Original

***In-Utero* and Lactational Exposure of 3,3',4,4',5-Pentachlorobiphenyl Modulate Dimethylbenz[a]anthracene-Induced Rat Mammary Carcinogenesis**

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Abstract: While polychlorinated biphenyls (PCB) are fat-soluble environmental pollutants stored breast fatty tissue and secreted in milk; the precise evidence of breast carcinoma from exposure to PCBs remains unclear. The aim of this study was to investigate the dose-response relationship *in-utero* and lactational exposure of PCBs congener for dimethylbenz[a]anthracene (DMBA)-induced rat mammary carcinogenesis. Female SD rats were injected (i.g.) with 25 pg, 2.5 ng, 250 ng, 7.5 μ g of 3,3',4,4',5-pentachlorobiphenyl (PCB126)/kg, or the vehicle, on days 13 to 19 post-conception. Fifty-day-old female offspring were injected (i.g.) with 20 mg DMBA. The concentration of PCB in the liver of the 50-day-old was compared to the vehicle-group, found to be 530-fold higher in the 7.5 μ g-group, nearly 21-fold higher in the 250 ng-group, nearly 2.4-fold in the 2.5 ng-group, and nearly 1.2-fold in the 25 pg-group. The expression of CYP1A1 in the liver of the 50-day-old was intensive in the 7.5 μ g- and 250 ng-groups, and slight in the 2.5 ng-group, and not observed in the other groups. The observation was terminated when the pups were 170-day-old or the tumor size reached 20mm in diameter. The 7.5 μ g-group showed a reduction of mammary carcinogenesis, but the 250 ng- and 2.5 ng-groups revealed increases incidence of carcinoma, while the 25 pg-group showed a similar incidence of the vehicle-group. The time course of tumor development of the 7.5 μ g-group was significantly lower than that of the other groups, but that of the 250 ng-group was significantly higher, and that of the 2.5 ng- and 25 pg-groups was similar to that of the vehicle-group. Moreover, the tumors of the 250 ng-group showed the highest cell proliferation, aneuploid tumor index, and nuclear grade when compared to those of the other groups. The present studies indicate that *in-utero* and lactational exposure of relatively low dose PCB acts as an enhancing agent toward DMBA-induced rat mammary carcinogenesis, but that of high dose PCB acts as an inhibiting agent. This effect partly may be due to the *in vivo* difference at the day of DMBA exposure (50-day-old); between the concentration of PCB and the expression of phase I drug-metabolize enzymes (CYP1A1) converting DMBA into the ultimate carcinogen. (J Toxicol Pathol 2001; 14: 213–224)

Key words: 3,3',4,4',5-pentachlorobiphenyl, dimethylbenz[a]anthracene, rat, mammary carcinogenesis

Introduction

Dioxin-type chemicals include serious environmental pollutants, such as polychlorinated biphenyls (PCB), polyhalogenated dibenzo-*p*-dioxins, dibenzofurans, naphthalenes, and polyaromatic hydrocarbons, all of which

possess similar toxic properties^{1,2}. PCBs constitute a large number of plasticizers and other common substances, and are found to be slowly bio-degradable and therefore bio-accumulate easily in food chains^{3–5}. Since PCBs are fat-soluble nature found in breast fatty tissue and can reach individuals via the placental circulation and breast milk, they possess a potential hazard to health including breast carcinoma^{4,6}. There are some reports that breast fat tissue and serum lipids of women with breast carcinoma contain higher levels of some organochlorine residues than non-carcinoma controls^{7,8}, but a formal link between PCBs and breast carcinoma has been unproved^{3,4}.

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There have been limited experiments reported to determine whether dioxins could influence development of chemical-induced mammary carcinogenesis⁹⁻¹³. One well established model for the study of mammary carcinogenesis involves the use of a single oral dose of 7,12-dimethylbenz(*a*)anthracene (DMBA) to initiate rats mammary carcinoma¹⁴, where interacting agents can be investigated for their ability to modulate growth of the tumors¹⁵. In animal models, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) given to adult rats just 3 day prior to administration of DMBA was found to delay the onset of mammary carcinoma⁹. Also, TCDD or 3,3',4,4'-Tetrachlorobiphenyl (PCB77) administered to adult rats having DMBA-induced mammary tumors has been reported to inhibit the growth of these tumors^{12, 13}. Meanwhile, rats treated *in-utero* with TCDD and then with DMBA at day 50 post partus developed more mammary carcinoma compared to the vehicle-group¹⁰. Moreover, when PCB77 was co-administrated with DMBA to adult rats, increased mammary carcinoma formation is reported¹¹. These results conflict with the previous reports which have been considered to be due to differences in the timing of compound administrations; prenatal or postnatal⁹⁻¹³.

It is also clear that the evaluation of tumorigenic/ anti-tumorigenic activities of dioxins should consider both the enhancing and protective effects of this compound in mammary carcinogenesis. However, no investigations have been carried out on the dose-response relationship for exposure of PCBs to alter development and predisposition to mammary carcinogenesis. Hence, we investigated how the dose-responsive potential of *in-utero* with lactational exposure of 3,3',4,4',5-pentachlorobiphenyl (PCB126) to modulate DMBA-induced rats mammary carcinogenesis.

Material and Methods

Animals, chemicals and treatments

Seventy-five female and fifteen male six-week-old Sprague-Dawley (slc) rats (Japan SLC) were housed, three per plastic cage, on hardwood-chip bedding in an environment-controlled room on a 12-hour light/12-hour dark cycle at $22 \pm 2^\circ\text{C}$ and $55\% \pm 5\%$ relative humidity, with a conventional diet (MF, Oriental Yeast Co. Ltd.). All experimental procedures were conducted following approval of the Animal Care and Use Committee of the Azabu University School of Veterinary Medicine. Guidelines set by the National Institute of Health and Public Health Service Policy on the Humane Use and Care of Laboratory Animals were followed at all times. The PCB126 was obtained from AccuStandard Inc., New Haven, CT, and the DMBA was obtained from Tokyo Chemical Industry Co. Ltd.. Seven-week-old rats were housed in five females and a male per plastic cage. A lifetime tolerable daily intake (TDI) of PCB126 has been described ranging from 10 to 100 pg/kg/day⁶. In this study, the four doses of PCB126 were settled; 25 pg/kg/day as to TDI dose, 10^2 -hold of TDI dose, 10^4 -hold of TDI dose, and 3×10^5 -hold of TDI dose. Timed pregnant,

each fifteen female rats were treated with 25 pg, 2.5 ng, 250 ng, 7.5 μg PCB126 /kg body (i.g.) or with an equivalent volume of corn oil (~ 0.5 ml/animal, i.g.), on day 13 through 19 post-conception. The offspring were sexed at birth and litters were reduced so that each dam was left with eight offspring (four females/dam). Weaning was carried out at day 21 post-partus. Each group (25 pg-, 2.5 ng-, 250 ng-, 7.5 μg -, or vehicle-group) was revealed sixty females. Five randomly selected females from each group were used at 30- or 50-day-old without DMBA administration for the evaluation of hepatic PCB126 and CYP1A1. For tumorigenesis experiments, 50-day-old female rats (forty-five females for each group) received 20 mg DMBA (in Con oil) body (i.g.), or (five females for each group) received an equivalent volume of corn oil (~ 0.5 ml/animal, i.g.). Rats were palpated every day for mammary tumors until they were 170-day-old, or until the tumors reached 20 mm in diameter. The location, size, and date of onset of each tumor were charted for each animal. The samples of the mammary tumors were obtained from rats under deep anesthesia with diethyl ether. In each group, the cumulative number of tumor masses was calculated by every day's palpation of rats, and the time course of tumor development was calculated by the following two factors; one was the tumor weight at dissection, and the other was the time length between the day of tumor discovery by palpation and the day of tumor dissection. The representative sections of each tumor were fixed 10% phosphate-buffered formalin and routinely processed for hematoxylin-eosin staining. Pathological diagnoses of the mammary lesions were classified as described previously¹⁶. Moreover, the other representative sections were frozen without fixation and stored at -70°C .

Chemical analysis

Analysis for PCB126 was carried out following the alkaline alcohol digestion method¹⁷. Aliquots of homogenized samples were refluxed in 1N KOH-ethanol solution for 1 hr. PCB126 thus extracted into ethanol were transferred to 100 ml of hexane by shaking in a separating funnel. Subsequently, the hexane layer was concentrated and cleaned up by passing through 1.5 g of silica gel (Wako gel S-1, Wako Co. Ltd.) packed in a glass column (10 mm inside diameter $\times$ 200 mm length). PCB126 was eluted with 200 ml of hexane at an elution rate of one drop a second. The eluate was concentrated to 5 ml in a Kuderna-Danish concentrator and further cleaned up with 5% fuming sulphuric acid. All samples were injected into a gas chromatograph-mass spectrometer (GC-MS: Shimadzu 9020 DF with SCAP-1123 data system, Shimadzu Co. Ltd. Tokyo) equipped with an electron-impact ion-source and moving needle-type injection system for the determination and identification of PCB126. The column consisted of 0.23 mm inner diameter $\times$ 30 m length glass capillary, coated with silicone. Operating conditions of the GC-MS were as follows: Column oven temperature was programmed from 190°C to 250°C at $0.5^\circ\text{Cmin}^{-1}$, injector and ion-source

temperatures were kept at 250°C and 280°C, respectively. PCB126 was determined by selected ion monitoring at *m/z* 326. The carrier flow of helium was controlled at 0.6 ml min⁻¹.

Immunohistochemistry

Immunohistochemical expression of CYP1A1 and Proliferating Cell Nuclear Antigen (PCNA) were analyzed. The avidin-biotin complex (ABC) method was used. After deparaffinization, sections, 4 µm thick, were treated sequentially with 0.3% H₂O₂ for 10 min, then blocked with 10% goat serum or horse serum in PBS for 20 min. On thawing, sections were rinsed in PBS and treated with primary antibodies of rabbit anti-rat-CYP1A1 (Affiniti Res. Inc.: diluted 1:1000), and mouse anti-PCNA (Novocastra Co. Ltd.: diluted 1:50). Bound IgG was detected with biotinylated goat anti-rabbit IgG (Vector Lab.:diluted 1:100) followed by avidin-biotin complex (ABC)-peroxidase (Vector Lab.) and diamino-benzidine (Sigma). Sections were then counter-stained with hematoxylin. As negative control, nonimmunized rabbit serum was substituted for the primary antibody. The numbers of PCNA-positive cells were measured at random in >1000 cells per each section and the proliferating indices were expressed as percentage values.

Comparative RT-PCR

Measurements of RNA levels were performed using an RT-PCR. RNAs were prepared for the semi-quantitation of CYP1A1 and β-actin. PCR primer pairs used to amplify these specific RNAs are identified in Table 1. Total RNA was isolated from frozen liver samples using an acid-guanidinium-phenol-chloroform method¹⁸. Total liver RNA (1 µg) was reverse-transcribed using Reverse Transcriptase Superscript II (Life Technologies, Inc.) in a total volume of 20 µl following the manufacturer's protocol. Controls included for each reaction were the RNA sample without

reverse transcriptase (RNA-RT) and no RNA with reverse transcriptase (no RNA+RT). PCR reactions were performed by first-strand cDNA synthesis (2 µl) from each sample with 1 × reaction buffer, 2 mM MgCl₂, 200 µM deoxynucleotide triphosphates, 0.5 U/µl Taq Gold (PE Biosystems) included both CYP1A1 and β-actin with 0.5 µM each forward and reverse primers. Amplifications were 94°C - 30s, 57°C - 30s, and 72°C - 30s for 30 cycles using Gene Amp PCR system 9700 (PE Biosystems). RNA-RT, no RNA+RT, and no DNA controls were included with each PCR run. PCR products were separated by 2% agarose gel electrophoresis in 1 × TBE buffer and stained with 1 µg/ml ethidium bromide, and exposed to film.

Real-time quantitative RT-PCR

For each RNA sample, 100 ng were then used as template for first strand cDNA synthesis using a TaqMan Reverse Transcription kit, following two-step RT-PCR manufacturer's protocol (PE Biosystem). Controls included for each reaction were the RNA sample without reverse transcriptase (RNA-RT) and no RNA with reverse transcriptase (no RNA+RT). The condition of final reaction for reverse-transcription was as follows: 1 × TaqMan RT buffer; 5.5 mM MgCl₂; 500 µM dATP, dGTP, and dCTP; 1 mM dTTP; Random Hexamers 0.25 µM; 1.25 U/µl MµLV reverse transcriptase and 0.4U (PE Biosystem). Quantitative analysis of CYP1A1 mRNA expression was performed by the real-time quantitative PCR using the ABI Prism 7700 Sequence Detection System (PE Biosystems) with TaqMan chemistry and probe. Forward and reverse primers and the fluorogenic TaqMan probe were designed using Primer Express software (PE Biosystems). The TaqMan probe is an oligonucleotide with fluorescent reporter and quencher dyes attached, designed from the mRNA sequence to hybridize to a region between the forward and reverse PCR primers (Table 1). Optimal primer, probe, and cDNA concentrations were determined in a separate set of experiments to insure

Table 1. Primer Pairs for Comparative RT-PCR Analysis

	Forward (5'-3')	Reverse (5'-3')	Product size (bp)
CYP1A1 (X00469)	CCATGACCAGGAAGTATGGG	TCTGGTGAGCATCCAGGACA	341
β-actin (V01217)	CAGCCTTCCTCCTGGGTATG	TAGAGCCACCAATCCACACAG	246
Primer pairs for real-time quantitative RT-PCR analysis			
	Forward (5'-3')	Reverse (5'-3')	
CYP1A1 TaqMan Probe	GCCTTCACATCAGCCACAGA	TTGTGACTCTAACCACCCAGAATC	
	CTGGCCGTCACCACATTCTGCCTT		
GAPDH TaqMan Probe	GAAGGTGAAGGTCGGAGTCAAC	CAGAGTTAAAAGCAGCCCTGGT	
	TTTGGTCCGTATTGGGCGCCT		

Product size (bp): CYP1A1=76, GAPDH=78.

that both CYP1A1 and GAPDH fragments were amplified with equal efficiency. PCR reactions were performed with first-strand cDNA synthesis (2 μ l) from each sample, Universal PCR Master Mix kit (PE Biosystems), 250 nM TaqMan probe, 0.16 U of AmpErase UNG (uracil N-glycosylase), and 900 nM forward- reverse primers of CYP1A1 and GAPDH. PCR parameters were 50°C - 2 min, 95°C - 10 mins, and 40 cycles at 95°C - 15s, 60°C - 1 min. Three measurements per sample were performed in each of two independent experiments. Results were analyzed with the ABI Sequence Detector software version 1.7 (PE Biosystems). For relative quantification of CYP1A1 expression, the calibrated standard curve of CYP1A1 cDNA and GAPDH amplification plots were examined at five different dilutions (containing 100, 50, 25, 10, or 5 ng) of total RNA samples.

Image-morphometrical analysis for quantitative DNA

Sections, 5 μ m thick, of 10% phosphate-buffered formalin fixed sample were stained by the Feulgen method (Bacus Lab. Inc. IL) and analyzed for DNA ploidy with the CAS200 Image Analyzer (Bacus Lab. Inc. IL). After the instrument was calibrated against tetraploid rat hepatocytes stained similarly, the DNA content of the mammary tumors in a minimum of 150 tumor cells, and the tumor DNA index was determined by comparison with the control diploid cells of the normal rat lymphocytes or normal mammary epithelial cells. All the tumor cell histograms were reviewed without knowledge of the tumor histology. The DNA index of 0.83–1.22 was considered to be diploid. Peaks in the tetraploid region containing less than 15% of the total cell population were considered to be the G2M components of diploid cell populations. Tumors with tetraploid peaks greater than 15% and hyperdiploid, nontetraploid peaks were considered to be aneuploid^{19, 20}.

Image-morphometric nuclear grading Z-score

Nuclear images were also extracted from the Feulgen-stained histological sections using the CAS200 Quantitative DNA software program²⁰. Nuclear features were measured on these individual nuclei using the Nuclear Grade software program (Bacus Lab., Inc. IL). The mean and standard deviations (SD) of the measurements were then used to transform subsequent measurements on tumor cells nuclei into Z-score scaled in SD units²¹. The method of combining the Z-scores of selected nuclear morphological feature measurements into a single nuclear grade for each nucleus. Using this method, the nuclear grades of normal mammary epithelium are always distributed about a mean value of zero. The individual feature Z-scores are weighted by a co-efficient and summed to obtain the final nuclear grade. The weighting coefficients are obtained by Fischer liner discriminate analysis of the n-dimensional mean differences of carcinoma nuclei compared with the references of population of normal nuclei. For the mammary carcinoma, the four nuclear features and their weighting coefficients

used were: area, 0.74; DNA, 0.62; entropy, 0.21; and Valley, 0.10^{21, 22}. The nuclear morphological feature with the greatest discriminating capacity has the highest weighting co-efficient. In many nuclei, the contributions of morphological features with smaller weighting co-efficient were still important because their measured values were high enough to over-ride their smaller weights^{21, 22}. Details of the mathematical-method in the nuclear grade were previously described by Bacus JW *et al.*²¹.

Statistical analysis

For each set the mean value, standard deviation and standard error of mean were calculated and compared using Scheffé's F-test or Chi-squared test with statistical computer analysis system Stat View-J 5.0 (Abacus Concepts).

Results

Body and organ weight, and sexual maturation

Prenatal PCB126 treatment of dams resulted in offspring with body weights at 21-, 30-day-old significantly lower, dose despondent, than those from dams treated with the vehicle (vehicle-group), but their weights in the 50-day-old were similar between groups (Table 2). Liver and uterine weights of 30-, 50-day-old were similar between groups (Table 2). Endometrial or vaginal hypoplasia were not histologically observed in each group (data not shown). As to sexual maturity vaginal opening in the 7.5 μ g-, the 250 ng-, and the 2.5 ng-groups was at 36-day-old, while that in the 25 pg- and the vehicle-group was at 34-day-old. Estrous cycle in each group was not investigated.

Concentration of PCB in liver

The concentration of PCB in the liver of the 50-day-old when compared to the vehicle-group was 530-fold higher in the 7.5 μ g-group, about 21-fold higher in the 250 ng-group, about 2.4-fold in the 2.5 ng-group, and about 1.2-fold in the 25 pg-group (Table 3).

Expression of CYP1A1 in the liver

Immunohistochemical analysis revealed expression of CYP1A1 in the liver of the 30-day-old was intensive at the centrilobular zone of the 7.5 μ g- and the 250 ng-groups, slight for the 2.5 ng-group, and was not observed in the other groups (Fig. 1). In the 50-day-old, expression of CYP1A1 in the liver was also intensive in the 7.5 μ g- and the 250 ng-groups, but was not observed in other groups (Fig. 1).

Comparative RT-PCR for CYP1A1 in the liver

The relative levels of CYP1A1 mRNA expression in the liver were observed by comparative RT-PCR. In the 30-day-old, relatively higher expression of CYP1A1 mRNA was revealed in the 7.5 μ g- and the 250 ng-groups, and slight expression in the 2.5 ng- and the 25 pg-groups (Fig. 2). In the 50-day-old, higher expression of CYP1A1 mRNA was only revealed in the 7.5 μ g-group (Fig. 2).

Table 2. Body, Liver and Uterine Weights in 21-, 30-, and 50-day-old Rats *In-Utero* and Lactational Exposure of PCB126

Group	Body weight (BW)(g)		
	(21-day-old)	(30-day-old)	(50-day-old)
7.5 μ g-	29.52 $\pm$ 0.91**	134.64 $\pm$ 4.32**	193.30 $\pm$ 5.01
250 ng-	34.03 $\pm$ 0.54**	159.92 $\pm$ 4.73	192.31 $\pm$ 4.18
2.5 ng-	36.84 $\pm$ 0.94	160.53 $\pm$ 2.77	192.26 $\pm$ 4.48
25 pg-	37.43 $\pm$ 0.67	167.43 $\pm$ 3.25	197.35 $\pm$ 5.12
Vehicle-	39.56 $\pm$ 0.91	167.21 $\pm$ 4.14	195.65 $\pm$ 5.43

Group	Liver weight (g)		Uterine weight (mg)	
	(30-day-old)	(50-day-old)	(30-day-old)	(50-day-old)
7.5 μ g-	3.52 $\pm$ 0.76	8.13 $\pm$ 0.37	101 $\pm$ 41	252 $\pm$ 47
250 ng-	4.50 $\pm$ 0.14	8.28 $\pm$ 0.36	156 $\pm$ 34	280 $\pm$ 56
2.5 ng-	4.50 $\pm$ 0.13	8.35 $\pm$ 0.30	177 $\pm$ 44	271 $\pm$ 48
25 pg-	4.58 $\pm$ 0.13	8.28 $\pm$ 0.55	149 $\pm$ 29	271 $\pm$ 57
Vehicle-	4.58 $\pm$ 0.11	8.45 $\pm$ 0.43	167 $\pm$ 33	275 $\pm$ 38

Values represent mean $\pm$ SEM. (**) Scheffé's F test $p < 0.05$.

Table 3. Concentration of PCB126 in Liver in 50-day-old Female Rats *In-Utero* and Lactational Exposure of PCB126

Group	
7.5 μ g-	64.08 $\pm$ 7.98**
250 ng-	2.47 $\pm$ 0.79
2.5 ng-	0.29 $\pm$ 0.08
25 pg-	0.14 $\pm$ 0.02
Vehicle-	0.12 $\pm$ 0.02

Values represent mean $\pm$ SEM ng/g. (**) Scheffé's F test $p < 0.05$.

Quantitative RT-PCR for CYP1A1 mRNA

The expression of CYP1A1 mRNA in the liver was analyzed by real-time quantitative RT-PCR. In the 30-day-old, quantification of CYP1A1 mRNA transcripts revealed significantly higher levels in the 7.5 μ g-, and the 250 ng-groups compared with the vehicle-group (Table 4). In the 50-day-old, a significant high level of quantification of CYP1A1 mRNA transcripts was revealed only in the 7.5 μ g-group (Table 4). RT-PCR products obtained were of the expected sizes (Table 1). No products were obtained when RT was carried out in the absence of the reverse transcriptase enzyme, indicating that there was no amplification from any genomic DNA that may be present in the RNA preparation (data not shown).

Tumorigenesis

The incidence of DMBA-induced mammary tumor was significantly low in the 7.5 μ g-group, while those in the other groups did not show significant differences from the vehicle-group (Table 5). Mean number of mammary tumors per individual was similar between all groups (Table 5). The cumulative number of tumor masses in each group of forty-

five rats is shown in Fig. 3. At the termination, the 7.5 μ g-group showed a reduction of mammary tumorigenesis, but the 250 ng- and the 2.5 ng-groups revealed increased incidence of mammary tumors, while the 25 pg-group showed a similar incidence as the vehicle-group (Fig. 3). The time course of tumor development of the 7.5 μ g-group was significantly lower than that of other groups; that of the 250 ng-group was the highest; those of the 2.5 ng- and the 25 pg-groups were similar to that of the vehicle-group (Fig. 4). In the 7.5 μ g-group 33/81 (41%) of mammary tumors were adenoma, while those observed other groups were all adenocarcinoma (Table 5). At autopsy, there were no nodules nor tumor mass in the liver in any of the rats used (data not shown).

Immunohistochemistry for PCNA

Cell proliferating activity of the tumor cells was examined by PCNA labeling index in the tissues diagnosed as carcinomas (Fig. 5). The significantly higher labeling index was observed in the 250 ng-group, and the other groups showed a similar index to each other (Table 6).

Image-morphometrical analysis for quantitative DNA

DNA ploidy of the mammary carcinomas was analyzed by image-morphometrical analysis. Examples of distribution of DNA mass are shown in Fig. 6. Aneuploid type mammary carcinoma incidence was significantly higher in 250 ng-group than the other groups (Table 7).

Image-morphometric nuclear grading Z-score

Image-morphometric nuclear grading Z-score of mammary carcinoma was analyzed following the method of Bacus *et al.*^{21, 22}. Examples of distribution of Z-score are shown in Fig. 7. Z-score of 250 ng-group was significantly higher than the other groups (Table 8).

Fig. 1. Immunohistochemical analysis for CYP1A1 in liver of 30- (A,B,C) or 50- (D,E,F) day-old rats, *in-utero* and lactational exposure of PCB126. At 30-day-old, abundant numbers of hepatocytes in the centrilobular zone were positive in the 7.5 μ g-group (A) and 250 ng-group (B), and a few number of positive hepatocytes was observed in the 2.5 ng-group (C). At 50-day-old, many hepatocytes were positive in the 7.5 μ g-group (D) and 250 ng-group (E), and no positive hepatocytes were observed in the 2.5 ng-group. ABC method, Mayer's hematoxylin counter stain. $\times 140$.

Discussion

The dioxins are ubiquitous environmental contaminants that produce a spectrum of biochemical and adverse biological effects in people and in a wide variety of animals, including carcinogenic effects²³. However, dioxins are considered non-genotoxic carcinogens, since they do not form DNA adducts and are negative *in vitro* tests for genetic toxicity²⁴. The PCB and TCDD possess high affinity to aryl hydrocarbon receptor (AhR), and their interactions with this cytosolic receptor are the essential first step in the resulting toxic actions^{1,5}. The AhR binding with dioxin detaches itself

from a complex with HSP90, translocates to the nucleus, where it forms a dimer with another helix-loop-helix protein "arnt" (a nuclear transcription factor), and eventually interacts with the promoters of specific genes²⁵. Several reports have demonstrated that PCB and TCDD exhibit anti-estrogenic and anti-tumorigenic activities via crosstalk between AhR and estrogen-receptors (ER) signaling pathway in human and rodent^{2,5,26}. There were some reports that TCDD or PCB77 administered to rats bearing DMBA-induced mammary tumors was able to inhibit growth of these tumors^{12,13}. These effects have been considered to be the anti-estrogenic and anti-promotional activity of these

Fig. 2. Comparative RT-PCR analysis of 30- (A) or 50- (B) day-old rats, *in-utero* and lactational exposure of PCB126. RT-PCR products were normalized to observe using the PCR products of β -actin as a quantitative control. (A) at 30-day-old, CYP1A1 mRNA levels in the 7.5 μ g- and 250 ng-group were increased in comparison to other groups. (B) at 50-day-old, CYP1A1 mRNA levels in the 7.5 μ g-group was only increased in comparison to other groups.

Table 4. Levels of CYP1A1 mRNA Expression Using Real-Time Quantitative RT-PCR Analysis in Liver in 30-, and 50-day-old Rats *In-Utero* and Lactational Exposure of PCB126

Group	30-day-old	50-day-old
7.5 μ g-	1.170 $\pm$ 0.076**	0.525 $\pm$ 0.345**
250 ng-	0.367 $\pm$ 0.027**	0.012 $\pm$ 0.001
2.5 ng-	0.010 $\pm$ 0.002	0.006 $\pm$ 0.002
25 pg-	0.007 $\pm$ 0.001	0.005 $\pm$ 0.001
Vehicle-	0.005 $\pm$ 0.002	0.005 $\pm$ 0.002

CYP1A1 mRNA expression levels were normalized by GAPDH mRNA expression levels. Three cases were analyzed in each group. Values represent mean $\pm$ SEM. (**) Scheffé's F test $p < 0.05$.

chemicals by way of inhibiting E2-induced responses *in vivo* model^{12, 13}.

One important action of dioxins binding with the AhR is the induction of cytochrome P450 1A1 enzymes (CYP1A1) in various tissues, particularly in the liver²⁷. Meanwhile, DMBA is a member of the polycyclic aromatic hydrocarbons, also binds to activate the AhR and induces the transcription of a number of genes including *CYP1A1*^{28, 29}. DMBA is a pro-carcinogen requiring oxidative metabolic conversion by CYP1A1 to the ultimate carcinogen (DMBA-3,4-diol-1,2-epoxide) which reacts with DNA to form adducts introducing mutations in key genes, and resulting in cellular transformation^{30, 31}.

In the present study, *in-utero* and lactational exposure of PCB126 modulated DMBA-induced rats mammary carcinogenesis. The 250 ng- and 2.5 ng-groups revealed increased incidence of carcinoma, while the 25 pg-group showed a similar incidence of the vehicle-group, but the 7.5 μ g-group showed a reduction of mammary carcinogenesis. The precise mechanism concerning the modulation of incidence of mammary tumor by PCB126 remains to be

understood, one possible mechanism could result from the speculation that the ability of highly oxidative metabolized DMBA by CYP1A1 to induce carcinogenesis was dominant in the 250 ng-group, while the ability of PCB126 to induce anti-estrogenic and anti-promotional effects was dominant in the 7.5 μ g-group. Since, at the day of DMBA exposure, the 250 μ g-group revealed low level hepatic PCB126 (about 26-fold lower than that of the 7.5 μ g-group) with high level hepatic CYP1A1, but the 7.5 μ g-group revealed high level of hepatic PCB126 and CYP1A1. This would be consistent with previous studies showing that benz(a)anthracene, another AhR agonist and inducer of cytochrome P450 mono-oxygenases, can increase metabolic activation of DMBA in rat cell lines^{29, 31}. Moreover, in the present study, at the day of DMBA exposure, hepatic CYP1A1 mRNA expression of the 250 ng-group was much lower, but high level of hepatic CYP1A1 was observed. Since at 20 days prior to DMBA exposure, hepatic CYP1A1 mRNA and CYP1A1 expression were similar both in the 7.5 μ g- and the 250 ng-groups. Then hepatic CYP1A1 observed at the day of DMBA exposure in the 250 ng-group might have been produced previously and stargazed in liver cells.

Moreover, mammary carcinomas of the 250 ng-group (relatively low dose of PCB126 treated group) showed the highest tumor malignancy; high levels of tumor incidence, tumor development, cell proliferation, aneuploid tumor index, and nuclear grade. While the 7.5 μ g-group apparently showed a reduction of mammary carcinogenesis with significant lower tumor malignancy. Although adenocarcinomas were predominant in DMBA-induced rat mammary carcinoma^{14, 15}, many benign tumors were observed in the 7.5 μ g-group. In this study, the precise mechanism of mammary tumor malignant modulation, further studies are required concerning the expression or mutation of cell-cycle regulated genes.

It has been previously reported that 10 μ g TCDD/kg given to rats 3 days prior to administration of DMBA show a decrease in mammary carcinoma formation⁹. We think that the high dose exposure of TCDD (10 μ g/kg) could inhibit the DMBA-induced mammary carcinogenesis by anti-estrogenic and anti-promotional activity. The toxic mechanism of action of dioxins form the basis for the risk assessment of these substances by using the toxic equivalency factor (TEF) concept, and TCDD as a reference compound^{3, 4, 6}. Recently, Nesaretnam *et al.*^{11, 32} reported that PCB77 bound to the ER and induced estrogenic response, and female rats were treated 10 mg PCB77/kg co-administrated with DMBA showed enhancement of mammary carcinoma formation. Since TEF of PCB77 has been described as 0.0001⁶, 10 mg PCB77/kg reveals the same toxic level of 10 μ g TCDD/kg. It was surprising that the high dose PCB77 enhanced rats mammary carcinogenesis. Although the exposure of 10 mg PCB77/kg probably would not be able to inhibit the anti-promotional activity by the effect of highly oxidative metabolized DMBA, unknown impurities or metabolites might play a role in this response. While, more recently, Ramamoorthy *et al.* re-examined Nesaretnam *et al.* studies^{11, 32}

Table 5. Incidence of DMBA-induced Mammary Tumors of Rats *In-Utero* and Lactational Exposure of PCB126

Group	No. of rats	No.(%) of rats with tumor	No. of tumors per rat
7.5 μ g-	45	35† (77.8%) **	2.25 $\pm$ 1.55♣
250 ng-	45	43 (95.6%)	5.09 $\pm$ 2.42
2.5 ng-	45	42 (93.3%)	3.98 $\pm$ 2.82
25 pg-	45	44 (97.8%)	2.77 $\pm$ 1.89
Vehicle-	45	42 (93.3%)	3.12 $\pm$ 0.74

† number of mammary tumor bearing rats, (**) Chi-squared test $p < 0.05$.♣ Values represent mean $\pm$ SEM. Scheffé's F test, NS.

Group	Total†	Fibroadenoma**	(Sec.	Adenoma**	(Cy-pap.)	(Pap.	Tubu.	Adenocarcinoma♣	Comedo-cribri.)
7.5 μg-	81	6 (7.4%)	10 (12.4 %)	17 (21.0%)	1 (1.2%)	20 (24.7%)	25 (30.9%)		
250 ng-	219	--	--	--	9 (4.1%)	92 (42.0%)	118 (53.9%)		
2.5 ng-	167	--	--	--	17 (10.1%)	67 (40.1%)	83 (49.7%)		
25 pg-	122	--	--	--	10 (8.2%)	32 (26.2%)	80 (65.6%)		
Vehicle-	128	--	--	--	11 (8.6%)	35 (27.3%)	82 (64.1%)		

† total number of mammary tumors, (**) Chi-squared test $p < 0.05$, (♣) Chi-squared test, NS, Sec.: Secretory pattern, Cy-Pap.: Cystic papillary pattern, Pap.: Papillary pattern, Tubu.: Tubular pattern, Comedo-Cribri.: Comedo-cribriiform pattern. **Fig. 3.** The time course of tumor appearance with effects of PCB126 for DMBA-induced rat mammary tumorigenesis. Each point represents the total number of palpable mammary tumors in each group of forty-five rats. **Fig. 4.** The time course of tumor development with effects of PCB126 for DMBA-induced rat mammary tumorigenesis. Values represent mean tumor weight $\pm$ SEM. (**) Scheffé's F test $p < 0.05$.

Fig. 5. Immunohistochemical analysis for PCNA of DMBA-induced mammary carcinomas of rats *in-utero* and lactational exposure of PCB126. A small number of them observed in the 7.5 µg-group (A), and a number of PCNA positive nuclei observed in the 250 ng-group (B). ABC method, Mayer's hematoxylin counter stain. $\times 240$.

Table 6. PCNA Labeling Index of DMBA-induced Mammary Carcinomas of Rats *In-Utero* and Lactational Exposure of PCB126

Group	PCNA labeling index (%)	Evaluated number of carcinomas
7.5 µg-	24.29 $\pm$ 1.02	48
250 ng-	39.48 $\pm$ 2.45**	110
2.5 ng-	29.23 $\pm$ 1.75	110
25 pg-	26.90 $\pm$ 1.65	105
Vehicle-	26.82 $\pm$ 1.64	105

Values represent mean $\pm$ SEM. (**) Scheffé's F test $p < 0.05$.

and reported that PCB77 did not bind to the ER and did not exhibit estrogenic activity¹².

Meanwhile, rats were treated 1 µg TCDD/kg on day 15 gestation to their dam with avoided lactational exposure of TCDD from dam, and then with DMBA at day 50 post partus developed twice as many tumors as compared to rats treated prenatally with vehicle only and then with DMBA¹⁰. It has been indicated that transfer of TCDD through the placenta is only about 0.01% of the total dose found in the fetus liver, and it has been considered that the offspring were

Fig. 6. Image-morphometrical analysis for quantitative DNA of DMBA-induced mammary carcinomas of rats *in-utero* and lactational exposure of PCB126. Distribution of DNA of (A) shows diploid type, and (B) shows an aneuploid type. Arrows show first DNA peak.

predominantly exposed by dioxins via lactation rather than through placental transfer³³. Then the report of Brown *et al.*¹⁰ also suggested that low level of dioxin exposure could enhance DMBA-induced rats mammary carcinogenesis.

An IARC working group²³ concluded that dioxins seen to act similarly in rodent and human, and several epidemiological studies have shown an association between dioxin exposure and adverse effects including increased overall cancer risk in human⁵. Using a threshold-based approach derived a lifetime TDI ranging from 1 to 10 pg/kg/day of TCDD for human and mammals⁶. The TEF of PCB126 has been described as 0.1, and TDI of PCB126 is considered to 10–100 pg/kg/day⁶. The present study revealed that pups from the dam exposed by 2.5 ng/kg PCB126 on day 13 to 19 post-conception increased the incidence of DMBA-induced mammary carcinogenesis compared to those from dams treated with the vehicle. The dose of 2.5 ng/kg/day was 25–250-fold higher than TDI of PCB126 in human and mammals⁶.

In summary, *in-utero* and lactational exposure to relatively low doses of PCB126 increased the risk to develop

Fig. 7. Image-morphometric nuclear grading Z-score of DMBA-induced mammary carcinomas of rats *in-utero* and lactational exposure of PCB126. Feulgen-stained histological sections of carcinomas ($\times 500$) and corresponding Z-score distributions are shown by red lines. Green lines show normalized distribution of Z-score in normal mammary epithelium (Gaussian). (A) 7.5 μg -group, (B) 250 ng-group, (C) 2.5 ng-group.

Table 7. DNA Ploidy Pattern of DMBA-induced Mammary Carcinomas of Rats *In-Utero* and Lactational Exposure of PCB126

Group	Aneuploid type carcinoma incidence (%)	Evaluated number of carcinomas
7.5 μg -	9.72	72
250 ng-	37.32**	142
2.5 ng-	18.80	133
25 pg-	11.43	105
Vehicle-	11.88	101

**Chi-squared test $p < 0.05$.

Table 8. Image-morphometrical Nuclear Grading Z-score of DMBA-induced Mammary Carcinomas of Rats *In-Utero* and Lactational exposure of PCB126

Group	Z-score	Evaluated number of carcinomas
7.5 μg -	2.39 ± 0.64	49
250 ng-	$6.96 \pm 0.42^{**}$	56
2.5 ng-	3.30 ± 0.70	50
25 pg-	2.62 ± 0.46	49
Vehicle-	2.87 ± 0.53	54

Normal mammary epithelium (48 cases) 0.00 ± 0.36

Values represent mean $\pm$ SEM. (**) Scheffé's F test $p < 0.05$.

DMBA-induced rat mammary tumors, but that exposure to the high dose decreased that risk. However, it remains to be determined whether such levels of PCB exposed during *in-utero* and lactational periods induce subtle changes in humans as determined in the present experimental animal studies.

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References

- Poland A, and Knutson JC. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: Examination of the mechanism of toxicity. *Ann Rev Pharmacol Toxicol* 1982; **22**: 517–554.
- Fielden MR, Chen I, Chittim B, Safe S, and Zacharewski TR. Examination of the estrogenicity of 2,4,6,2',6'-pentachlorobiphenyl (PCB104), its hydroxylated metabolite 2,4,6,2',6'-pentachloro-4-biphenylol (HO-PCB104), and a further chlorinated derivative, 2,4,6,2',4',6'-hexachlorobiphenyl (PCB155). *Environ Health Perspect* 1997; **105**: 1238–1248.
- Safe S, Astroff B, Harris M, Zacharewski T, Dickerson R, Romkes M, and Biegel L. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds as antioestrogens: characterization and mechanism of action. *Pharmacol Toxicol* 1991; **133**: 400–409.
- Safe S. Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic response, and implications for risk assessment. *CRC Crit Rev Toxicol* 1994; **24**: 87–149.
- Safe S. Modulation of gene expression and endocrine response pathways by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related compounds. *Pharmacol Therap* 1995; **67**: 247–281.
- van den Berg M, Birnbaum L, Bosveld ATC, Brunstrom B, Cook P, Feely M, Giesy JP, Hanberg A, Hasegawa R, Kennedy SW, Kubiak T, Larsen JC, Rolaf van Leeuwen FX, Liem AKD, Nolt C, Peterson RE, Poellinger L, Safe S, Schrenk D, Tillitt D, Tysklind M, Younes M, Waern F, and Zacharewski T. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for human and wildlife. *Environ Health Perspect* 1998; **106**: 775–792.
- Falck F, Ricci A, Wolff MS, Bodbold J, and Deckers P. Pesticides and polychlorinated biphenyl residues in human breast lipids and their relation to breast cancer. *Arch Environ Health* 1992; **47**: 143–146.
- Krieger N, Wolff MS, Hiatt RA, Rivera M, Vogelmann J, and Orentreich N. Breast cancer and serum organochlorines: a prospective study among white, black and Asian women. *J Natl Cancer Inst* 1994; **86**: 589–599.
- Holcomb M, and Safe S. Inhibition of 7,12-dimethylbenzanthracene-induced rat mammary tumor growth by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin. *Cancer Lett* 1994; **82**: 43–47.
- Brown NM, Manziolillo PA, Zhang JX, Wang J, and Lamartiniere CA. Prenatal TCDD and prediosition to mammary cancer in the rat. *Carcinogenesis* 1998; **19**: 1623–1629.
- Nesaretnam K, Hales E, Sohail M, Krausz T, and Darbre P. 3,3',4,4'-Tetrachlorobiphenyl (TBC) can enhance DMBA-induced mammary carcinogenesis in the rat. *Eur J Cancer* 1998; **34**: 389–393.
- Ramamoorthy K, Gupta MS, Sun G, McDougal A, and Safe SH. 3,3',4,4'-Tetrachlorobiphenyl exhibits antiestrogenic and antitumorigenic activity in the rodent uterus and mammary cells and in human breast cancer cells. *Carcinogenesis* 1999; **20**: 115–123.
- DeVito MH, and Maier WE, Diliberto JJ, and Birnbaum LS. Comparative ability of various PCBs, PCDFs and TCDD to induce cytochrome P-450 1A1 and 1A2 activity following 4 weeks of treatment. *Fundam Appl Toxicol* 1992; **20**: 125–130.
- Huggins C, Grand LC, and Brillantes FP. Mammary cancer induced by a single feeding of polynuclear hydrocarbons, and its suppression. *Nature* 1961; **189**: 204–2–7.
- Clement I. Mammary tumorigenesis and chemoprevention studies in carcinogen-treated rats. *J Mammary Gland Biol Neoplasia* 1996; **1**: 37–47.
- Mann PC, Boorman GA, Lolline LO, McMartin DN, and Goodman DG. Proliferative lesion of the mammary gland in rats., IS-2. In: *Gudies for Toxicologic Pathology*. STP/ARP/AFIP, Washington, DC, 1–11, 1996.
- Tanabe S, Tatsukawa R, and Phillips D. Mussels as bioindicators of PCB pollution: a case study on uptake and release of PCB isomers and congeners in Green-lipped Mussels (*Perna viridis*) in Hong Kong Waters. *Environ Pollut* 1987; **47**: 41–62.
- Chomczynski P, and Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extration. *Anal Biochem* 1987; **162**: 156–159.
- Korabiowska M, Brinck U, Kotthaus I, Berger H, and Droese M. Analysis of DNA content in the progeression of recurrent and metastatic melanomas. *Anticancer Res* 2000; **20**: 2791–2794.
- Bacus JW, and Bacus JV. A method of correcting DNA ploidy measurements in issue sections. *Mod Pathol* 1994; **7**: 562–664.
- Bacus JW, Boone CW, Bacus JV, Follen M, Kelloff GJ, Kagan V, and Lippman SM. Image morphometric nuclear grading of intraepithelial neoplastic lesions with applicaitons to cancer chemoprevention trials. *Cancer Epidemiol Biomarkers Prev* 1999; **8**: 1087–1094.
- Bacus JW, Bacus JV, Stoner GD, Moon RC, Kelloff GJ, and Boone CW. Quantitation of preinvasive neoplastic progression in animal models of chemical carcinogenesis. *J Cell Biochem Suppl* 1997; **28/29**: 21–38.
- IARC Working Group on the Evaluation of Carcinogenic Risks to Humans: Polychlorinated Dibenzo-Para-Dioxins and Polychlorinated Dibenzofurans. *IARC Monogr Eval Carcinog Risks Hum* 1997; **69**: 1–631.
- Turteltaub KW, Felton JS, Gledhill BL, Vogel JS, Southon JR, Caffee MW, Finkel RC, Nelson DE, Procter ID, and David JC. Accelerator mass spectrometry in biomedidal dosimetry: Relationship between low-level exposure and covalent binding of heterocyclic amine carcinogens to DNA. *Proc Natal Acad Aci USA* 1990; **87**: 5288–5292.
- Enan E, and Matsumura F. Evidence for a second pathway in the action mechanism of 2,3,7,8-Tetrachlorodibenzo-*p*-

- dioxin (TCDD). *Biochem Pharmacol* 1995; **49**: 249–261.
26. Zacharewski T, and Safe S. Antiestrogenic activity of TCDD and related compounds. In: *Reproductive and Developmental Toxicology*, Korach KS (ed), New York: Marcel Dekker Inc, 431–448, 1998.
27. Tritscher AM, Goldstein JA, Portier CJ, McCoy Z, Clark GC, and Lucier GW. Dose-response relationships for chronic exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin in a rat tumor promotion model: quantification and immunolocalization of CYP1A1 and CYP1A2 in the liver. *Cancer Res* 1992; **52**: 3436–3442.
28. Angus WG, Larsen MC, and Jefcoate CR. Expression of CYP1A1 and CYP1B1 depends on cell-specific factors in human breast cancer cell lines: role of estrogen receptor status. *Carcinogenesis* 1999; **20**: 947–955.
29. Christou M, Moore CJ, Gould MN, and Jefcoate CR. Induction of mammary cytochromes P-450: an essential first step in the metabolism of 7,12-dimethylbenz(a)anthracene by rat mammary epithelial cells. *Carcinogenesis* 1987; **8**: 73–80.
30. Bolongesi C, Parrini M, Aiello C, and Rossi L. DNA damage induced by 7,12-dimethylbenz[a]anthracene in the liver and the mammary gland of rats exposed to polycyclic aromatic hydrocarbon enzyme inducers during perinatal life. *Mutagenesis* 1991; **6**: 113–116.
31. Christou M, Savas U, Schroeder S, Shen X, and Thompson T. Cytochromes CYP1A1 and CYP1B1 in the rat mammary gland: cell-specific expression and regulation by polycyclic aromatic hydrocarbons and hormones. *Mol Cell Endocrinol* 1995; **115**: 41–50.
32. Nesaretnam K, and Darbre P. 3,5,3',5'-Tetrachlorobiphenyl is a weak oestrogen agonist *in vitro* and *in vivo*. *J Steroid Biochem Mol Biol* 1997; **62**: 409–418.
33. Faqi BJ, Dalsenter PR, Merker HJ, and Chahoud I. Preproductive toxicity and tissue concentrations of low doses of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin in male offspring rats exposed throughout pregnancy and lactation. *Toxicol Appl Pharmacol* 1998; **150**: 382–392.