle (stripped corn oil; 5 ml/kg body weight; Acros Chemical Company, Pittsburgh, PA), or vehicle with 1 μmol/kg body weight (326 μg/kg body weight) or 5 μmol/kg body weight (1.63 mg/kg body weight) of PCB 126 (6 rats per dose). Two weeks after the PCB treatment the rats were euthanized using carbon dioxide asphyxiation followed by thoracotomy. Femurs were excised and bone marrow was flushed out.

Bone marrow cells isolation, culture and treatment

Bone marrow nucleated cells were isolated from femurs of Sprague-Dawley rats as Zhang et al described (Zhang et al. 2008). The cells were washed with DMEM supplemented with 10% heat inactivated FBS (FBS incubated at 56 ºC for 30 min) and 1% P/S (complete DMEM) and passed through a 70 μm nylon strainer to remove cell clumps and debris. The red blood cells were lysed by incubating the cells with Flow Cytometry Mouse Lyse Buffer (R&D Systems, Minneapolis, MN) for 5 min at room temperature. After washing with DMEM, 5×10^6 cells suspended in complete DMEM with 10 ng/ml recombinant rat M-CSF (Peprotech, Rocky Hill, NJ) were seeded in 10 cm plastic petri dishes and cultured in a humidified 37 ºC, 5% CO₂ incubator for 8 days. In each treatment group, there were six animals. The cells from the first three animals in each treatment group were treated with 0.05% DMSO as vehicle control and the cells from the last three animals were treated with 5 μM PCB126 during the 8 days differentiation. By the end of the differentiation, the supernatants were discarded and the remaining adherent cells were washed with PBS. Then the cells were detached by adding 3 ml Cell stripper

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nonenzymatic cell dissociation solution (Corning, Manassas, VA) to each dish and incubated for 5 min at 37 °C.

**Figure 3-1. Experimental design.**

Cell morphology of bone marrow derived macrophages

Bone marrow cells were flushed out and washed with complete DMEM. After counting, the cells were grown on autoclaved microscopy slides with induction to differentiate into macrophages by adding M-CSF (10 ng/ml) into the medium. After 8 days differentiation, the cells on slides were washed with PBS twice, stained with the Hema 3 Staining system and observed 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).
Measurement of telomerase activity

Telomerase activity was measured according to the method described previously in Chapter 2 (Page 22) with slight modification. The primers used for rat samples are TS primer (5’-AATCCGTCGGAGCAGGTT-3’) and CX primer (5’-CCCTTACCCTACCTTACCCTAA-3’) (Banerjee et al. 1998). All the other steps were the same as described in Chapter 2 Method for telomerase activity measurement (Page 22).

Quantitative real-time PCR

The methods for total RNA extraction, cDNA synthesis and qPCR analysis are the same as described in Chapter 2 (Page 28). The primers for the quantification of selected genes are as follows: CYP1A1: Forward, 5’-GGGTCCTAGAGAACACTCTTCA-3’, and reverse, 5’-CAAGGCAGAATGTGGTGACG-3’ (Designed by Primer 3); TERT: Forward, 5’-TCCGCACGTTGGTTGCCAG-3’, and reverse, 5’-CCTCTCACCACGCTCGCACA-3’ (Wang et al. 2014); GAPDH: Forward, 5’-TGGAGAAACCTGCCAAGTATG-3’, and reverse, 5’-GGGTGGTCCAGGGTTTCTTA-3’ (Li et al. 2010). 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: $\Delta C_T$ (sample) = $C_T$ (mRNA of interest) − $C_T$ (GAPDH); $\Delta \Delta C_T = \Delta C_T$ (treatment) − $\Delta C_T$ (control); relative expression = $2^{-\Delta \Delta C_T}$.
Immunofluorescent staining

Rat bone marrow nucleated cells were isolated, washed twice with PBS and cytospun onto the microscopic slides. Then cells were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized by 0.25% Triton X-100 for 25 min and blocked with 5% goat serum for 30 min at room temperature. After two times washing with PBS, the cells were incubated at 4°C overnight with rabbit anti-CYP1A1 (+CYP1A2) (1:50, Acris Antibodies, San Diego, CA) or anti-TERT antibodies (1:50, Bioss, Woburn, MA), washed twice with PBS, incubated with Alexa Fluor 568-conjugated goat anti-rabbit (1:200), or Alexa Fluor 488-conjugated goat anti-rabbit (1:200; Both from Life Technologies) secondary antibodies for 4 h, and mounted coverslips with VECTASHIELD mounting medium for fluorescence with DAPI (Vector Laboratories, Inc., Burlingame, CA) for cell nuclei staining for 10 min. Fluorescence was observed by Zeiss LSM710 Confocal Microscope (Carl Zeiss AG, Jena, Germany) in Central Microscopy Research Facility, The University of Iowa. Negative control without primary antibody was used to confirm the specificity of staining.

Colony forming cell (CFC) assay

Bone marrow cells were flushed out from femurs and washed with IMDM supplemented with 2% FBS. The cells were then passed through a 70 μm nylon strainer and the red blood cells were removed as mentioned earlier. After washing with IMDM/2% FBS, cell numbers were counted by hemacytometer and 10× stock cell suspensions were made in IMDM/2% FBS. 0.3 ml of the stock cell suspension was added into 3 ml Rat Methycellulose Complete Media Without Epo (R&D Systems, Minneapolis, MN) to
make the final cell number at $3 \times 10^4$ cells/ml. Then the cells were plated in duplicate in 35-mm untreated culture dishes. Dishes were incubated in a humid incubator at 37°C, 5% CO$_2$ for 12 days prior to enumeration. Colonies were counted via microscopy and gridded scoring dishes. This assay was done twice at the same time. One was done without additional PCB126 treatment and the other was done with a PCB126 treatment during the 12 days incubation time. In the later one, the cells from the first three animals in each treatment group were treated with vehicle control DMSO and the cells from the last three animals were treated with 5 μM PCB126.

Flow cytometry analysis for cell surface marker expression

For cell surface marker staining about $1 \times 10^6$ were washed twice with cold staining buffer (PBS with 0.2% BSA), blocked the Fc Receptor with Rat CD32 (BD Pharmingen, San Diego, CA) for 10 min, and resuspended in 100 μl staining buffer, and incubated for 30 min with anti-CD34 PE-conjugated antibody (Acris Antibodies, San Diego, CA), anti-rat myeloid lineage FITC-conjugated antibody (Biolegend, San Diego, CA), and anti-CD11b Alexa Fluor 647-conjugated antibody (AbD Serotec, Raleigh, NC) on ice in the dark. After two washings with cold staining buffer, the cell pellet was re-suspended in 100 μl staining buffer and analyzed using an Accuri C6 flow cytometer (BD, San Jose, CA).

Statistical analysis

All experiments were performed in triplicate and every individual qRT-PCR sample was assayed in duplicate. All data are expressed as the mean ± SEM or SD 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 t test. \( p<0.05 \) was considered to be statistically significant.

**Results**

PCB126 induced CYP1A1 expression at both mRNA and protein levels in rat bone marrow cells

We determined CYP1A1 gene expression to confirm the activation of the AhR pathway in bone marrow cells by the *in vivo* PCB126 exposure. As shown in Figure 3-2, both low and high dose PCB126 significantly induced CYP1A1 gene expression to almost 200 fold more than control. CYP1A1 protein level was determined by immunofluorescent staining. As shown in Figure 3-3, CYP1A1 protein expression was significantly increased by PCB126 treatment. Low dose of PCB126 was already enough to induce CYP1A1 expression (Figure 3-3B), and the high dose of PCB126 induced its expression more significantly compared to the control group (Figure 3-3C, extremely high red signal). This suggested that the AhR was activated by PCB126 treatment.

*Figure 3-2. PCB126 induced CYP1A1 gene expression in rat bone marrow cells.* Male Sprague-Dawley rats were exposed to low or high dose of PCB126 by i.p. injection.
Two weeks after the injection, the rats were euthanized and the bone marrow nucleated cells were isolated. qRT-PCR was performed to analyze gene expression. PCB126 low: 1 μmol/kg body weight; PCB126 high: 5 μmol/kg body weight. * p<0.05, compared with the control group.

**Figure 3-3. CYP1A1 protein expression in rat bone marrow nucleated cells.** Bone marrow cells were isolated from bone marrow and filtered to eliminate the clumps. Red blood cells were removed and the cells were washed with ice cold PBS twice. After fixation with 4% of paraformaldehyde (PFA) and permeabilization with 0.25% Triton X-100, the cells were blocked with 5% goat serum and incubated with primary antibody anti-CYP1A1 overnight at 4 °C. Then the samples were incubated with secondary antibody for 4h at room temperature. The slides were sealed after adding mounting medium with DAPI. The red color is CYP1A1 staining with Alex Fluor 568 and blue is nucleus staining with DAPI. A. Control. B. PCB126 low dose (1 μmol/kg body weight). C. PCB126 high dose (5 μmol/kg body weight).
PCB126 decreased telomerase activity and downregulated TERT expression

Then we measured telomerase activity and TERT expression to explore the effects of PCB126 on telomerase in bone marrow hematopoietic stem cells and progenitor cells. As shown in Figure 3-4, PCB126 significantly decreased telomerase activity (Figure 3-4A) and downregulated TERT gene expression (Figure 3-4B) in a dose-response manner, with the effect on TERT gene more pronounced than telomerase activity. We performed immunofluorescent staining to detect the protein level of TERT. Figure 3-5 showed that in the control group, TERT protein was highly expressed in all the cells (Figure 3-5A). However, the high dose of PCB126 significantly decreased its expression (low green signal), as shown in Figure 3-5C. A low dose of PCB126 had no obvious effect on TERT protein expression (Figure 3-5B).

**Figure 3-4. PCB126 decreased telomerase activity and down regulated TERT gene expression.** Male Sprague-Dawley rats were exposed to low or high dose of PCB126 by i.p. injection. Two weeks after the injection, the rats were euthanized and the bone marrow nucleated cells were isolated. Telomerase activity and TERT gene expression were analyzed. A. Telomerase activity. B. TERT gene expression. PCB126 low: 1 μmol/kg body weight; PCB126 high: 5 μmol/kg body weight. * p<0.05, compared with the control group.
Figure 3-5. TERT protein expression in rat bone marrow nucleated cells. Bone marrow cells were isolated from bone marrow and filtered to eliminate the clumps. Red blood cells were removed and the cells were washed with ice cold PBS twice. After fixation with 4% of paraformaldehyde (PFA) and permeabilization with 0.25% Triton X-100, the cells were blocked with 5% goat serum and incubated with primary antibody anti-TERT overnight at 4 ºC. Then the samples were incubated with secondary antibodies for 4h at room temperature. The slides were sealed after adding mounting medium with DAPI. The green color is TERT staining with Alexa Fluor 488 and the blue is nucleus staining with DAPI. A. Control. B. PCB126 low dose (1 μmol/kg body weight). C. PCB126 high dose (5 μmol/kg body weight).
PCB126 decreased stem cell number but increased the differentiation

To test the effects of PCB126 on different phenotypes of the bone marrow cells, we detected cell surface marker expression using a flow cytometer. The three markers we chose to analyze were: CD34 which is a marker for hematopoietic stem cells, a myeloid lineage marker for myeloid progenitor cells, and CD11b, a marker of more mature differentiated cells. As shown in Table 3-1, in the control group, the CD11b positive population was about 35% of the total cell number, while the stem cells (CD34 positive) and progenitor cells (myeloid lineage marker positive) were only about 1% and 5%, respectively. After exposure to PCB126, both low dose and high dose PCB126 significantly increased CD11b positive cell number and myeloid lineage positive cell number, but decreased the number of CD34 positive cells. This indicated that PCB126 can reduce stem cell numbers and promote the differentiation of bone marrow stem cells into downstream myeloid cells.

Table 3-1. Cell surface marker expression in rat bone marrow cells from rats with PCB126 exposure

<table>
<thead>
<tr>
<th>Exposures</th>
<th>Cell surface marker expression (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD34 %</td>
</tr>
<tr>
<td>In vivo (Two weeks)</td>
<td></td>
</tr>
<tr>
<td>Control (0.05% v/v DMSO)</td>
<td>0.96 ± 0.15</td>
</tr>
<tr>
<td>PCB126 low (1 μmol/kg b.w.)</td>
<td>0.69* ± 0.11</td>
</tr>
<tr>
<td>PCB126 high (5 μmol/kg b.w.)</td>
<td>0.69 ± 0.17</td>
</tr>
</tbody>
</table>

Note: * p<0.05, compared with the control group.
PCB126 modified the differentiation pattern of HSCs

The colony forming cell (CFC) assay, also referred to as the methylcellulose assay, is an *in vitro* assay used in the study of hematopoietic stem cells. The assay is based on the ability of hematopoietic progenitors to proliferate and differentiate into colonies in a semi-solid media in response to cytokine stimulation. The colonies formed can be enumerated and characterized according to their unique morphology as colony forming unit (CFU, 1 colony = 1CFU). To directly measure the absolute number of viable and functional progenitor cells in the bone marrow, we performed a CFC assay. As shown in Figure 3-6, there are three different types of CFUs formed for rat samples in this assay: CFU-G (colony forming unit-granulocyte), CFU-M (colony forming unit-macrophages), and CFU-GM (colony forming unit-granulocyte, macrophage). PCB126 treatment increased the total CFU numbers compared to the control group, but only the high dose PCB126 treatment (5 µmol/kg body weight) showed significance (Figure 3-7A). PCB126 significantly decreased the number of CFU-M, while significantly increasing the numbers of CFU-G and CFU-GM (Figure 3-7B). The CFU numbers did not show significant changes after additional PCB126 treatment for 12 days. Only the high dose PCB126 treatment significantly decreased the number of CFU-M compared to the control group (Table 3-2). These results indicated that the effects of PCB126 on the differentiation pattern of stem cells were due to the *in vivo* exposure rather than the extra *in vitro* treatment.
**Figure 3-6.** Different types of colony forming unit from rat bone marrow.  
(Magnification 40×)

**Figure 3-7.** CFU numbers were changed by PCB126 treatment.  
3×10⁴ cells were seeded per 35 mm dish and cultured at 37 °C for 12 days. The colony numbers were counted using microscopy and gridded scoring dish.  
A. Total CFU numbers. B. Different types of CFU numbers. *p<0.05, compared with the control group.
Table 3-2. CFU numbers with additional PCB126 treatment during the colony forming incubation

<table>
<thead>
<tr>
<th>Exposures</th>
<th>In vivo (Two weeks)</th>
<th>In vitro (12 days)</th>
<th>CFU (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Control</td>
<td>CFU-M %</td>
</tr>
<tr>
<td>(0.05% v/v DMSO)</td>
<td>Control</td>
<td>PCB126</td>
<td>32 ± 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>29 ± 3</td>
<td>48 ± 4</td>
</tr>
<tr>
<td></td>
<td>PCB126 low</td>
<td>Control</td>
<td>31 ± 4</td>
</tr>
<tr>
<td>(1 μmol/kg b.w.)</td>
<td></td>
<td>27 ± 4</td>
<td>48 ± 7</td>
</tr>
<tr>
<td></td>
<td>PCB126 high</td>
<td>Control</td>
<td>28* ± 3</td>
</tr>
<tr>
<td>(5 μmol/kg b.w.)</td>
<td></td>
<td>29 ± 6</td>
<td>49 ± 4</td>
</tr>
</tbody>
</table>

Note: * p<0.05, compared with the control group.

PCB126 exerted no effect on cell morphology of bone marrow derived macrophages

Bone marrow cells were induced to differentiate into macrophages and grown on glass microscope slides. No obvious differences between vehicle control and PCB126 treated cells were observed (Figure 3-8). There were mature activated macrophages (big cells with two nuclei) and immature macrophages (cells with one nucleus) in both the control and PCB126 treated groups. Cell morphology did not change obviously, which suggested that PCB126 had almost no effect on the cell morphology of bone marrow derived macrophages.
Figure 3-8. Cell morphology of bone marrow derived macrophages. Vehicle control: cells from control animal with vehicle control DMSO treated during differentiation; PCB126: cells from high dose PCB126 treated animal with 5 μM PCB126 treated during differentiation.

Telomerase activity and gene expression in macrophages were decreased after differentiation

After the differentiation into macrophages, we measured telomerase activity and TERT gene expression to investigate the effect of PCB126 on the telomere maintenance in differentiated macrophages. Both telomerase activity and TERT gene expression were reduced after differentiation (compared to the Ct value of the undifferentiated stem cells), but we did not observe significant effects of the additional in vitro PCB126 treatment. Only low dose PCB126 with additional PCB treatment significantly increased telomerase activity after differentiation into macrophages (Figure 3-9A). And in the control group, PCB126 treatment during differentiation significantly down regulated TERT gene expression (Figure 3-9B). These results indicated that PCB126 showed no effect on the macrophage differentiation.
Figure 3-9. Telomerase activity and TERT expression in bone marrow derived macrophages. A. Telomerase activity in macrophages derived from bone marrow after additional exposure to PCB126 during the differentiation for 8 days. B. TERT gene expression in macrophages derived from bone marrow after additional exposure to PCB126 during the differentiation for 8 days. PCB126 low: 1 µmol/kg body weight; PCB126 high: 5 µmol/kg body weight. * p<0.05, compared with the control group.

Discussion

Higher chlorinated PCBs, like PCB126, bioaccumulate readily in fatty tissues in both animals and humans. They are found two to four fold higher in bone marrow compared to adipose or fat tissue in children (Scheele et al. 1992, 1995). This indicates that bone marrow could be an important target tissue for PCBs in humans. Human hematopoietic cells were reported to be more sensitive to environmental compounds including PCB126 and lead compared to mice (Van Den Heuvel et al. 2001). Retention of telomerase activity is an important characteristic of stem cells and progenitor cells for maintaining longer telomeres. Interference of telomere maintenance of these cells may lead to premature aging and different types of cancer, such as leukemia and NHL. Previously we reported that PCBs reduced telomerase activity and shortened telomere length in HaCaT (Jacobus et al. 2008; Senthilkumar et al. 2011; Senthilkumar et al. 2012) and HL-60 cells (Xin et al. 2015). However, no study has been performed to explore the
effects of PCBs on telomere maintenance of HSCs. In this study, we investigated the hypothesis that PCB126 interferes with hematopoietic stem cells by disturbing telomerase and modifying the differentiation pattern of these cells.

We previously used HL-60, a hematopoietic progenitor cell line, as a study model to determine the effects of PCBs on telomerase and telomere maintenance and differentiation of these cells. In the current study, we used hematopoietic stem cells from rat bone marrow to study how PCB126 affects the telomerase and differentiation of HSCs in the bone marrow. Bone marrow nucleated cells were isolated from the rats that received one single i.p. injection of two doses of PCB126 (1 or 5 μmol/kg body weight) two weeks before euthanization. In order to see if PCB126 affects bone marrow cells through activation of AhR pathway, we determined the mRNA level and protein level of CYP1A1, one of the AhR target genes. Our results clearly showed that PCB126 highly induced CYP1A1 expression at both mRNA level and protein level, which confirmed that the AhR pathway was activated by PCB126 in bone marrow cells. The AhR is reported to have an important function in the regulation of HSCs. AhR null-allele mice showed altered characteristics and function of HSCs (Yoon et al. 2002), and abnormal activation of the AhR by xenobiotics also resulted in altered numbers and function of HSCs in mouse bone marrow (Gasiewicz et al. 2010). TCDD affects HSCs numbers and function directly through the AhR of these cells, whereas increased HSCs proliferation could protect the cells from the toxic effects of TCDD (Singh et al. 2009). The AhR was postulated to be a negative regulator of growth and proliferation (Mulero-Navarro et al. 2006), which means that when PCB126 activates AhR, the proliferation of HSCs will be inhibited. This may explain our results that PCB126 reduced telomerase activity in bone
marrow cells because telomerase activity normally is reduced when cells stop proliferating or when cells differentiate into mature tissue cells. In our experiments this effect was also dose-dependent. The effect of PCB126 on telomerase activity is consistent with our previous results with progenitor-like HL-60 cells where PCB126 significantly decreased telomerase activity during exposure. This was accompanied with a down regulation of TERT mRNA and protein expression.

PCB126 significantly decreased the number of stem cells (CD34 positive), and increased the numbers of CD11b positive cells (differentiation marker for granulocytes and macrophages) and myelocytic progenitor cells (myeloid lineage positive). The colony forming cell assay results showed that PCB126 modified the differentiation pattern of the HSCs by significantly increasing the total number of the CFUs and shifting the colony types from CFU-M to CFU-G and CFU-GM, which indicated that PCB126 could promote the differentiation of hematopoietic stem cells into downstream myelocytic cells. However, Luster et al. reported that a decrease in bone marrow CFU-GM was observed in mice exposed to TCDD (Luster et al. 1985). This could be due to the different species and different test compounds, even though they are both AhR agonists. Also, the exposure time for the animals was different in the two studies. They exposed mice to a single dose of TCDD (1 μg/kg body weight) by gavage and isolated the bone marrow cells after three to five days exposure, whereas we used rats that received one single i.p. injection of PCB126 and all the analyses were done two weeks after the injection. These differences in the experimental procedures could be the reason that we observed differing results. In addition, we also performed CFC assays with an additional PCB126 treatment during the colony forming period. However, we did not observe any significant effects
due to the additional PCB126 exposure. The reasons for this could be that the effects of PCB126 needs an *in vivo* environment which cannot be replicated *in vitro*, and/or it also could be due to the high viscosity of the methylcellulose medium, in which bioavailability of PCB may be different compared to normal culture medium.

We also induced the rat bone marrow cells *in vitro* to differentiate into macrophages, and during the differentiation the cells were treated with 5 μM PCB126 for 8 days. The overall levels of telomerase activity and TERT expression in macrophages were lower than those in bone marrow cells (compare the Ct values), which is reasonable because in differentiated cells telomerase activity is normally lower compared to the undifferentiated stem cells. However, cell morphology did not show a significant difference between the vehicle control and PCB126 treated groups. Also PCB126 did not cause further effects on telomerase activity and TERT expression. These results suggest that the effects of PCB126 on the telomerase and the differentiation of HSCs have to occur *in vivo* rather than in the *in vitro* condition. Therefore, other factors and pathways may also be involved in the regulation of PCB126 on HSCs.

**Conclusion**

In this study, we have shown that PCB126 decreased telomerase activity and TERT gene and protein levels of rat bone marrow cells *in vivo*, which could be a reason for the decreased number of stem cells observed in these HSC samples. PCB126 also modified the differentiation pattern of the rat bone marrow cells and promoted the granulocytic differentiation of HSCs. But PCB126 did not show much effect on the differentiation of macrophages and on the telomerase activity of macrophages derived from the bone
marrow in vitro. These results together suggest that PCB126 has hematotoxicity through regulation of telomerase activity and TERT expression and modification of the differentiation pattern of the HSCs in the bone marrow of living rats. Such effects could be a contributing factor to immune suppression, leukemia and other types of tumors which have been observed.

Acknowledgments

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CHAPTER 4 INVESTIGATE THE MECHANISM OF PCB126 REGULATION OF hTERT GENE EXPRESSION

Abstract

PCB126 is the most potent dioxin-like PCB congener, which has been shown to affect transcription via AhR. Upon ligand binding, AhR translocates into the nucleus, heterodimerizes with the aryl hydrocarbon nuclear translocator (ARNT) protein and then binds to the XRE of target genes to regulate their expression. There is a crosstalk between AhR and the hypoxia pathway because both AhR and HIF-1α need to form a heterodimer with ARNT to play their role in gene regulation. We previously reported that PCB126 down regulated hTERT expression in human HL-60 cells, but the mechanism remains unclear. Here we hypothesized that PCB126 regulates hTERT expression through the AhR/ARNT signaling pathway and hypoxia could affect the regulation. We first exposed HL-60 cells with AhR antagonists prior to PCB126 treatment and found the antagonists attenuated the adverse effects of PCB126 on hTERT mRNA expression, but caused an up-regulation of hTERT in the solvent control group. Then we exposed the cells to CoCl₂ to induce the hypoxia-like condition and observed a decrease of hTERT gene and protein expression in a dose-response manner. We further used siRNA to knock down AhR or ARNT or both. The results support the previous findings and also showed that both AhR and ARNT are important for the regulation of hTERT and that this was a down regulation. Next, we examined the hTERT promoter activity and found that both PCB126 and CoCl₂ inhibited the promoter activity while CH223191, an AhR antagonist, increased the promoter activity of hTERT despite the absence of any AhR/ARNT binding sites in the
promoter region of hTERT. These results together indicated that PCB126 regulates hTERT expression through activation of the AhR/ARNT pathway and AhR and ARNT work together to play a negative role of regulation. However, these effects may be indirect, through regulation of other factors which then consequently regulate hTERT rather than by directly binding to the promoter of hTERT.

**Introduction**

Telomerase is a reverse transcriptase that adds telomeric repetitive DNA sequence TTAGGG to the ends of chromosomes which are called telomeres. The telomeres maintain chromosome end integrity in eukaryotic cells (Greider and Blackburn 1985, 1989). In normal somatic cells, this enzyme is repressed, which limits cell divisions. However, telomerase is abnormally activated in most cancer cells and hTERT, the rate-limiting enzymatic component of telomerase, is encoded by a gene that is activated or overexpressed in cancer cells which thereby are able to maintain the telomere length and unlimited proliferative capacity (Holysz et al. 2013; Zhang et al. 2015). Recent reports show that mutation in the hTERT promoter region might be the most important reason for hTERT overexpression in different cancer cells (Horn et al. 2013; Killela et al. 2013; Tallet et al. 2014), and the transcriptional regulation of hTERT seems to be the key mechanism of cancers. Accumulating evidence has indicated that several transcription factors are involved in the regulation of hTERT gene, but AhR/ARNT has not yet been implicated (Daniel et al. 2012; Poole et al. 2001).

PCBs are a group of persistent organic pollutants (POPs), which bioaccumulate in the environment and biomagnificate in the food chain (Safe 1994). From 1929 to the
early 1980s PCBs were manufactured to use as insulating fluids in transformers, adhesives, plastics and building materials such as caulking. Its production was banned in most countries by the late 1980s due to PCBs broad range of adverse health effects to human and animals. The International Agency for Research on Cancer (IARC) recently classified PCBs as human carcinogens and dioxin-like PCBs were classified because of the evidence of an AhR-mediated mechanism of carcinogenesis (Lauby-Secretan et al. 2013).

PCB126 is the most potent dioxin-like PCB congener and has been shown to affect transcription of genes via the AhR (Fukuzawa et al. 2003; Hassoun et al. 2001). The AhR is a transcription factor known to regulate transcription of drug-metabolizing enzymes, such as cytochrome P450 1A1 (CYP1A1), CYP1A2, CYP1B1, and the expression of genes involved in cell growth control (e.g., transforming growth factor-α and -β, B-Jun, and Jun-D), among others. Upon ligand binding, the AhR translocates into the nucleus, dissociates from several cytoplasmic chaperone proteins including heat shock protein 90 (HSP90), XAP-2 and p23 (Carver and Bradfield 1997; Lees and Whitelaw 1999; Perdew 1988), heterodimerizes with the aryl hydrocarbon nuclear translocator (ARNT) protein (Lees and Whitelaw 1999) and then binds to DNA response elements known as the xenobiotic/dioxin response element (XRE/DRE) on the promoters of the target genes mentioned above (Kewley et al. 2004; Lees and Whitelaw 1999). In Chapter 2, we reported that PCB126 down regulated hTERT expression in HL-60 cells (Xin et al. 2015), but the mechanism remains unclear.

HIF-1α is a sensor of oxygen level in cells that regulates responses to hypoxia. Similar to the AhR pathway, during hypoxia, HIF-1α also binds with ARNT to serve as a
transcriptional regulator of hypoxia-inducible genes (Kewley et al. 2004). In normal oxygen condition, prolyl hydroxylase domain-containing enzymes (PHDs) continuously hydroxylate HIF-1α to degrade it. During hypoxia, HIF-1α escapes from hydroxylation, translocates into the nucleus, dimerizes with ARNT and binds to the hypoxia response element (HRE) of target genes to regulate their expression (Majmundar et al. 2010). HIF-1 as a regulator of hTERT has been reported to be either an inducer (Nishi et al. 2004; Yatabe et al. 2004) or a repressor (Koshiji et al. 2004).

Since heterodimerization with ARNT is a prerequisite for both the AhR and hypoxia pathways to play their roles in gene regulation, we hypothesized that a competition for ARNT between the two pathways exists that can affect the regulation of gene expression. In this study, we investigated the PCB-induced AhR function and its potential regulatory ability on hTERT. We used dioxin-like PCB126 as an AhR ligand to study its regulation of hTERT gene expression and CoCl₂ to induce a hypoxia-like condition to investigate how hypoxia affected this regulation. Our results demonstrated that AhR/ARNT acts like a potential regulator of the hTERT gene and the hypoxia pathway was also involved in the regulation. The regulation of AhR/ARNT on hTERT may be indirect and was suppressing.

Materials and methods

Materials

All cell culture media and components, i.e. RPMI 1640 medium, DMEM, FBS, PS, 0.25% trypsin with EDTA, and PBS were obtained from Invitrogen (Carlsbad, CA, USA). DMSO and CH223191 were purchased from Sigma-Aldrich (St. Louis, MO, USA).
Alpha-Naphthoflavone (α-NF) was purchased from Acros Organics (Pittsburgh, PA, USA). Cobalt chloride (CoCl₂) was obtained from MP Biomedicals (Santa Ana, CA, USA). PCB126 was prepared by an improved Suzuki-coupling method of 3,4,5-trichlorovromobenzene with 3,4-dichlorophenyl boronic acid utilizing a palladium-catalyzed cross-coupling reaction (Luthe et al. 2009). PCR reagents and kits were obtained from Qiagen (Valencia, CA, USA).

Cell culture and treatment

The HL-60 and HaCaT cell lines were obtained from the American Tissue Culture Collection (ATCC) (Manassas, VA, USA). HL-60 Cells and HaCaT cells were maintained in RPMI 1640 or DMEM, respectively, with 10% FBS and 1% P/S 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. Cells were pretreated with AhR antagonists α-NF or CH223191 for 4 h, or hypoxia mimetic CoCl₂ (1 – 100 μM) for 24 h prior to PCB126 treatment.

Measurement of telomerase activity

Telomerase activity was measured following the protocol described in Chapter 2 Materials and methods (Page 27).
Quantitative real-time PCR

The methods for total RNA extraction, cDNA synthesis and qPCR analysis are the same as described in Chapter 2 (Page 28). The primers used in this study are shown in Table 4-1.

Table 4-1. Primer pairs used in qRT-PCR experiments

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
</table>
| AhR  | Forward: CTTCCAAGCCGGCATAGAGAC  
Reverse: AGTTATCCTGGCCTCCGTTT | Designed using Primer 3 |
| ARNT | Forward: AACCTCACTTCGTTGGTGTC  
Reverse: CAATGTTGTGTCCGGAGATG | |
| hTERT| Forward: TGTACTTCTGTCAAGGGGATGTGA  
Reverse: GCTGGAGGTCTGTCAAGGTAGAG | (Gourronc et al. 2010) |
| HPRT | Forward: AGATGGTGCAAGGGCAAGC  
Reverse: GGACTCCAGATTTCCAAAACCTCAAC | Pre-designed primers from IDT (Coralville, IA, USA) |
| RPLP0| Forward: AGACTGGAGACAAGGTGGGA  
Reverse: CAGACAGACACTGGCAACA | |
| GAPDH| Forward: AAGGTCATCCATGACAACTTTG  
Reverse: GTAGAGGCGAGGATGTGTCTT | (Gourronc et al. 2010) |

Immunofluorescent staining

HL-60 cells were pretreated with 5 μM or 50 μM CoCl₂ for 24 h, then treated with 5 μM PCB126 for another 24 h. Treated cells were washed twice with PBS and cytopun onto the microscopic slides. Then the cells were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.25% Triton X-100 for 25 min, and blocked with 5% goat serum for 30 min at room temperature. After incubation at 4 °C
overnight with mouse anti-AhR (1:50, Abcam, Cambridge, MA), anti-ARNT (1:50, Novus Biologicals, Littleton, CO), and rabbit anti-telomerase antibodies (1:250, Abcam, Cambridge, MA), the slides were washed with PBS, incubated with Alexa Fluor 488-conjugated goat anti-mouse (1:200, Life Technologies), Alexa Fluor 647-conjugated goat anti-mouse (1:200; Life Technologies), or Alexa Fluor 568-conjugated goat anti-rabbit (1:200; Life Technologies) secondary antibodies for 4 h at room temperature, and mounted coverslips with VECTASHIELD mounting medium for fluorescence with DAPI (Vector Laboratories, Inc., Burlingame, CA) for cell nuclei staining for 10 min. Fluorescence was observed by confocal microscopy using a Leica LMD7000 Laser Capture Microdissection System (Leica Microsystems, Wetzlar, Germany) at the Central Microscopy Research Facility, The University of Iowa. Negative control, omitting primary antibody, was used to confirm the specificity of staining.

Transient Transfection and RNA Interference

We used small interfering RNAs (siRNAs) directed against AhR, ARNT, HPRT (positive control) and non-human related mRNA as a negative control, all obtained from IDT (Coralville, IA). Briefly, HaCaT cells with 60-80% confluence, cultured in 24-well plates, were transiently transfected with siRNAs, 5 pmol per well, using Lipofectamine RNAiMAX reagent (Invitrogen, Foster City, CA) for 24 h. Then the transfected cells were treated with 100 μM CoCl2 for 24 h or 5 μM PCB126 for 3 h. RNA samples were isolated from the cells and gene expression was investigated by qRT-PCR. siRNA transfection showed no effect on cell viability, as demonstrated by microscopic examination (data not shown).
hTERT promoter activity

An hTERT-luciferase reporter vector (pGL3-hTERTp1375) containing the 5’-flanking region (-1375 to +78) of the hTERT gene sub-cloned into the pGL3-basic vector upstream of the firefly luciferase reporter gene (Takakura et al. 1999) was kindly provided by Dr. Takakura (Department of OB/GYN, Kanazawa University, Japan). HaCaT cells around 80% confluent, in 60 mm dishes were transfected with the pGL3-hTERTp1375 vector (5 μg) and Renilla luciferase reporter vector (pRL-TK, 2 μg) according to the Lipofectamine 3000 transfection reagent protocol (Invitrogen, Foster City, CA). Transfection medium was removed after 5 h and replaced with fresh medium. Transfected cells were split the next day, pretreated with 50 μM CoCl₂ or 5 μM CH223191 for 24 h, and subsequently exposed to 5 μM PCB126 for 24 h. Cells were lysed according to the Dual-Glo Luciferase Reporter Assay System protocol (Promega, Madison, WI) and luminescence was measured from whole cells lysates using a microplate reader (GeniosPro from TECAN, Switzerland).

ChIP assay for binding of ARNT or AhR with the hTERT promoter

ChIP assay was performed using the SimpleChIP Enzymatic Chromatin IP Kit (Magnetic Beads) (#9003, Cell Signaling Technology, Danvers, MA) according to the manufacturer’s protocol with slight modification. After pretreatment with 5 μM PCB126 or 100 μM CoCl₂ for 24 h, HaCaT cells at 90% confluence in 10 cm dishes were crosslinked with 1% formaldehyde for 10 min at room temperature. The crosslinking reaction was stopped by adding glycine for 5 min at room temperature. After washing twice with ice-cold 1×PBS, cells were harvested in ice-cold 1×PBS with PMSF (1mM)
and pelleted by centrifugation. Then cells were lysed and nuclei were pelleted by centrifugation. Chromatin was digested by adding Micrococcal Nuclease and 0.5 M EDTA was added to stop the digestion. The lysate was sonicated for 3×20 seconds by Microson XL 2000 ultrasonic liquid processor (Qsonica, LLC, Newtown, CT, USA) to obtain chromatin pieces at a length of 200 bp and bigger. The sheared chromatin was centrifuged and the supernatant was collected. Samples were diluted and incubated with 3 μg of anti-AhR (Abcam, Cambridge, MA), or anti-ARNT (Novus Biologicals, Littleton, CO) antibodies overnight at 4 °C. After the bound chromatin was washed, the DNA-protein crosslinks were reversed using proteinase K and the AhR, ARNT or HIF-1α-enriched fraction of genomic DNA was purified. qPCR was used to analyze ChIP DNA for enrichment at the hTERT promoter region. Normal Rabbit IgG and Histone H3 (D2B12) XP Rabbit mAb were used as negative control and positive control, respectively. CYP1B1 primers were used as a treatment positive control. hTERT ChIP qPCR primers were purchased from Qiagen [GPH1024036(+)01A] and qPCR reaction was performed following the manufacturer’s protocol by Eppendorf RealPlex Thermal Cycler (Eppendorf, Hamburg, Germany). The HotStart DNA polymerase was activated at 95 °C for 10 min followed by 40 cycles, denaturing at 95 °C for 15s, annealing and elongating at 60 °C for 60s. Data were collected and calculated by the equation: Percent Input = 2% × 2(CT(2% Input)−CT(ChIP sample))

Statistical analysis

All experiments were performed in triplicate and each individual qRT-PCR sample was assayed in duplicate. All data are expressed as the mean ± SEM of triplicate. 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 t test. \( p<0.05 \) was considered to be statistically significant.

**Results**

Telomerase activity was decreased with PCB126 treatment and reversed by AhR antagonists

In order to examine the role of AhR in the regulation of PCB126 on telomerase and the hTERT gene, we used the AhR antagonist \( \alpha \)-naphthoflavone (\( \alpha \)-NF) to pretreat the cells and then treated with 5 \( \mu \)M PCB126 for six days. As shown in Figure 4-1, PCB126 decreased telomerase activity while \( \alpha \)-NF increased it significantly. When cells were pretreated with \( \alpha \)-NF prior to PCB126 treatment, the telomerase activity was slightly increased compared to PCB126 treatment alone, reaching almost back to the untreated control levels.

![Figure 4-1. Telomerase activity in HL-60 cells treated with AhR antagonist and PCB126. HL-60 cells were treated with \( \alpha \)-NF for 4h before treatment with PCB126 for 6 days. *\( p<0.05 \), compared to control group; \#\( p<0.05 \), PCB126 treated group compared to \( \alpha \)-NF treated group.](image-url)
Gene expression after AhR antagonist treatment

To examine the effect of PCB126 activated AhR on hTERT expression, we first used qRT-PCR to determine the mRNA level of the CYP1B1 gene to confirm that PCB126 had activated the AhR and the antagonists were efficiently working. As shown in Figure 4-2A, CYP1B1 expression was induced by PCB126 while repressed by antagonist α-NF. Then we found that hTERT gene expression, as shown in Figure 4-2B and C, was down-regulated by PCB126 exposure for 6 days and the effect was weakened by the antagonists α-NF or CH223191 pretreatment.

Figure 4-2. CYP1B1 and hTERT gene expression in HL-60 cells treated with AhR antagonists and PCB126. HL-60 cells were treated with AhR antagonists for 4h prior to the treatment with 5 μM PCB126 for 6 days. *p<0.05, compared to control group; #p<0.05, compared to α-NF or CH223191 treated group.
hTERT gene expression after CoCl₂ treatment

To investigate whether activation of the hypoxia pathway also affects the hTERT gene expression, we employed CoCl₂ to mimic the hypoxic condition. First, we used different concentrations of CoCl₂ (1 µM to 100 µM) to treat cells for 48 h and measured hTERT gene expression. As shown in Figure 4-3A, hTERT expression was significantly reduced by CoCl₂ treatment in a dose-response manner. Then, we determined hTERT gene expression after combining treatment with 5 µM CoCl₂ and 5 µM PCB126, or either one of them alone. hTERT mRNA level was significantly reduced by 5 µM CoCl₂ or 5 µM PCB126 and the effect was enhanced by the combining treatment (Figure 4-3B).

Figure 4-3. hTERT expression in HL-60 cells treated with CoCl₂ and/or PCB126. A. HL-60 cells were treated with different concentrations of CoCl₂ (1 µM to 100 µM) for 24 h and hTERT expression was determined. B. HL-60 cells were treated with 5 µM CoCl₂ for 24 h prior to the treatment with PCB126 for 24 h. *p<0.05, compared to control group; #p<0.05, compared to PCB126 treated group.

siRNA knock down of AhR and/or ARNT in HaCaT cells

We employed siRNA transfection to knock down the AhR or ARNT gene or both of them to see if AhR/ARNT is the regulator of hTERT gene. siRNA of HPRT was used as positive control. After 24h transfection, HPRT was knocked down about 80% (Figure 4-4A). ARNT and AhR were knocked down to around 65% and 60%, respectively. After
double transfection with both siAhR and siARNT, the transfection efficiency was around 50% for both AhR and ARNT (Figure 4-4B). CYP1A1 gene expression was used to confirm the AhR pathway was activated by PCB126 treatment. CYP1A1 was induced in each group with PCB126 treatment for 3h, but the induction was significantly lowered in siRNA knock down groups (Figure 4-4C). siRNA transfection had no effect on the basal level of CYP1A1 expression. As shown in Figure 4-4D, hTERT expression was down regulated by both PCB126 treatment and ARNT gene knock down, while upregulated after the AhR gene was knocked down. When both ARNT and AhR were knocked down, hTERT was upregulated about 5-fold. When combining PCB126 treatment with AhR or ARNT knockdown or both knockdown, hTERT expression was not changed very much compared to the corresponding knockdown groups without PCB treatments. In Figure 4-4E, CoCl$_2$ treatment alone significantly decreased hTERT expression. When CoCl$_2$ exposure was combined with siAhR transfection, hTERT induction was lower than in the siAhR transfection alone group. CoCl$_2$ treatment reversed the decrease of hTERT expression due to ARNT knock down and it further enhanced the increase of hTERT mRNA observed with both AhR and ARNT knock down.
Figure 4-4. Gene expression after siRNA transfection and PCB126, CoCl₂ treatment. HaCaT cells were seeded in 24-well plate and transfected with siHPRT as positive control, siAhR or siARNT for 24 h. Then the cells were treated with 100 µM CoCl₂ or 5 µM PCB126 for 21 h. Total RNA was isolated and reverse transcribed to cDNA and used for qPCR. A. HPRT expression, serving as a positive control to check if the transfection is working well. B. AhR and ARNT mRNA expression to check the transfection efficiency. C. CYP1A1 expression to make sure that PCB126 activated AhR pathway. D. hTERT expression after siRNA transfection and 5 µM PCB126 treatment. E. hTERT expression after siRNA transfection and 100 µM CoCl₂ treatment.

Immunofluorescent staining

To examine the protein levels of AhR, telomerase and ARNT, we performed three colors immunofluorescent staining. There were five treatment groups: vehicle control, 5 µM PCB126, 5 µM CoCl₂, 5 µM CoCl₂ + 5 µM PCB126, and 50 µM CoCl₂. CoCl₂ was added 24 h prior to the 24 h PCB126 treatment. The grey color is DAPI staining the
nuclei; the green color stains AhR; the red color indicates telomerase; the blue color shows ARNT. As shown in Figure 4-5, telomerase protein expression was decreased by both concentrations of CoCl$_2$, especially the 5 µM concentration. The ARNT protein level was increased by both PCB126 and CoCl$_2$ treatment, with the most in the 5 µM CoCl$_2$ treated group. However, the AhR protein level was not changed much after PCB126 or CoCl$_2$ treatment. Also, the telomerase was not changed significantly by PCB126 treatment, which could be because the half-life of telomerase protein is longer than 24h.

Figure 4-5. AhR, telomerase and ARNT protein expression by immunofluorescent staining. HL-60 cells were treated with 100 µM CoCl$_2$ for 24 h prior to the 24 h 5 µM
PCB126 treatment. Treated cells were washed, fixed, permeabilized, blocked and incubated with primary and secondary antibodies overnight. Then slides were sealed after adding mounting medium with DAPI. Grey: nuclei; Green: AhR; Red: telomerase; Blue: ARNT.

hTERT promoter activity

To investigate the mechanism of inhibition of hTERT gene expression after PCB126 treatment and the role of AhR/ARNT in the regulation of its expression, we transfected HaCaT cells with an hTERT luciferase reporter construct containing 1.4 kb of the hTERT promoter. After transfection, HaCaT cells were treated with 50 μM CoCl₂ for 24 h or 5 μM CH223191 for 24 h prior to PCB126 treatment for 24 h, and luminescence was measured. As shown in Figure 4-6A, hTERT promoter activity was significantly decreased by PCB126 while increased by CH223191, which was consistent with our previous results that PCB126 inhibited hTERT gene expression and CH223191 increased its expression. In Figure 4-6B, hTERT promoter activity was decreased by PCB126, CoCl₂ or the combination treatment of these two compounds.

![Graph showing hTERT promoter activity](image)

**Figure 4-6. hTERT promoter activity in HaCaT cells with CH223191 or CoCl₂ treatment prior to PCB126 treatment.** HaCaT cells were seeded in 60 mm petri dishes and transfected with pGL3-hTERTp1375 luciferase reporter vector and Renilla luciferase reporter vector. After 5h transfection, the medium was replaced by fresh medium. After overnight culture, cells were split to different treatment groups and treated with CH223191 or CoCl₂ for 24h prior to PCB126 treatment for 24h. The luminescent signal
was measured using a plate reader. A. hTERT promoter activity with AhR antagonist CH223191 pretreated. B. hTERT promoter activity with CoCl₂ pretreated. * $p < 0.05$, compared to control group; # $p < 0.05$, compared to CH223191 treated group.

**ChIP assay**

To check for AhR and/or ARNT binding sites in the promoter of the hTERT gene, we performed ChIP assay. The results shown in Figure 4-7A and B indicates that there is AhR and ARNT binding at the promoter of CYP1B1. Enrichment at the loci was increased after PCB126 treatment and only slightly increased after CoCl₂ treatment. DMSO treated control cells showed only small enrichment of the AhR or ARNT at the analyzed CYP1B1 promoter region. However, there is no detectable enrichment of the proteins at the hTERT promoter region after PCB126 or CoCl₂ treatment, which indicated that there is no binding site for AhR or ARNT in the analyzed hTERT promoter region.

**Figure 4-7. AhR and ARNT binding to CYP1B1 and hTERT promoters after PCB126 or CoCl₂ treatment.** HaCaT cells were treated with 5 µM PCB126 or 100 µM CoCl₂ for 24 h. ChIP assay was performed using anti-AhR and anti-ARNT antibodies. Enriched DNA was analyzed using qPCR primers designed to regions in the promoters of CYP1B1 and hTERT. The enrichment levels were calculated by the equation: Percent Input $= 2\% \times 2^{(C_T(\text{Input}) - C_T(\text{ChIP sample}))}$. A. Enrichment levels of AhR. B. Enrichment levels of ARNT.
**Discussion**

PCB126 is the most potent AhR agonist of the dioxin-like congeners and it is reported to affect transcription via AhR (Fukuzawa et al. 2003; Hassoun et al. 2001). We previously found that it reduces telomerase activity in HL-60 cells. However, the underlying mechanism is not well understood. TERT is the enzymatic subunit of telomerase and it is the rate-limiting factor of the enzyme. The transcriptional regulation of TERT is very important to the regulation of telomerase. No study has ever reported how PCB126 regulates TERT gene expression. In this study, our objective is to investigate if AhR/ARNT signaling pathway is involved in the regulation of PCB126 on hTERT and if hypoxia pathway will affect this regulation.

Since PCB126 is an AhR agonist, we first used AhR antagonists to examine if the effect of PCB126 on telomerase and hTERT is associated with the activation of the AhR pathway. Our results show that both antagonists, α-NF and CH223191, attenuated the down regulation of hTERT by PCB126 and also increased the expression of hTERT, which indicates that AhR might be a negative regulator of hTERT. This was consistent with our results from the siRNA transfection experiment. After using siRNA to knock down AhR, hTERT expression was significantly increased and PCB126 treatment can slightly attenuate the increase of hTERT expression. Together, these data suggest that AhR may be a repressor of hTERT gene.

When AhR is activated, it has to dimerize with ARNT to play its role in gene regulation. We also knocked down ARNT to test if it is also necessary for the regulation of hTERT. Our results show that when ARNT was knocked down, hTERT expression was also down regulated. But when both AhR and ARNT were knocked down, hTERT
mRNA level was increased even more than when AhR was knocked down alone. This suggests that both AhR and ARNT are important for the transcription of hTERT, but their roles are different. AhR could be a negative regulator whereas ARNT is only a cofactor of the AhR, and the role of AhR is likely more important. On the other hand, when the hypoxia pathway is activated, HIF-1α also needs to heterodimerize with ARNT to play its role in regulation of hypoxic response genes. Accordingly, we used CoCl₂ to induce the hypoxia-like condition, and interestingly we found that CoCl₂ down regulated hTERT gene expression in a dose-dependent manner and telomerase protein expression was also decreased by CoCl₂. Also, when combining the treatment with CoCl₂ and PCB126, hTERT expression was decreased even more than with CoCl₂ or PCB126 alone. These results indicate that ARNT could play a positive role in the regulation of hTERT, which is consistent with the siRNA knock down results. In addition, we also observed similar results when examining the hTERT promoter activity in plasmids containing the hTERT promoter in front of the luciferase gene. These experiments suggest that changes of hTERT mRNA levels were likely due to the change of transcription of hTERT because we observed that PCB126 and CoCl₂ inhibited the promoter activity while AhR antagonist CH223191 increased the activity. All these results reveal that AhR is a negative regulator while ARNT is a positive regulator of hTERT. However, we did not observe any binding site at the analyzed promoter region of hTERT for AhR or ARNT. But although we saw an enrichment of AhR and ANRT at the promoter of CYP1B1 after PCB126 treatment, the level of enrichment was not significant. One of the possible reasons for this could be that the antibodies for AhR and ARNT were not efficient for ChIP assays. Alternatively, the promoter region we analyzed did not have binding sites
for these two factors. More promoter regions could be tested in future experiments. Moreover, AhR and ARNT might not directly bind to the promoter of hTERT. They might first bind to other transcription factors of hTERT and consequently indirectly regulate hTERT expression.

Many factors are reported to regulate TERT expression, including c-myc, a proto-oncogene product that activates telomerase by direct binding to the E-boxes of the TERT promoter (Wang et al. 1998; Wu et al. 1999). TCDD has been shown to regulate TERT and telomerase activity through c-myc signaling (Fiorito et al. 2013; Sarkar et al. 2006). Fiorito et al. showed that TCDD down regulated TERT and telomerase activity through activation of AhR and down regulation of c-myc in Madin-Darby bovine kidney (MDBK) cells. Sarkar et al. demonstrated that TCDD induced telomerase activity and hTERT by inducing c-myc in human choriocarcinoma (BoWo) cells. This suggested that dioxin-like PCB126 might regulate hTERT through the activation of AhR, which regulates c-myc, one of the transcription factors of hTERT.

Taken together, our findings suggest that both AhR and ARNT are important for the regulation of hTERT and their regulation is negative (down-regulating). AhR could be the main repressor of hTERT transcription with ARNT as a cofactor to play its role. They probably regulate other transcription factors of hTERT and consequently regulate hTERT gene expression.

Conclusions

The results in the present study show that PCB126 regulates hTERT expression through activation of AhR. Both AhR and ARNT are important for the regulation of
hTERT, but their roles are negative and indirect. They probably need to interact with other transcription factors of hTERT to play their role in the regulation of hTERT.

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CHAPTER 5 CONCLUSIONS AND FUTURE PERSPECTIVES

PCBs pose a significant health risk to both humans and animals in the environment. Since PCBs are reported to have various adverse health effects and are classified as human carcinogens, it is of public health relevance to study their effects and mechanisms of action. Because telomerase and telomeres maintenance are very important to the function of stem cells and progenitor cells and also are related to many diseases such as cancer and premature aging in humans, I reasoned that PCBs could be toxic to stem cells and progenitor cells. Thus, in this dissertation I assessed the effects of PCBs on telomere maintenance and differentiation of hematopoietic stem cells and progenitor cells.

First, the results presented in this dissertation clearly show that long term exposure of HL-60 cells to PCB126 resulted in a down regulation of telomerase activity and shortening of telomere length, accompanied by a 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. I further induced HL-60 cells to differentiate into granulocytes or monocytes after they had been exposed to PCBs for 6 days. The differentiated HL-60 cells showed G0/G1 phase arrest and almost non-detectable telomerase activity and hTERT mRNA levels. But hTR, TRF1/2 and POT1 were upregulated in granulocytes and TRF2 in monocytes. Both PCBs further reduced telomerase activity in differentiated cells, but had different effects on the shelterin gene expression. These results together indicated that different PCB congeners have differential effects on telomere/telomerase-related genes and could possibly influence the longevity of progenitor or stem cells. However, I did not explore the underlying mechanism of how these two congeners have different effects. It would be
interesting to perform further experiments to investigate this. This could be focused on the different receptor-activating activity of PCB126 and PCB153. Since they activate AhR or CAR/PXR, respectively, I would like to see if regulation factors that are correlated to the proliferation and differentiation of stem cells are altered by the abnormal activation of AhR and CAR/PXR. Also, because telomerase has been reported to have other regulatory activities that are telomere maintenance independent, it would be worthwhile to determine if telomerase could directly regulate factors that are involved in stem cell functions.

Second, the results in this dissertation show that PCB126 significantly decreased telomerase activity and down regulated TERT gene and protein expression in rat bone marrow cells. It could also promote bone marrow HSCs to differentiate into a downstream granulocytic direction, visible by the increased numbers of CFU-G and CFU-GM, and increased number of CD11b positive cells. These results suggested that PCB126 has hematotoxicity through regulation of telomerase activity and differentiation of HSCs. To further analyze the hematotoxicity of PCB126, I would like to investigate the effect of PCB126 on the immunophenotyping of stem cells. Even though I analyzed the cell surface markers expression in our study, the antibodies developed for the rat model are much fewer than those for human and mice. It would be worthwhile to employ mouse bone marrow or human samples to see if PCB126 has an adverse effect on the phenotyping of HSCs. It would also be interesting to explore the effects of PCB126 on the functions of HSCs by transplanting the HSCs from the animals that are exposed to PCBs to the leukemia animals to examine if the HSCs still have normal functions for the therapy of leukemia. Since AhR plays an important role in the regulation of HSCs
development and proliferation, it would also be interesting to use an AhR knock-out mouse or rat model to investigate if PCB126 regulates the function of HSCs through the activation of AhR.

Lastly, I exposed HL-60 cells with AhR antagonists prior to PCB126 treatment and found that the antagonists attenuated the adverse effects of PCB126 on hTERT mRNA expression, which indicated that the AhR pathway is involved in the regulation of hTERT expression. Then I exposed the cells to CoCl₂ to induce a hypoxia-like condition and observed a decrease of hTERT gene and protein expression, and when treatment with both CoCl₂ and PCB126 the effect was more pronounced, which suggested that ARNT could be important for the regulation of hTERT. Further I used siRNA to knock down AhR or ARNT or both of them and the results confirmed that both AhR and ARNT are involved in the regulation of hTERT and their regulation was negative. However, I did not see direct binding of AhR or ARNT in the promoter region of hTERT, though there are predicted binding sites for them. This might indicate that AhR and ARNT could bind to other factors and regulate them first and consequently regulate the expression of hTERT. To further investigate this, I would like to do a RNA array to analyze the genes that are changed due to the activation of AhR by exposure to PCB126. Through these altered genes, I would focus on those which are correlated to the regulation of hTERT expression and further investigate how PCB126 affects hTERT expression through these factors.

The long term goal of this dissertation was to gain new insights into the mechanisms and regulation of stem cells proliferation and differentiation mediated by the AhR activation due to PCB126 exposure. Ultimately, the studies in this dissertation have
helped to gain these insights into the regulation of PCB126 on telomerase and telomeres maintenance in hematopoietic stem or progenitor cells. I also gained understanding that the hematotoxicity of PCB126 could be associated with the activation of AhR. I hope that these results help to elucidate the detrimental biological effects of PCB126 and the mechanism of PCB126 carcinogenesis.
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