DISSERTATION

MECHANISMS OF HEPATIC TUMOR PROMOTION BY POLYCHLORINATED BIPHENYL MIXTURES

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY CHARLES E. DEAN JR. ENTITLED "MECHANISMS OF HEPATIC TUMOR PROMOTION BY POLYCHLORINATED BIPHENYL MIXTURES" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

MECHANISMS OF HEPATIC TUMOR PROMOTION BY POLYCHLORINATED BIPHENYL MIXTURES

Polychlorinated biphenyls (PCBs) are environmental pollutants that exist as mixtures and are potential humans carcinogens. Despite this, there are relatively few relevant data on interactive biological effects and risk estimation for carcinogenesis remains based on data for individual PCB congeners. Mechanisms by which PCBs exert their promotional carcinogenic effects are unknown.

Our studies evaluated the role of alterations of TGFβ signaling pathways in PCB-induced promotion stage of hepatocarcinogenesis. Rats were administered the planar PCB 126 (3,3',4,4',5-pentachlorobiphenyl) and the non-planar PCB 153 (2,2',4,4',5,5'-hexachlorobiphenyl) alone and in combination in a medium term bioassay that included diethylnitrosamine iniation and partial hepatectomy. For PCB 126 and 153, the doses ranged form 0.1-10 and 10-10,000 μg/kg body weight, respectively. Treatment with PCB 126 or 153 alone resulted in a significant ($P<0.01$) dose dependent increase in preneoplastic foci area and number compared with controls. Treatment with combined PCB 126 and PCB 153 increased preneoplastic focus formation but resulted in a less than additive promotional effect. The antagonistic effect was present at all five PCB 126/PCB

153 dose combinations, including the low doses of PCB 126 and 153 that did not show significant promotional activity alone.

An eight-week time course study without the partial hepatectomy was conducted to evaluated alterations in TGFβ signaling, cell proliferation and apoptosis. Treatment with individual and combined PCBs throughout the study did not significantly alter cell proliferation, apoptosis or TGFβ receptor expression compared to initiated and non-initiated controls nor did the formation of altered hepatic foci occur.

Immunohistochemistry for alterations in TGFβ, TGFβ II receptor, cell proliferation and apoptosis were evaluated in preneoplastic foci in animals treated with the highest PCB 126 and PCB 153 doses alone and in combination. TGFβ-positive preneoplastic foci increased in PCB 126-treated animals and this correlated with a reduced TGFβ II receptor expression. Treatment with PCB 153 and the PCB mixture did not result in significant changes in receptor expression. Our results show expressions of TGFβ and TGFβ II receptor are significantly altered during the promotion stage of PCB 126-induced hepatocarcinogenesis and suggest that these changes might contribute to the development and progression of preneoplastic lesions.

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DEDICATION

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CHAPTER 1

GENERAL INTRODUCTION

Polychlorinated Biphenyls: Structure, Environmental Contamination and Human Exposure

PCBs constitute a group of 209 different congeners and sites of chlorine substitution determine the chemical and biological characteristics of a specific congener. Ballschmiter and Zell arranged the 209 congeners in ascending numeric order and assigned what are commonly termed "Ballschmiter or "BZ" numbers to identify individual congeners. PCBs have 2–10 chlorine atoms are attached to the biphenyl molecule. The general chemical structure of chlorinated biphenyls is shown in Figure 1.1 (top). The benzene rings can rotate around the bond connecting them resulting in planar and the non-planar configurations in which the benzene rings are at a 90-degree angle to each other. The number of substitutions in the *ortho* positions largely determines the degree of planarity. The replacement of hydrogen atoms in the *ortho* positions with larger chlorine atoms forces the benzene rings to rotate out of the planar configuration as demonstrated by PCB 153 in figure 1.1 (middle). The benzene rings of non-*ortho* substituted PCBs, as well as mono-*ortho* substituted PCBs, may assume a planar configuration and are referred to as planar or

co-planar congeners as demonstrated by PCB 126 in figure 1.1 (bottom). Benzene rings of other congeners that cannot assume a planar configuration and are referred to as non-planar.

Some commercial PCB mixtures are known in the United States by their industrial trade name, Aroclor which is followed by a four digit number. In trade names, the last two digits indicate the approximate chlorine content by weight percent. For example, Aroclor 1242 is a chlorinated biphenyl mixture of varying amounts of mono through penta chlorinated congeners with average chlorine content of 42%. The trade names of some commercial PCB mixtures manufactured in other countries are Clophen (Germany), Fenclor (Italy), Kanechlor (Japan), and Phenoclor (France) (Erickson, 2001).

PCBs don't burn easily and are good insulating materials and, as a result, were used widely as coolants and lubricants in transformers, capacitors, and other electrical equipment. The manufacture of PCBs stopped in the United States in August 1977 because there was evidence that PCBs build up in the environment and may cause harmful effects. Consumer products that may contain PCBs include old fluorescent lighting fixtures, old microscope oil, hydraulic oil, and electrical devices or appliances containing PCB capacitors made before PCB use was stopped.

The use of PCBs is now banned in many countries and the environmental levels have been reported to be decreasing in temperate industrialized areas (Liem *et al.*, 2000), although they have increased in other areas (Faroon and Olsen, 2000). Nevertheless, there is great concern for toxic effects of PCBs and other dioxin-like compounds since, despite the decline, human

exposure is still close to or in excess of the recommended tolerably daily intake (Ahlborg *et al.*, 1992; Liem *et al.*, 2000). Their physical properties, which made them applicable to their varied industrial applications, also allowed them to persist in nature, leading to their accumulation in the environment and biological systems (McFarland and Clarke, 1989; Robertson and Hansen, 2001; Safe, 1994). Despite cleanup efforts, the persistence of PCBs can be expected to persist and impact humans and the environment into the foreseeable future.

PCBs in the environment are mixtures of congeners with differing physical and biological characteristics and their impact on biological systems may be due to interactions between themselves and other classes of chemical pollutants thereby making them good candidates for the study of chemical mixtures. Chemical mixture contamination is associated with an estimated 30,000 hazardous waste disposal sites nationally (Upton *et al.*, 1989). Polychlorinated biphenyls (PCBs) have been identified in at least 500 of the 1,598 hazardous waste sites that at have been proposed for inclusion on the EPA National Priorities List (Faroon and Olsen, 2000). Despite this, there are few relevant data on interactive biological effects of PCB congeners and risk estimation is currently based on toxicological data on individual PCBs. (Safe, 1990; Safe, 1994).

Humans have been exposed to PCB mixtures accidentally and occupationally, as well as environmentally through contaminated food, air, and water. Analyses of human breast milk, blood, and adipose tissue have demonstrated that probably all individuals are exposed to PCBs to some degree (Kimbrough, 1995). Food consumption has been and continues to be

the major source of body burden of PCBs in the general population. The estimated dietary intake of PCBs for an average adult was 0.027 μg/kg/day in 1978 and had declined to <0.001 μg/kg/day by 1991 (Gunderson, 1995). There is evidence that diets high in fish from PCB-contaminated waters, such as in the Great Lakes-St. Lawrence River basins, can significantly increase a person's dietary intake of PCBs. Breast-fed infants of mothers who have diets high in contaminated fish may have a particularly increased risk for PCB exposure due to its presence in the milk. In recent years, mean serum PCB levels range from 0.9–1.5 ppb (μg/L) in individuals who do not have diets high in fish from waters contaminated with PCBs (Faroon and Olsen, 2000).

Metabolism of Polychlorinated Biphenyls

The metabolism of PCBs has been extensively reviewed (Faroon and Olsen, 2000; Hu and Bunce, 1999; James, 2001). Differential accumulation and retention of PCBs is related to exposure and the relative biological stability of each congener. Limited excretion of parent PCBs does occur, but biotransformation is necessary for the majority of PCB excretion. The initial step in the biotransformation of PCBs involves cytochrome P-450 enzyme (CYP) (CYP1A1, 1A2, and CYP2B1/2B2) mediated oxidation of arene oxides, which readily undergo further metabolism (Matthews and Dedrick, 1984;Preston *et al.*, 1983). Planar PCBs are dioxin-like inducers of CYP1A and are preferentially oxidized by these isozymes. Many *ortho*-substituted, non-planar, PCBs are inducers of CYP 2B isozymes and are also metabolized by these enzymes (Brown, 1994). Mono-*ortho* PCBs are often mixed inducers of CYP1A and CYP2B isozymes (Safe *et al.*, 1985a). Some congeners induce P-450s from the 3A and 4A families, but the structure-activity relationships are incomplete (Huang and

Gibson, 1992; Schuetz *et al.*, 1986). Arene oxides are mainly transformed to hydroxylated aromatic compounds but also to sulfur-containing metabolites via the mercapturic acid pathway (Haraguchi *et al.*, 1999). Depending on the number and position of the chlorine-substitutions, one or more arene oxide intermediates may be formed from a given PCB. Unsubstituted *meta* and *para* carbon atoms are the preferred site for oxidation (Borlakoglu and Wilkins, 1993). Hydroxylation of planar PCBs usually predominates at the para position in the least chlorinated phenyl ring, and the rate of metabolism generally decreases with increasing chlorine substitution (Hu and Bunce, 1999). Non-planar PCBs are generally hydroxylated at an open *meta* position. Despite physicochemical differences, it appears that higher chlorinated PCBs and congeners that lack unsubstituted *meta*-*para*-vicinal positions are better candidates for bioaccumulation. Less chlorinated PCBs (1–4 chlorines) are readily taken up by organisms, but are also readily eliminated and metabolized. The penta-, hexa-, and hepta-PCBs are both bioavailable and resistant to degradation in organisms and these PCB homologs bioaccumulate in organisms to the greatest extent (McFarland and Clarke, 1989).

Toxicity of Polychlorinated Biphenyls: Humans and Laboratory Animal Studies

Mechanisms by which the broad array of toxic effects observed in humans and animals orally exposed to PCB mixtures develop are incompletely understood, but there is evidence to suggest that PCB congeners differ qualitatively and quantitatively in biological activities and that multiple and diverse mechanisms are involved in responses to PCB mixtures.

There are 209 congeners of PCBs, possessing a wide spectrum of toxic effects.

The biological effects induced by PCBs are dependent on the molecular structure of individual congeners. Planar PCBs and other halogenated hydrocarbons such as 2,3,7,8-tetra-chlorodibenzo-p-dioxin (TCDD) produce their biological effects through a receptor-mediated response, binding to the cytosolic aryl hydrocarbon receptor (Ah-r). This is followed by changes in gene expression (Robertson *et al.*, 2000; Safe, 1994). PCBs that are substituted in both *para* and at least two *meta* positions, but not in any of the *ortho* positions, may form a planar configuration similar to TCDD and bind strongly to the Ah-r. These non-*ortho* substituted PCBs appear to have TCDD-like activity and induce a variety of reproductive, immunologic and hepatic toxic effects (Safe, 1994). Non-*ortho*-chlorinated PCB congeners induce CYP1A1 and CYP1A2. CYP1A forms are the major enzymes responsible for the metabolic activation of pro-mutagens and procarcinogens. PCB congeners that bind the Ah-r and elicit the previous described effects will be referred to in this text as planar or as Ah-r agonist congeners. Introduction of two or more chlorines in an ortho position results in decreased planarity between the two phenyl rings due to stearic interactions. Such PCBs congeners exhibit liver metabolic activity that is "phenobarbital-like." These compounds are not as potent as the Ah-r agonist congeners but, like phenobarbital (PB), can induce hepatic tumor promotion. PCBs of this less planar group induce CYP2B1 and CYP2B2 or both CYP1A1/2 and CYP2B1/2 (Safe *et al.*, 1985b; Safe, 1994). The induction of CYP2B1 by phenobarbital and, putatively, by non-planar phenobarbital-like PCB congeners is mediated by the nuclear receptor constitutive androstane (CAR) (Muangmoonchai *et al.*, 2001; Oliver and Roberts, 2002). These congeners will be referred to as non-planar or CAR agonists in this text.

Adverse health effects of PCBs in occupationally exposed workers have included chloracne and related dermal lesions, diverse hepatic effects, decreased birth weight in the offspring of occupationally exposed mothers, decreased pulmonary function, and eye irritation. Specific effects on liver include increased serum liver enzymes including increases in serum levels of γ -glutamyltranspeptidase [GGT], aspartate aminotransferase [AST], and/or alanine aminotransferase [ALT]), induction of hepatic drug-metabolizing enzymes, and hepatomegaly (Faroon and Olsen, 2000). Nearly all human epidemiologic data on the carcinogenicity of PCBs have been equivocal. While no overall increases in cancer-related mortality could be correlated with occupational PCB exposure, some studies reported non-statistically significant increases of specific cancers, including the grouping of liver, gall bladder, and biliary tract cancers (Bertazzi *et al.*, 2001; Brown, 1987; Gustavsson and Hogstedt, 1997). However, one analysis that combined results from 3 cohorts having different durations and levels of exposure, latencies, and follow-up did find statistically significant increases for liver/biliary tract/gall bladder cancer (Nicholson WJ, 1994). The Environmental Protection Agency (EPA) determined that human studies examining the cancer-causing effects of PCBs are inadequate, but are suggestive of carcinogenicity (IRIS, 2000) and, as a result, has listed them as potential carcinogens. The National Toxicology Program (NTP) similarly concluded that PCBs are reasonably anticipated to be carcinogenic in humans based on sufficient evidence of carcinogenicity in animals.

Numerous toxic responses observed in laboratory animals following acute high dose and long term low dose exposure to PCBs include acute lethality, hepatomegaly, fatty liver and other indicators of hepatotoxicity, porphyria, body weight loss, dermal toxicity, thymic

atrophy and immunosuppressive effects, reproductive and developmental toxicity, neurotoxicity, and carcinogenesis (Faroon and Olsen, 2000; Robertson and Hansen, 2001). Although the carcinogenicity of PCBs in humans has not been established, the carcinogenic effects of certain mixtures of PCBs in laboratory animals have been amply demonstrated (Faroon *et al.*, 2001; Safe, 1989; Silberhorn *et al.*, 1990a). The liver is a common target organ. Originally it was thought that all individual PCB congeners and mixtures were non-genotoxic carcinogens. However, weight of evidence from rodent studies indicates that PCBs can be complete carcinogens, acting as initiators (via oxidative stress mechanism) (Robertson and Hansen, 2001) and promoters (Faroon *et al.*, 2001).

PCBs as Tumor Promoters: Individual congeners and mixture results

The process of carcinogenesis is multi-step in nature and can be divided into three phases, initiation, promotion, and progression. Initiation involves the induction of an irreversible, heritable change in the genome of the cell. In promotion, subsequent cell division "fixes" the genomic alteration and the cell undergoes clonal expansion, resulting in an identifiable preneoplastic or neoplastic lesion. PCBs are presumed to function as tumor promoters by activating one or more signal transduction pathways that increase cell proliferation, or inhibit negative growth control, or induce apoptosis. Some research does suggest that TCDD causes activation of the growth factor signal transduction pathway through an Ah-receptor-mediated process which, in turn, induces increased levels of proto-oncogene products like *c-fos* and *c-jun* and a mitogenic response (Matsumura, 1994). Some of the non-planar PCBs exhibit liver metabolic that is "phenobarbital-like." These compounds are not as potent as the co-planar congeners but, like phenobarbital (PB), they can induce cell

proliferation, which plays a role in the promotion process (Mills *et al.*, 1995). Understanding the control of cell proliferation is therefore vital to understanding the mechanisms of action of promoters.

Numerous studies utilizing liver medium term bioassays have demonstrated the carcinogenicity of PCBs in rodents (Faroon *et al.*, 2001), as well as their potent tumor-promoting capabilities (Glauert *et al.*, 2001; Mayes *et al.*, 1998)Findings that both planar and non-planar PCB congeners can act as promoters of carcinogenesis individually have been reported by numerous investigators (Berberian *et al.*, 1995b; Buchmann *et al.*, 1991; Cogliano, 1998), V. J. 1998; (Haag-Grönlund *et al.*, 1997; Haag-Grönlund *et al.*, 1998) (Dean, Jr. *et al.*, 2002; Tharappel *et al.*, 2002). Additionally, liver medium term bioassay results obtained for individual PCBs are similar to those reported for other tumor promoters, including phenobarbital, TCDD, and other halogenated aromatic compounds (Buchmann *et al.*, 1991; Park *et al.*, 2001; Sargent *et al.*, 1991; Silberhorn *et al.*, 1990b; Whitlock, Jr., 1986; Whysner and Williams, 1996).

Mixtures of planar and non-planar PCB congeners have been reported to influence the occurrence of altered hepatic foci in different ways. A mixture of PCB 77 and PCB 47 was reported by Sargent *et. al* (1991) to give a seemingly synergistic effect in one dose combination. In a study by Bager *et al.* (1995), three different doses of PCB 126 in combination with one dose of PCB 153 also responded in a synergistic manner. A study by Berberian *et. al.* (1995), however, did not observe any synergistic effects on foci following PCB 77 and PCB 153 exposure, but did report that the number and volume of altered hepatic

foci were increased by both congeners individually. This study was recently duplicated (Tharappel *et al.*, 2002a) and reported similar antagonistic results. Findings of antagonistic or less than additive foci formation following treatment with planar and non-planar mixtures have been reported (Berberian *et al.*, 1995b; Dean, Jr. *et al.*, 2002; Haag-Grönlund *et al.*, 1998). While a study by van der Plas *et. al.* (1999) reported that addition of PCB 153 to a mixture of planar polyhalogenated aromatic hydrocarbons, including TCDD and PCB 126, resulted in a higher mean foci volume and volume fraction when compared to the mixture without PCB 153, thus suggesting additivity (van der Plas *et al.*, 1999).

Although it is known that both co-planar and non-planar PCBs can act as promoters of carcinogenesis, particularly in the liver, their mechanism of action is not known. While there has been extensive research on carcinogenicity of dioxins and dioxin-like Ah-r agonist PCBs, the actions of the non-planar PCB congeners are less well studied, as are potential interactions between the two. Although it is accepted that dioxin-like PCBs mediate their toxic effects via the Ah-r little is known about the mechanisms of action of non-planar congeners. The mechanism of possible synergism between planar and non-planar congeners is of interest for human risk assessment purposes, but at present remains undetermined. The varied results obtained from mixtures of planar and non-planar PCBs challenge the hypothesis that the Ah-r route is the only mechanism involved in tumor induction by PCBs and suggest that other mechanisms may play an important role in the interactive tumor promoting effects. A number of mechanisms have been proposed, including the induction of changes in gene expression, the induction of oxidative damage, effects on vitamin A metabolism, and effects on intercellular communication (Glauert *et al.*, 2001).

One mechanism by which PCBs may promote hepatic tumors is by acting directly on TGFβ signal transduction pathways in the liver. Some studies have shown that TCDD treatment can interfere with expression of functional TGFβ receptors (Personal communication, Dr. F. Matsamura, University of California, Davis, 1996). Functional receptors are required for TGFβ-induced growth inhibition (Feng *et al.*, 1995) and some cancer cells that are not inhibited by TGFβ lack functional receptors (MacKay *et al.*, 1995). Tumor cells promoted by PB have reduced TGFβ receptor expression and reduced TGFβ activation from its latent form (Jirtle *et al.*, 1994). One might explain planar/non-planar PCB synergism if PCB 126 induced a nonfunctional TGFβ receptor, in conjunction with putative reduced TGFβ receptor expression and reduced TGFβ activation that would occur with the PB-like activity of PCB 153.

This is relevant to human health since both PCB 126 and 153 are among the most environmentally important congeners (Kimbrough, 1995; McFarland and Clarke, 1989). It has even been suggested that PCB 126 might pose a greater threat to humans than TCDD itself (Faroon and Olsen, 2000; McFarland and Clarke, 1989). Finally, since the non-planar PCBs with "PB-like" metabolic activity comprise a high percentage of the higher chlorinated PCB mixtures found in the environment, the need for further risk assessment on these compounds has been stressed (Faroon and Olsen, 2000; Safe, 1990; Safe, 1994).

Transforming Growth Factor Beta: Normal Function and Alterations in Hepatocarcinogenesis.

Transforming growth factor-beta (TGFβ) is a member of a large and growing family of growth factors with regulatory properties affecting a multitude of cellular processes including cell proliferation, differentiation, motility, adhesion, and apoptosis. Virtually every cell in the body, including epithelial, endothelial, hematopoietic, neuronal, and connective-tissue cells, produces TGFβ and has receptors for it. TGFβ regulates the proliferation and differentiation of cells, embryonic development, wound healing, apoptosis and angiogenesis.

To elicit biological effects, the TGFβ ligand must become activated after its initial secretion. TGFβ is modified intracellularly by the cleavage of the C-terminus. The C-terminal region is designated the latency-associated peptide, while the N-terminus is active TGFβ. The latency-associated peptide noncovalently associates with a 25-kDa active dimer to form the 100-kDa secreted inactive precursor. To become active TGFβ, the latency-associated peptide must be released or undergo a conformational change to expose the receptor binding site (Khalil, 1999; Koli *et al.*, 2001) *In vivo*, TGFβ is released from the complex by the multifunctional matrix glycoprotein thrombospondin-1, which appears to act by changing the conformation of the latent TGFβ binding protein (Sinha *et al.*, 1998). TGFβ may also be activated by plasmin-mediated cleavage of the complex. Thrombospondin-1 is a major activator of TGFβ *in vivo* (Crawford *et al.*, 1998) .

Once TGFβ ligand is activated, it binds to its receptors that are a family of transmembrane serine/threonine kinases to initiate a signal transduction cascade. These kinases are divided into two subfamilies, type I and type II receptors, which are distinguished based on their relative molecular weight. The type I receptor has a molecular weight of 55 kDa while the type II receptor has a molecular weight of 70 kDa (Hata, 2001). Although the type I and II receptors are the most important receptors in TGFβ signaling, there is also a class of accessory receptors. Betaglycan (termed the type III receptor) appears to bind TGFβ and present it to type I and II receptors to enhance signaling (Rich *et al.*, 2001a).

The TGFβ signal transduction cascade is initiated through the binding of the ligand to the type II receptor. Subsequently, the type I receptor is recruited and binds TGFβ within a receptor complex. Complexes of the type I and type II receptors are required for the type II receptor to phosphorylate the type I receptor. The activated type I receptor then acts as a kinase on the pathway specific receptor Smads (R-Smads) proteins (Derynck *et al.*, 1998). The Smads are a family of at least nine genes, each bearing an identifying number (e.g., Smad1, Smad2, and Smad9), and each encoding a transcription factor protein involved in mediating intracellular responses to TGFβ and related polypeptides. The Smads have been given their unified name "Smad" based on their homology to Sma and Mad intracellular signaling proteins. Once activated by phosphorylation, Smad protein complexes migrate to the nucleus, where they recruit other transcription factors and stimulate the expression of genes. Several proteins that bind to the Smad complex in the nucleus have been identified, and these most likely help determine which genes will be activated in specific types of cells

and whether the TGFβ signal is turned into a stimulus or inhibitor of growth. (see Figure 1.2)

All epithelial cells are highly sensitive to TGFβ growth inhibitory properties. Hence, in normal cells, TGFβ acts as a tumor suppressor by inhibiting cell growth or by promoting cellular differentiation or apoptosis. The growth inhibitory effects of TGFβ are frequently mediated through the control of cell cycle machinery that is required for the G1-S transition of the cell cycle. Induction of the cyclin-dependent kinase inhibitors p15, p21, p27, increased phosphorylation of Cdc25a, down-regulation of Cdk4 levels, or inhibition of Cdk2 activating kinase activity have all been observed. (Rossmanith and Schulte-Hermann, 2001). These changes cause maintenance of the retinoblastoma protein Rb in the hypophosphorylated state, which prevents the transcription factor E2F from inducing expression of genes, such as c-myc, required for G1-S phase transition. Therefore, alterations of the targets of TGFβ can prevent TGFβ-mediated growth inhibition. In hepatocytes, TGFβ-mediated growth inhibition and apoptosis both occur (Oberhammer *et al.*, 1991). It is unknown how the decision between G1-arrest and cell death in response to TGFβ is regulated. The apoptosis-inducing capacity has been investigated in many cell types and liver cell apoptosis has been confirmed in many studies (Schuster and Krieglstein, 2002). The final execution of TGFβ triggered apoptosis has been shown to proceed via decreases in mitochondrial transmembrane potential, cytochrome c release, and activation of caspase 3 (Buchmann *et al.*, 1999).

The function of TGFβ in cancer biology is rather complex since it appears that TGFβ acts as a growth inhibitor in the early stages of tumorigenesis but may promote tumor growth at later stages (Blobe *et al.*, 2000). At some time during the stepwise transition towards malignancy, virtually all cells become, at least partially, resistant to TGFβ growth inhibition. The resistance is due to inactivating mutations or loss of expression of the genes for one or more known components of the TGFβ signaling pathway. A wide variety of cancers have mutations in TGFβ receptors. Particularly, the type II receptor is most commonly altered. The type II receptor is frequently mutated in esophageal, gastric, colorectal carcinomas, and hepatocellular cancers (Pasche, 2001; Rich *et al.*, 2001b; Rossmanith and Schulte-Hermann, 2001). Cancer cells can secrete larger amounts of TGFβ than their normal counterparts. This can inhibit proliferation or stimulate apoptosis of normal cells but the tumor cells are resistant because of reduced receptor expression. The association of TGFβ secretion with cancer is strongest in the most advanced stages of tumor progression (Blobe *et al.*, 2000).

Animal studies of the TGFβ signaling pathway in hepatocarcinogenesis have revealed derangements at the level of the IGF-II/M6P-R, the TGFβ receptors, as well as post-receptor signaling (Rossmanith and Schulte-Hermann, 2001). In the TGFβ/c-*myc* transgenic mouse model, in which the expression of both TGFβ/c-*myc* humans genes are targeted to the liver, accelerated hepatic tumor growth occurred. Additionally, TGFβ II receptorexpression was down regulated during liver tumor progression (Factor *et al.*, 1997). The accelerated neoplastic progression and TGFβ over expression in these transgenic mouse models appears associated with an early loss of TGFβ responsiveness during tumor development. The results suggest that overexpression of TGFβ may contribute to liver carcinogenesis and that

loss of TGFβ receptor type II inhibitory growth signals and up-regulation of c-myc are critical steps in liver tumor progression.

In DEN-initiated, phenobarital-induced hepatocarcinogenesis models in rats, decreased levels of the IGF-II/M6P-R and of all three TGFβ receptors were observed in liver tumors when compared to surrounding normal parenchyma (Jirtle *et al.*, 1994; Reisenbichler *et al.*, 1994). Based on these data, it was proposed that phenobarbital may selectively promote initiated cells with reduced levels of TGFβ receptors (Mansbach *et al.*, 1996). A recent rat hepatocarcinogenesis study reported that TGFβ positive preneoplastic hepatocytes increased with time, and this correlated with a reduced TGFβ II receptors expressed in these preneoplastic lesions. Additionally, apoptotic cells increased with time and were more numerous in the adjacent liver parenchyma than preneoplastic lesions (Park *et al.*, 2001). This data suggests that the alteration in the expression of TGFβ and TGFβ II receptors might contribute to the development of preneoplastic lesions.

In human hepatocellular carcinoma (HCC), the IGF-II/M6P-R, TGFβ receptors, as well as Smad2 and Smad4, have been identified as targets in the disruption of TGFβ signaling (De Souza *et al.*, 1997). In HCC, TGFβ is frequently overexpressed in association with a loss of responsiveness to TGFβ (Farber, 1982; Feng *et al.*, 1995; Rossmanith and Schulte-Hermann, 2001a). TGFβ was also increased in the plasma of patients with hepatocellular carcinoma (Ito *et al.*, 1991). Expression of the TGFβ receptors in human HCCs has been analyzed in a number of studies, and either down regulation or mislocalization was observed frequently. Using immunohistochemistry, Bedossa *et al.* (1995) found a diffuse cytoplasmic staining

with perinuclear accumulation for TGFβ II receptor in neoplastic hepatocytes instead of the membrane labeling observed in normal liver, suggesting defective processing of the receptor (Bedossa *et al.*, 1995). In another study, the mRNAs for both TGFβ receptor I and II were shown to be reduced by a factor of two in a small number of HCCs (Sue *et al.*, 1995), and down regulation of TGFβ II receptor was also demonstrated in 50% of yet another larger sample of HCCs (Kiss *et al.*, 1997).

Alternative Methods for Carcinogenicity Testing

Carcinogenicity testing normally includes two-year studies in rats and mice of both sexes, following widely accepted standardized procedures. These studies are usually preceded by tests for genetic toxicity and subchronic toxicity studies to select dose levels for two-year studies. Although these data are used for quantitative risk assessment, the mechanistic basis is usually unknown. Disadvantages of this approach include the high costs of two-year studies, the large numbers of animals required, the long duration of the studies, and the uncertainty of extrapolation of results to humans. Alternative approaches are being developed that would provide more mechanistic information and permit decisions to be made about carcinogenic potential without the necessity to conduct two-year studies in rats and mice.

In vivo medium-term initiation promotion liver bioassays for carcinogens have been recommended as alternatives to two-year carcinogenicity tests (Spielmann, 2003). Several medium-term carcinogenesis assays that capitalize on the multistage nature of hepatocarcinogenesis using foci of altered hepatocytes (FAH) as an endpoint have been

proposed (Bannasch *et al.*, 2003). Medium-term liver bioassays have been suggested to become part of a general testing strategy taking its place between *in vitro* mutagenicity tests and the chronic rodent bioassay (Enzmann *et al.*, 1998). Many of these protocols have shown good correlations with long-term results for individual carcinogens (Hothorn, 2002;Huff *et al.*, 1991). The histogenetic pathways leading from FAH to neoplasms in the liver have been well characterized (Altmann, 1994). One liver medium-term bioassay in particular has been developed as a very useful tool for detection of hepatocarcinogenicity and chemopreventive potential of chemicals. Using an experimental protocol originally proposed by Tsuda *et al.* (1984) the most extensive medium-term carcinogenesis bioassay has been conducted by Ito and colleagues (Ito *et al.*, 1994). This bioassay employs glutathione S-transferase placental form (GST-P) positive foci of altered hepatocytes as an end point. The assay protocol consists of initiation with a single dose of diethylnitrosamine (DEN) followed by the test compound for 6 weeks (from week 2 to week 8) and partial hepatectomy stimulating cell proliferation at week 3 (Figure 1.3). In a summary report (Ito *et al.*, 1992) including more than 170 test compounds (with and without previously determined DNA-reactivity), it was found that 90% of the hepatocarcinogens gave positive results. Additionally, results from the medium-term liver foci bioassay have correctly identified 97% of genotoxic hepatocarcinogens and 86% of the known nongenotoxic hepatocarcinogens, when results are compared with the two-year chronic bioassay (Hasegawa and Ito, 1992). It is appropriate for assessment of low-dose effects of hepatocarcinogens because of its high sensitivity using immunohistochemical detection of glutathione S-transferase placental form positive foci (GST-P+) (Ogiso *et al.*, 1990; Shirai,

1997). GST-P+ foci are putative preneoplastic lesions and have been shown to be the most accurate marker available (Tatematsu *et al.*, 1988).

The limitation of these *in vivo* test systems to the liver is an obvious disadvantage for the identification of cancer risk factors in general, but the liver is the prevailing target organ of chemical carcinogens in rodents, being affected by greater than 30% of all chemicals positively tested in long-term carcinogenesis bioassays in the National Toxicology Program (Huff *et al.*, 1991). Testing strategies based on the medium-term assays allow for evaluation of information related to nongenotoxic mechanisms of carcinogenicity. This includes data on mechanisms such as modulation of target cells by hormones, perturbation by growth factors, changes in cell proliferation, apoptosis, and other mechanisms of carcinogenicity that do not depend on primary damage to DNA. Due to the significant savings in time, animal lives, and resources, medium-term liver bioassays have become a widely used alternative to the two-year bioassay for evaluating carcinogenic potential.

The advantage of using FAH in medium-term carcinogenesis bioassays has been discussed, but quantitative morphometric approaches appear to be indispensable for an appropriate evaluation these bioassays. Adding all focal transactions areas and dividing the result by the total liver section area yields the area fraction occupied by foci. The area fraction is an unbiased estimate of the volume fraction whenever the foci are randomly distributed in the liver. Due to the stereological problems inherent in the medium-term bioassay data, a statistical analysis that concentrates on the evaluation of area fraction will provide clear results whereas the analysis of focal transection density and size distribution can produce

misleading results. In addition, the area fraction is a valid variable even in the presence of confluent foci (Kopp-Schneider, 2003).

Hepatic Preneoplasia in Rodents and Humans

The significance of hepatic preneoplasia in humans and rodents has recently been reviewed (Bannasch *et al.*, 2003; Su and Bannasch, 2003). Morphological and molecular biological approaches *in-situ* have provided evidence for striking similarities in specific changes of the cellular phenotype of preneoplastic FAH emerging in experimental and human hepatocarcinogenesis, irrespective of whether this was elicited by chemicals, hormones, radiation, viruses or, in animal models, by transgenic oncogenes or *Helicobacter hepaticus* (Bannasch *et al.*, 2003). This is significant considering hepatocellular neoplasms are the most common hepatic tumor type in animals and man. Hepatic preneoplasia represents an early stage in neoplastic development, preceding both benign and malignant neoplasia. Earliest types of FAH are composed of well-differentiated hepatocytes that show characteristic metabolic and molecular aberrations and gradually progress through intermediate forms to the poorly differentiated neoplastic phenotypes. Although there is a considerable phenotypic diversity and instability of FAH, results from several sequential studies in different models of hepatocarcinogenesis suggest that the various types of FAH do not occur at random (Bannasch *et al.*, 2003).

Preneoplastic foci of altered hepatocytes have been known as the earliest emerging distinct phenotypic parenchymal changes in chemical hepatocarcinogenesis in rodents, and have

been discussed as potential end-points in carcinogenicity testing (Bannasch, 1986). As previously mentioned, various research groups have used these lesions in mechanistic studies on hepatocarcinogenesis, and some groups have developed medium-term carcinogenesis bioassays based on the predictive value of FAH Bannasch, 1986; Bannasch *et al.*, 2003; Ito *et al.*, 1992; Oesterle *et al.*, 1989; Pitot, 1990). Comparative studies in serial sections revealed that the vast majority of FAH show an abnormal expression of a variety of enzymes, especially enzymes of energy and drug metabolism, including an increased expression of GST-P (Ittrich, 2003). This enzymatic alteration was first described by Sato *et al.* (Sato *et al.*, 1984) and has turned out to be one of the most reliable and easily detectable cytochemical markers for FAH (Ito *et al.*, 1992;Sato, 1989). Although the number of liver cancers induced in rats by chemicals is generally believed to be orders of magnitude lower than that of liver foci in the usual timeframe of these experiments, there is a clear correlation between the two endpoints (Tsuda *et al.*, 2003). Focal hepatic preneoplasia in rodents represents a sensitive marker of carcinogenic response.

Different lesions have been suggested to represent preneoplastic conditions in human liver. They include liver cell dysplasia, including in large-cell change (LCC) and small-cell change (SCC), adenomatoid hyperplasia, and the more recently identified FAH and nodules of altered hepatocytes (Su and Bannasch, 2003). These phenotypically similar preneoplastic lesions were identified in human chronic liver diseases associated with, or pre-disposing to, HCC. This was irrespective of their etiology including chronic viral infections and a few defined chemicals (Bannasch *et al.*, 2003; Su and Bannasch, 2003). In an extended study on 163 explanted or resected livers, various types of FAH were characterized regarding their

morphological phenotype and proliferation kinetics (Su *et al.*, 1997). The results provided evidence for a high incidence of different types of FAH in various kinds of liver diseases. These results strongly support the view that FAH and the resulting hyperproliferative nodular lesions are associated with HCC development in humans.

Research Objectives

Rarely are chemical pollutants encountered in the environment as single chemicals and multiple chemical exposures are more likely to occur, as with polluted domestic water supplies in heavily industrialized regions or near hazardous waste disposal sites. Consequently, the most realistic and accurate assessment of toxicologic effects of environmental contaminants should be not based on the toxicological properties of individual chemicals, but on the toxicological interactions of chemical mixtures.

The long-term objectives of this project are: 1) to evaluate and quantitate the interactive hepatic tumor promoting activity of sub-chronic exposure to two polychlorinated biphenyls (PCBs) which are common hazardous waste contaminants and 2) to explore the role of TGFβ signaling as a possible mechanism for interactive tumor promoting activity.

Non-planar PCBs can synergistically enhance the biochemical and toxic responses elicited by Ah-receptor agonists such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or co-planar PCBs (Bannister and Safe, 1987;Denomme *et al.*, 1986). Nonadditive (synergistic) (Bager *et al.*, 1995;Hemming *et al.*, 1995;Sargent *et al.*, 1991) and antagonistic interactions (Berberian *et al.*, 1995a; Dean, Jr. *et al.*, 2002; Haag-Grönlund *et al.*, 1998; Tharappel *et al.*,

2002) of the co-planar 3,3', 4,4', 5-pentachlorobiphenyl (PCB 126) and the non-planar 2,2', 4,4', 5,5'-hexachlorobiphenyl (PCB 153) as promoters of hepatic preneoplastic lesions in rats have been observed. The co-planar PCB 126 has potent TCDD-like activity, while the non-planar PCB 153 has strong phenobarbital (PB)-like metabolic activity. The mechanisms of interaction of these congeners are unknown.

TGFβ inhibits hepatic cell proliferation, enhances differentiation, and induces apoptosis. TCDD treatment can interfere with TGFβ receptor configuration (Personal communication, Dr. F. Matsamura, University of California, Davis, 1996) and some cancer cells that are not inhibited by TGFβ lack functional receptors (Faroon *et al.*, 2001). Tumor cells promoted by PB have reduced TGFβ receptor expression and reduced TGFβ activation from its latent form (Jirtle *et al.*, 1994). One might explain planar/non-planar PCB synergism if PCB 126 induced a nonfunctional TGFβ receptor, in conjunction with putative reduced TGFβ receptor expression and TGFβ activation with the PB-like PCB 153.

We hypothesize that compared to the promoting activities of each compound individually, enhancement of tumor promoting activity would occur in a mixture of PCBs 126 and 153. It was further hypothesized that the increased tumor promoting activity would correlate with increased cell proliferation and decreased apoptosis within preneoplastic foci. Finally, it was hypothesized that changes in tumor promotion, cell proliferation, and apoptosis would be mediated through decreased TGFβ receptor expression and signaling.

The **Specific Aims** of this project were to:

1. Evaluate and quantify the tumor promoting ability of PBC 126 and PCB 153 individually and the combined interactive effect in an initiation/promotion assay for preneoplastic foci.

2. Evaluate levels of cellular proliferation and apoptosis in individual and combined PCB exposures and correlate results with promoting activity.

3. Evaluate levels of TGFβ after individual and combined PCB exposures and correlate results with promoting, cell proliferation, and apoptotic activity.

4. Assess whether individual or combined PCB exposures alter hepatic TGFβ receptor expression and how this relates to promoting cell proliferation, and apoptotic activity.

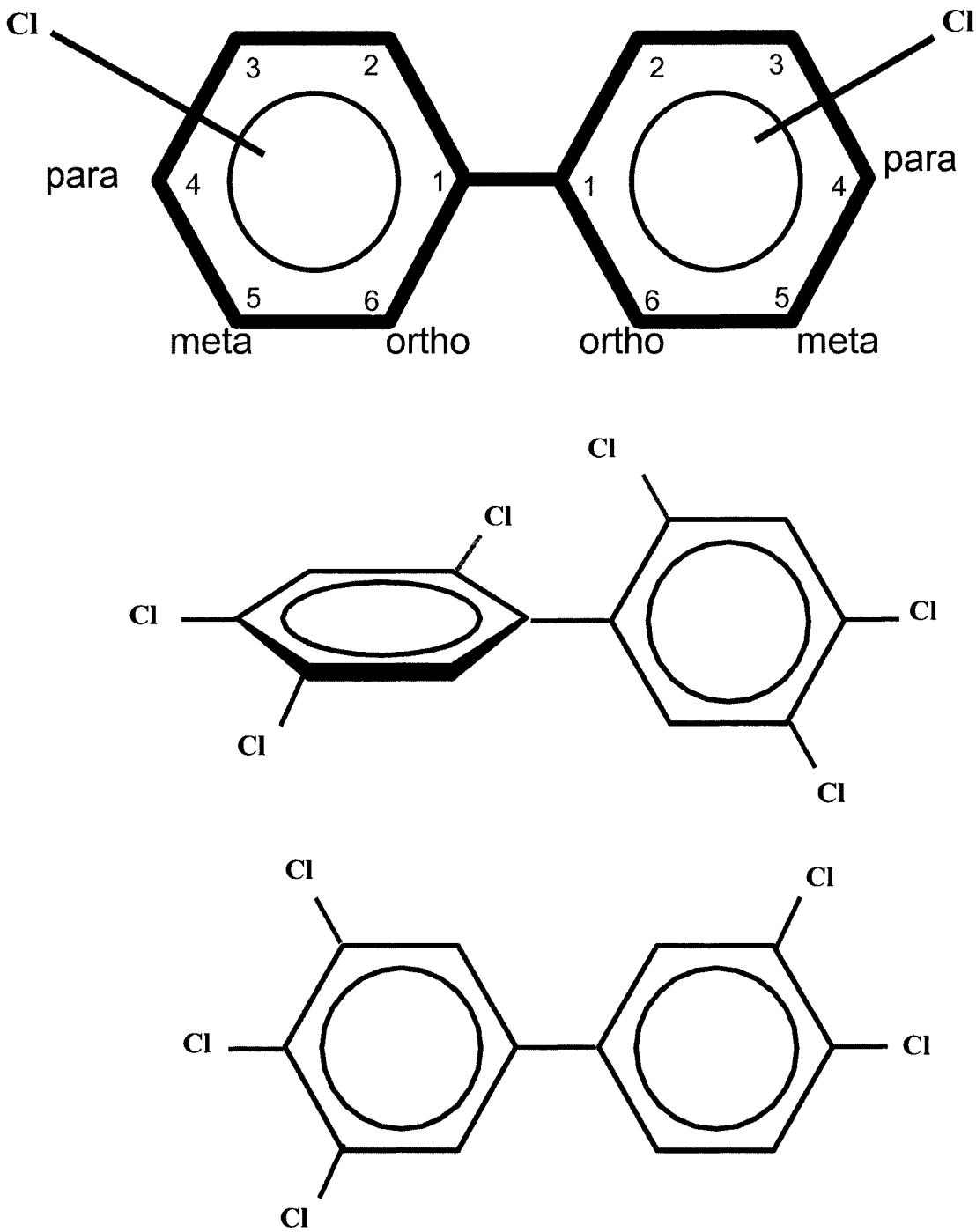

Figure 1.1 Generalized structure of polychlorinated biphenyl (top), a non-planar congener PCB 153 2,2',4,4',5,5'-hexachlorobiphenyl (middle), and a planar congener PCB 126 3,3',4,4',5-pentachlorobiphenyl (bottom).

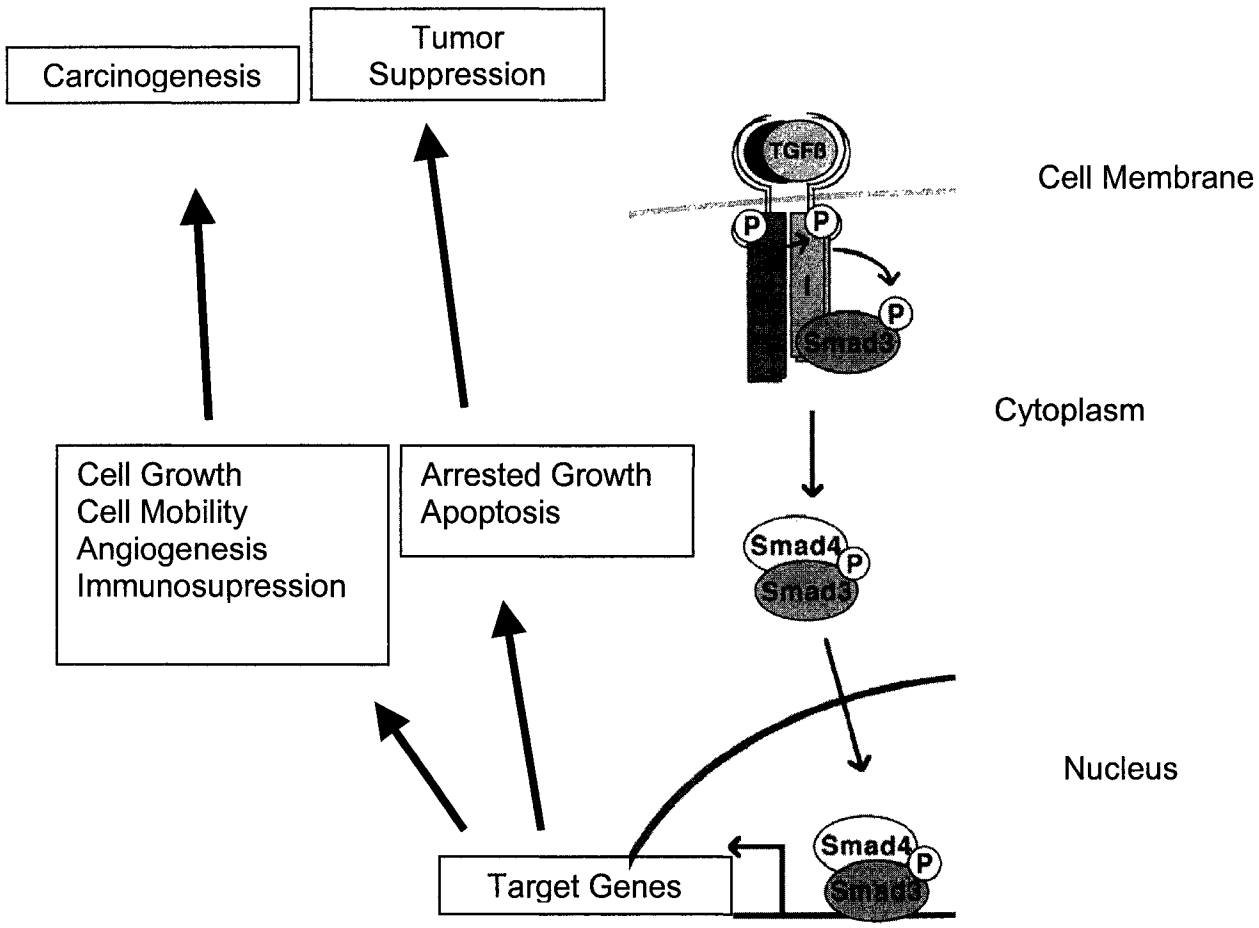

Figure 1.2 The binding of TGFβ to RII leads to binding of the type I receptor to the complex and the phosphorylation of RI. This phosphorylation activates the RI protein kinase, which then phosphorylates the transcription factor Smads. The resulting Smad complex moves from the cytoplasm into the nucleus. In the nucleus, the Smad complex interacts in a cell-specific manner with various other transcription factors to regulate the transcription of TGFβ-responsive genes and mediate the effects of TGFβ at the cellular level.

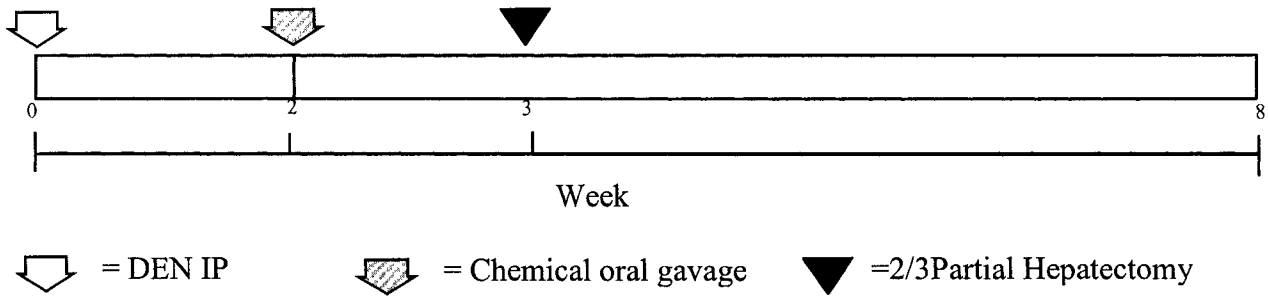

Figure 1.3 Experimental protocol for Ito's Medium Liver Bioassay. Den IP = 200 mg/kg Diethylnitrosamine intraperitoneal injection. Animals are sacrificed at the end of 8 weeks.

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CHAPTER 2

Non-additive Hepatic Tumor Promoting Effects by a Mixture of Two Structurally Different Polychlorinated Biphenyls in Female Rat Livers

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ABSTRACT

This study evaluates and quantifies the interactive hepatic tumor promoting effects of two PCBs, the aryl hydrocarbon receptor agonist PCB 126 (3,3',4,4',5-pentachlorobiphenyl) and the constitutive androstane receptor agonist PCB 153 (2,2',4,4',5,5'-hexachlorobiphenyl). Promotion of altered hepatic foci was evaluated utilizing a medium-term eight-week bioassay for promoters of hepatocarcinogenesis. The assay employs placental glutathione-S-transferase positive (GST-P+) liver cell foci as markers of preneoplasia in female Fischer 344 rats treated with the known initiator diethylnitrosamine followed by partial hepatectomy and by gavage exposure to test chemicals. GST-P+ foci were quantified by histomorphometry and were reported as areas and numbers of GST-P+ foci within area of the liver examined. For PCB 126, the doses were 0.1, 1.0, and 10 μg/kg body weight. For PCB 153, the doses were 10, 100, 1,000, 5,000, and 10,000 μg/kg body weight. Combined PCB 126 and 153 exposures were; 0.1+10, 1+100, 10+1,000, 10+5,000, and 10+10,000 μg/kg, respectively.

Individual PCB treatment resulted in dose dependent increases in liver and adipose concentrations. Hepatic PCB 153 levels were significantly increased ($p<0.01$) after combined exposure. Treatment with PCB 126 or PCB 153 alone resulted in a significant ($P<0.01$) dose dependent increase in GST-P+ foci area and number compared with controls. Treatment with the mixture of PCB 126 and 153 resulted in antagonistic GST-P+ focus formation ($p<0.001$) for both foci area and number. The less than additive effect was present at all five PCB 126/PCB 153 dose combinations, including the low doses of PCB 126 and 153 that did not show significant promotional activity alone.

BACKGROUND

To date, most toxicological and carcinogenesis data have been generated from studies performed on single chemicals. Yet in reality, humans are simultaneously and continuously exposed to a myriad of chemicals in their daily environment. A large number of agents capable of initiating and/or promoting carcinogenesis are recognized, many of which are present as mixtures at hazardous waste sites (Yang and Rauckman 1987). For these reasons, it is important to understand the possible toxicologic interactions that can occur in chemical mixtures.

Polychlorinated biphenyls (PCBs) are halogenated aromatic hydrocarbons that have been used widely in industry and are now recognized as worldwide environmental pollutants (Hansen 1987; Kimbrough 1995; McFarland and Clarke 1989; Safe *et al.,* 1985b; Safe *et al.,* 1987; Safe 1994). The biological effects induced by PCBs are dependent on the molecular structure of individual congeners (McFarland and Clarke 1989; Safe *et al.,* 1985a; Safe. 1992; Safe 1994). Congeners lacking or having one chlorine substitution in the ortho

position with an additional chlorine in the para position of the biphenyl ring have a more co-planar conformation. PCB congeners substituted in both para positions and at least two meta positions are structurally related to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and they elicit comparable biological effects including binding with the aryl hydrocarbon receptor (Ah-r), induction of the monooxygenase enzymes cytochrome P450 1A1 and 1A2 (CYP1A1 and CYP1A2), thymic involution, the induction of a wasting syndrome, increased mortality, and a variety of toxic effects in the liver including carcinogenesis (Safe 1990). PCB congeners which bind the Ah-r and elicit the previous described effects will be referred to as Ah-r agonist congeners in this section. Introduction of two or more chlorines in an ortho position results in decreased co-planarity between the two phenyl rings due to stearic interactions. Such PCBs congeners exhibit liver metabolic activity that is "phenobarbital-like." These compounds are not as potent as the Ah-r agonist congeners but, like phenobarbital (PB), can induce hepatic tumor promotion. PCBs of this less planar group induce CYP2B1 and CYP2B2 or both CYP1A1/2 and CYP2B1/2 (Safe, Safe and Mullin 1985b; Safe 1994). The induction of CYP2B1 by less planar PCBs congeners is putatively mediated by the nuclear receptor constitutive androstane (CAR) (Muangmoonchai *et al.*, , 2001) and such congeners be referred to as CAR agonists in this section.

Human epidemiologic data on the carcinogenicity of PCBs have been equivocal. While no overall increases in cancer-related mortality could be correlated with occupational PCB exposure, some studies reported non-statistically significant increases of specific cancers, including the grouping of liver, gall bladder, and biliary tract cancers (Brown 1987; Kimbrough 1995). Although the carcinogenicity of PCBs in humans has not been

established, the carcinogenic effects of individual PCBs and mixtures of PCBs in laboratory animals have been amply demonstrated (Safe 1989; Silberhorn *et al,*. 1990; Mayes and McConnell, *et al.,* 1998).

PCBs in the environment are mixtures of congeners with differing physical and biological characteristics and their impact on biological systems may be due to interactions between different congeners. Despite this, there are relatively few relevant data on such interactive biological effects and risk estimation remains based on toxicologic data for individual PCB congeners (Safe 1990; Safe 1994). Understanding the interactions between PCB 126 and PCB 153 is important due to the potent hepatotoxicity of PCB 126 (Kimbrough 1995; McFarland, V. A. and Clarke, J. U. 1989) and the ubiquitous nature of PCB 153. (Cogliano 1998; Hansen 1998). This study was designed to evaluate and quantify the interactive hepatic tumor promoting effects of these two environmentally relevant PCBs.

MATERIALS AND METHODS

Chemicals

PCB 126 was purchased from AccuStandard (New Haven, CT), PCB 153 was purchased from Ultra Scientific (North Kingstown, RI), and diethylnitrosamine (DEN) was purchased from Sigma (St. Louis, MO). All other reagents were of analytical grade.

Animals

Thirty-day-old, female F344 rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and allowed to acclimate to altitude (Fort Collins, CO 5000 feet) for four weeks. Following acclimation, rats were randomized by weight and divided into treatment groups. All animals were housed (three per cage) in polycarbonate cages with corncob bedding and stainless steel wire tops and were maintained at 25°C with 55% humidity with lighting maintained on a 12-h light-12-h dark cycle. Control and exposed animals were given food (Harland Tedkad NIH-07 diet; Madison, WI) and deionized water *ad lib*. Rats were observed for clinical state twice daily and food and water consumption and body weight were evaluated three times weekly. This study was conducted in accordance with the National Institutes of Health guidelines for the care of laboratory animals. Animals were housed in facilities fully accredited by the Association for Accreditation of Laboratory Animal Care.

Experimental Design

To evaluate promotional potential we used a medium-term bioassay for promoters of hepatocarcinogenesis (Ito *et al.,* 1989). This assay employs placental glutathione-S-transferase positive (GST-P+) liver cell foci as markers of preneoplasia in the rat after treatment with the known initiator diethylnitrosamine (DEN) followed by partial hepatectomy and exposure to a test chemical or chemical mixture. In contrast to some other known rat hepatic pre-neoplastic markers such as glucose-6-phosphatase, adenosine triphosphatase, γ-glutamyltransferase and glucose-6-phosphatase dehydrogenase, GST-P appears to be more stable after withdrawal of carcinogens from the diet. Ease of

visualization has also established GST-P as one of the best markers for detection of early liver lesions for analysis of hepatocarcinogenesis and in rapid bioassay methods for carcinogens. Although not all the liver foci detected may necessarily develop into tumors, good correlation (93% concordance) is obtained between the induction of GST-P positive foci and the incidence of hepatocellular carcinomas in parallel long-term assays. (Hasegawa and Ito 1994). At the start of the study, rats received either a single intra peritoneal (IP) injection of DEN (200 mg/kg) dissolved in 0.9% saline or an injection of saline only. Fourteen days after initial DEN/Saline treatment, oral gavage administration of PCBs dissolved in 1 ml of corn oil vehicle or corn oil alone was begun. Several dose levels of the two PCBs in corn oil, either alone or in combination were used (Figure 2.1). Gavages were administered 3 times weekly through the remainder of the 8-week study. The low PCB doses were aimed at achieving levels roughly comparable to what has been found in human tissues. The high PCB doses (PCB 126 at 1.0 and 10 μg/kg and PCB 153 at 1000-10000 μg/kg) were based on reported studies with PCB promotion of liver foci (Bager *et al.,* 1995; Hemming *et al.,* 1993; Hemming *et al.,* 1995). Groups 1 and 2 consisted of DEN-initiated single PCB treated animals. Group 3 contained DEN-initiated PCB combination treated animals. Group 4 contained non-DEN-initiated high dose individual and combined PCB treated animals. This group served to assay the potential of high dose PCBs alone and in combination to induce GST-P foci without prior DEN exposure. Groups 5 and 6 contained the DEN control and vehicle control animals respectively. On day 21 after initial DEN/Saline treatment, a 2/3 partial hepatectomy was performed on all animals. Post-operative pain management consisted of Tylenol® and codeine (200 mg/kg and 60 mg/kg, respectively) administered in the drinking water for 72 hours. On day 56 after initial

treatment, rats were sacrificed by exsanguination via the abdominal aorta while under isoflorane anesthesia.

Immunohistochemical staining and liver foci analysis

A total of four liver sections were taken for examination from each animal; one each from the right posterior lobe and caudate lobe and two from the right anterior lobe. Sections were fixed in 10% neutral buffered formalin for immunohistochemical examination for GST-P+ foci. Liver sections were embedded in paraffin and sectioned at 5 μm. For antigen retrieval, liver sections were microwaved on high power until boiling in a 1:10 dilution citrate buffer (Biogenex Labs, San Ramon, CA). After boiling, sections were microwaved at a reduced power for 10 min. A standard avidin biotin (ABC) protocol was followed using an immunoperoxidase kit (Vector Laboratories, Burlingame, CA). GST-P primary antibody (Binding Site, San Diego, CA) incubations were performed at 37°C for one hour. A final incubation with 3-amino-9-ethyl carbazole (AEC; Biomeda, Foster City, CA) was then carried out. Slides were counterstained with hematoxylin for histologic evaluation.

Number and area of GST-P+ foci greater than 0.2 mm diameter per liver sections examined were quantified. GST-P+ foci were measured using a Leitz light microscope coupled with the Bioquant® IV computerized histomorphometry program (B and M Biometrics, Nashville, TN). These measurements consisted of manually outlining the GST-P+ foci on the liver sections and allowing software to compute the enclosed area. Reference areas of liver sections were measured on a separate system using a Dage CCD72 MTI camera (Dage Corporation, Michigan City, IN) connected to a Bioquant® for Windows system (Version

2.6). In this system, images of the liver sections were projected on a computer terminal and automatically outlined and measured by the computer software. Results for the treatment and control groups were expressed as foci area in mm^2 of GST-P+ foci more than 0.2 mm in diameter per area of liver sections examined in cm^2. The use of foci >0.2 mm has been shown to provide the best correlation with the development of hepatocellular carcinoma (Tatematsu *et al.,* 1988).

Analysis of PCBs in the liver and adipose tissue

Samples of liver and fat from animals were analyzed for their PCB content. Tissues were extracted by a modification of published procedures (de Faubert Maunder *et al.,* 1964; Mills. *et al.,* 1963). PCB isomer determinations were by gas chromatography and ^{63}Ni pulsed electron-capture detector operated at 320°C. Analysis was performed with dual column confirmation using a 0.25 mm X 60m DB-5 capillary column and a 0.32mm X 30m DB-608 capillary column. Each set of ten samples included one reagent blank and a control spike of calibration standard. Evaluation of data was based on residues found in the reagent blank, recovery of a matrix spike of all analytes, and recovery of a PCB (BZ#183) spike from all samples.

Statistical Analysis

Numbers and areas of GST-P foci greater than 0.2 mm in diameter were analyzed using two-way analysis of variance (ANOVA) for unequal group size, with PCB 126 dose and PCB 153 dose as the two main effects including their interaction. This analysis was followed by posthoc comparisons of the PCB treatment group means compared to DEN- corn-oil control

via Dunnett's multiple comparison procedure. PCB tissue concentrations and changes in liver and body weights were analyzed by one-way analysis of variance (ANOVA) using Tukey's pairwise comparisons procedure where DEN- initiated, PCB- treated animals were compared to DEN- corn oil controls. All statistical tests were performed using the Minitab statistical software package, release 10.5 (Minitab, State College, PA).

RESULTS

Body and liver weight

No adverse clinical effects were observed in any of the treatment groups during the experimental period. Table 2.1 summarizes final body weight and absolute liver weights at the end of the eight-week study. Body weights were significantly decreased ($p<0.01$) in the PCB 126-treated animals (10 μg/kg) when compared to DEN/Corn Oil-treated controls. Body weights of PCB 153 treated rats were decreased, ($p<0.05$) only in the group dosed at 100 μg/kg. Body weights were decreased in the two highest PCB 126/ PCB 153 mixture-treated groups, (10/5,000 and 10/10,000 μg/kg) when compared to DEN/Corn Oil treated controls. Liver weights were increased ($p<0.01$) in the highest dose PCB 126 group (10 μg/kg), the two highest dose PCB 153 groups (5,000 and 10,000 μg/kg) and the three highest PCB 126/PCB 153 mixture groups (10/1,000, 10/5,000 and 10/10,000 μg/kg). Relative liver weights (liver weight as % of body weight) demonstrated similar changes as absolute liver weights (data not shown).

Liver and Adipose tissue concentrations of PCBs

Tables 2.2-2.5 summarize liver and adipose tissue PCB concentrations at the time of sacrifice. Liver concentrations increased in a dose dependent manner in rats treated with PCB 126 alone (Table 2.2). After combined PCB 126/153 exposure, PCB 126 liver concentrations were not significantly different at any dose compared with PCB 126 exposure alone (Table 2.2). Similarly, liver concentrations also increased in a dose dependent manner in rats treated with PCB 153 alone (Table 2.3). However, after combined PCB 126/153 exposure (Table 2.3 bottom row) PCB 153 levels were significantly increased ($p<0.01$) at the three highest mixture doses compared with PCB 153 exposure alone. Adipose tissue levels increased in a dose dependent manner in rats treated with PCB 126 or PCB 153 alone (Tables 2.4 & 2.5). PCB 126 fat concentration was significantly increased ($P<0.05$) compared with PCB 126 exposure alone only after one mixture exposure (PCB 126 1.0 μg/kg + PCB 153 100 μg/kg) (Table 4). PCB 153 fat concentrations after combined PCB 126/153 exposure were not significantly different at any dose compared with PCB 153 exposure alone (Table 2.5).

Quantitative morphometric evaluation of GST-P positive foci

Treatment of non-DEN initiated rats with PCBs alone or in combination did not result in formation of GST+ foci (Group 4, data not shown). Treatment DEN-initiated rats with PCB 126 or PCB 153 alone resulted in a significant ($p<0.01$) dose dependent increase in GST-P+ foci area and number compared with DEN controls (Figures 2.2 and 2.3) at the highest doses. Treatment with the mixture of PCB 126 and 153 resulted in significantly increased foci area and number ($p<.01$) in the three highest dose groups (Figure 2.4). When the two

PCBs were combined PCB 126 and PCB 153 had an antagonistic effect on GST-P formation. The interaction between the two PCBs after subtraction of the DEN control responses from the PCB-induced responses is shown in (Figure 2.5). This response was less than additive and analysis (two way ANOVA using a general linear model) showed this negative interaction to be significant ($p<.001$). PCB mixture responses were less than the predicted additive values of the individual PCBs for all dose groups.

DISCUSSION

In carcinogen-treated rats, single hepatocytes that express the placental form of glutathione-S-transferase (GST-P) represent putative initiated cells that have not yet been promoted (Dragan *et al.,* 1995; Dragan *et al.,* 1994a; Dragan *et al.,* 1994c; Moore *et al.,* 1987; Tsuchida and Sato 1992). Altered hepatocellular foci which consist of hyperplastic enzyme-altered cells that stain for GST-P are considered to be preneoplastic in nature (Farber and Sarma 1987; Pitot 1990) and are the most likely to progress to form true neoplasms in the rodent (Dragan *et al.,* 1994b; Dragan *et al.,* 1994a; Hasegawa and Ito 1994; Ogiso *et al.,* 1990). Although preneoplastic foci are reversible and most regress, some foci continue to progress to adenomas and carcinomas (Grisham 1997). The formation of foci can be accelerated using protocols which combine treatment with an initiator, followed by cell proliferation stimulated by partial hepatectomy, and other chemicals as agents of promotion (Dragan *et al.,* 1993; Ito 1989; Pitot and Dragan 1993). This study evaluated hepatic tumor promoting activity induced by the Ah-r agonist PCB 126 and CAR agonist PCB 153. Treatment of non-DEN initiated rats with PCBs alone or in combination did not result in formation of GST+ foci (Group 4, data not shown). This suggests that the PCBs alone did

not have initiating potential in our study. After DEN initiation, the two PCB congeners alone enhanced GST-P+ altered hepatic foci formation, while there was an antagonistic effect on PCB 126 GST-P formation when the two PCBs were combined.

Mixtures of Ah-r agonist and CAR agonist PCB congeners have been reported to influence the occurrence of foci in different ways. A mixture of PCB 77 and PCB 47 was reported by Sargent *et al* (1991) to give a seemingly synergistic effect in one dose combination. In a study by Bager *et al.,* (1995), three different doses of PCB 126 in combination with one dose of PCB 153 also responded in a synergistic manner. Berberian *et. al* (1995), however did not observe any synergistic effects on foci following PCB 77 and PCB 153 exposure, but did report the number and volume of altered hepatic foci were increased by both congeners individually. Our findings that both Ah-r agonist and CAR agonist PCB congeners can act as promoters of carcinogenesis individually are consistent with the findings of Berberian *et al* (1995) and other investigators (Buchmann *et al.,* 1991; Cogliano 1998; Haag-Grönlund *et al.,* 1997; Haag-Grönlund *et al.,* 1998; Safe 1994; Silberhorn *et al.,* 1990; Buchmann *et al.,* 1986). Our finding of antagonistic foci formation differed from reported studies of synergism between these two PCB congeners and other mixtures studies with more planar and less planar congeners (Bager *et al.,* 1995; Sargent *et al.,* 1991). This finding was surprising because our studies were based on similar reported dosing schemes (Bager *et al.,* 1995). A more recent publication, however, is in accordance with our findings of antagonism (Haag-Grönlund *et al.,* 1998). Haag-Grönlund *et. al.*, (1998) reported a combination of PCB 126 and PCB 153 resulted in a weak antagonistic effect for volume fraction of altered hepatic foci. Interestingly, van der Plas *et. al.* (1999) report that addition of PCB 153 to a mixture of Ah-r agonist polyhalogenated aromatic hydrocarbons, including

TCDD and PCB 126, resulted in a higher mean foci volume and volume fraction when compared to the mixture without PCB 153, thus suggesting additivity (van der Plas *et al.*, , 1999).

While there has been extensive research on carcinogenicity of dioxins and dioxin-like Ah-r agonist PCBs, the actions of the CAR agonist PCB congeners are less well studied, as are potential interactions between the two. Although it is accepted that dioxin-like PCBs mediate their toxic effects via the Ah-receptor little is known about the mechanisms of action of CAR agonist congeners. The varied results obtained from mixtures of Ah-r agonist and CAR agonist PCBs challenge the hypothesis that the Ah-r route is the only mechanism involved in tumor by PCB and suggest that other mechanisms may play an important role in the interactive tumor promoting effects of PCBs.

It has been stressed that non-additive interactions between Ah-r agonist PCBs or TCDD with CAR agonist PCBs appear to be affected by toxicokinetic interactions (van den Berg *et al.*, 1994) and that knowledge of actual tissue retention is critical both for understanding mechanisms and for providing realistic risk assessments (Giesy and Kannan, 1998). TCDD binding in liver is related to induction of a specific hepatic binding protein, CYP1A2, and by binding the Ah-r (van Birgelen *et al.*, 1996). Selective binding of the Ah-r by PCB 126 also occurs (Chu *et al.*, 1996). High doses of PCB 153 are reported to alter the distribution of TCDD, increasing the concentration in liver and decreasing the concentration in adipose tissues via induction of hepatic CYP1A2, which subsequently binds TCDD (De Jongh *et. al.*, 1995). PCB 153 concentration can be increased in liver by a high dose of TCDD (van

Birgelen *et al.,* 1996). PCB 153 is lipophilic and, since TCDD appears to increase PCB 153 levels in the liver, it has been suggested that this is related to increased fat in liver cells due to toxicity of the TCDD (van Birgelen *et al.,* 1996).

Our data indicate an increase in PCB 153 concentrations in liver when administered in combination with PCB 126 compared with PCB 153 alone. Increased levels of PCB 153 in the liver would be expected to increase promotional effects based on the effect of the chemical alone, rather than to decrease them, as we have seen. Thus, distribution changes alone could not account for the antagonistic promotional effects. While the mechanism of this distribution change is not clear, the rats in the highest dose groups (PCB 126 at 10 μg/kg and PCB 153 at 5,000 and 10,000 μg/kg) did have significant weight loss compared to controls. Loss of adipose tissue could have led to redistribution of the PCB 153 from the fat to the liver. As previously mentioned, one study suggested that TCDD toxicity caused hepatic lipidosis and this increased PCB 153 levels in liver because of its lipophilicity (van Birgelen *et al.,* 1996). This is unlikely in our study, since we saw no histologic evidence of hepatic toxicity or visible fat increase after exposure to individual congeners or mixtures. PCB 126 fat concentration after combined PCB 126/153 exposure increased only at one dose combination (PCB 126 1.0 μg/kg + PCB 153 100 μg/kg) and this was a minimal change. The specific congeners in the mixture, the dose levels of the individual congeners, route of administration, duration of exposure, and species and strain of animal no doubt affect such toxicokinetic interactions. Regardless, our results suggest toxicokinetic interactions alone cannot explain the antagonistic promotional response seen with the PCB mixture

Understanding the mechanism of interaction between PCB 126 and PCB 153 is important because they are among the most environmentally important congeners (Kimbrough 1995; McFarland and Clarke 1989) and are found in significant quantities in human tissues (Cogliano 1998; Hansen 1998). Currently risk estimation is based on toxicologic data on individual PCBs, mainly the dioxin-like congeners (Giesy and Kannan 1998; Safe 1994; van den Berg *et al.,* 1998; Whysner and Williams 1996). The toxic equivalency factor (TEF) concept is used to facilitate the risk assessment of PCBs and related substances, the potency of the most toxic dioxin, TCDD, is used as a standard and set to one, and other dioxin-like chemicals are assigned TEFs that are fractions thereof. The total toxic equivalency (TEQ) of a mixture is the sum of the individual potencies. Several assumptions are made when utilizing the TEF scheme. First, all interactions are presumed to be additive in nature even though non-additive PCB interactions are reported. TEFs are assigned based on a chemical's affinity to bind to the Ah-r, which CAR agonist PCB congers are unable to do. Therefore, the current system does not include the potential impact of CAR agonist PCB congeners even though they are known promoters. It has been stressed that without consideration of the toxicity of the CAR agonist PCBs, current risk assessment for the PCBs is incomplete (Ahlborg *et al.,* 1994; Fischer *et al.,* 1998; Hansen 1998; van den Berg *et al.,* 1998). With respect to this, our lowest dose of PCB 153 (10 μg/kg body weight) resulted in adipose tissue levels of 872 ±107 ng/g, only 2-3 times those reported in human tissues (Hansen 1998; Stellman *et al.,* 1998). Our low dose of PCB 126 (0.1 μg/kg body weight) resulted in tissue levels that were at the lower limit of detection by our analysis system, which is highly sensitive (capable of detecting 8 ng/g and above), again emphasizing the

environmental relevance of these studies. Our data demonstrates antagonistic hepatic tumor promoting activity in a mixture of Ah-r agonist and CAR agonist PCBs, which results in a less than additive GST-P foci formation. The less than additive response was present in all five PCB 126/PCB 153 combinations, including low doses of PCB 153 (10 and 100 μg/kg) that alone did not show any evidence of promotional activity (Figure 2.5).

In summary, results from this study confirm that individual treatment with either the CAR agonist PCB 153 or Ah-r agonist PCB 126 under DEN initiation protocol lead to the formation of GST-P+ altered enzymatic hepatic foci and that both congeners can act as promoters of hepatocarcinogenesis. Additionally, treatment with a mixture of PCB 126 and 153 resulted in less than additive promotional interactions. These results suggest that risk estimation for mixtures containing multiple PCBs cannot be based on a model of simple additivity as has been proposed.

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Table 2.1

Body and liver weights in diethylnitrosamine initiated rats treated with polychlorinated biphenyls 126 and 153 alone and in combination.

Dose (μg/kg bw)		Body Weight (g) ± SD ✦	Liver Weight (g) ± SD ✦
DEN Corn Oil	0	165.66±10.63	5.65 ± 0.47
PCB 126	0.1	158.14±9.31	5.36 ± 0.38
	1.0	160.74±10.48	5.94 ±0.5
	10	155.07±17.71 *	7.56 ±1 *
PCB 153	10	157.25±9.68	5.21 ±0.56
	100	156.92±12.46 †	5.63 ±1.32
	1000	158.9±8.62	5.58 ±0.35
	5000	160.56±7.93	7.02 ±0.40 *
	10000	167.04±7.72	7.57 ±0.86 *
PCB Mixture 126/153	0.1/10	174.58±7.76	6.27±1.97
	1/100	171.29±8.49	6.01 ±0.34
	10/1000	163.89±8.51	7.78 ±0.57 *
	10/5000	153.38±8.39 *	10.14 ±0.81 *
	10/10000	150.04±10.07 *	10.57±1043 *

* Significant difference ($p<0.01$) from initiated control group
† Significant difference ($p<0.05$) from initiated control group
✦ Weights at time of eight week sacrifice.
Data expressed as mean ± standard deviation

Table 2.2

Liver concentrations of polychlorinated biphenyl 126 after treatment with polychlorinated biphenyl alone and in combination with polychlorinated biphenyl 153 in diethylnitrosamine-treated rats.

		PCB 126 (ng/g liver)		
Dose (μg/kg)		0.1	1.0	10
PCB 153	0	7.2 ± 8.3†	110.9 ± 34.1	909 ± 141.3
	10	15.2 ± 2.0		
	100		101.6 ± 14.5	
	1000			1030 ± 131.5
	5000			764.8 ± 76.7
	10000			970 ± 403

†PCB 126 liver concentrations after combined PCB 126/153 exposure were not significantly different ($P>0.05$) at any dose compared with PCB 126 exposure alone.

Individual boxes represent individual doses of PCB 126 (first row) or combinations of PCB 126 and PCB 153. Blank boxes represent PCB combined doses that were not included in this study.

Table 2.3

Liver concentrations of polychlorinated biphenyl 153 after treatment with polychlorinated biphenyl alone and in combination with polychlorinated biphenyl 126 in diethylnitrosamine-treated rats.

		PCB 153 (ng/g liver)				
Dose (μg/kg)		10	100	1000	5000	10000
PCB 126	0	38.7 ± 7.0	250.6 ± 87.1	3843 ± 451	14483 ± 3243	29850 ± 6148
	0.1	43.3 ± 7.6				
	1.0		326 ± 82.5			
	10			5913 ± 972*	28200 ± 6148*	75900 ± 19107*

*PCB 153 liver concentrations after combined PCB 126/153 exposure significantly increased ($P<0.01$) compared with PCB 153 exposure alone.

Individual boxes represent doses of PCB 153 alone (first row) or combinations of PCB 126 and PCB 153. Blank boxes represent PCB combined doses that were not included in this study.

Table 2.4

Adipose tissue concentrations of polychlorinated biphenyl 126 after treatment with polychlorinated biphenyl 126 alone and in combination with polychlorinated biphenyl 153 in diethylnitrosamine-treated rats.

	PCB 126 (ng/g fat)		
Dose (μg/kg)	0.1	1.0	10
PCB 153 0	2.2 ± 4.4	13.7 ± 0.6	91.4 ± 26.6
PCB 153 10	4.0 ± 3.6		
PCB 153 100		15.5 ± 1.5*	
PCB 153 1000			53.1 ± 11.3
PCB 153 5000			64.1 ± 12.8
PCB 153 10000			64.4 ± 4.1

*PCB 126 fat concentration after combined PCB 126/153 exposure significantly increased ($P<0.05$) compared with PCB 126 exposure alone.

Individual boxes represent doses of PCB 126 alone (first row) or combinations of PCB 126 and PCB 153. Blank boxes represent PCB combined doses that were not included in this study.

Table 2.5

Adipose tissue concentrations of polychlorinated biphenyl 153 after treatment with polychlorinated biphenyl 153 alone and in combination with polychlorinated biphenyl 126 in diethylnitrosamine-treated rats.

		PCB 153 (ng/g fat)				
Dose (μg/kg)		10	100	1000	5000	10000
PCB 126	0	872 ± 107†	7245 ± 1524	72050 ± 11468	325167 ± 98146	651667 ± 241137
	0.1	716 ± 128				
	1.0		6358 ± 416			
	10			54860 ± 10992	407500 ± 180495	648333 ± 181112

† PCB 153 fat concentrations after combined PCB 126/153 exposure were not significantly different ($P>0.05$) at any dose compared with PCB 153 exposure alone.

Individual boxes represent doses of PCB 153 alone (first row) or combinations of PCB 126 and PCB 153. Blank boxes represent PCB combined doses that were not included in this study.

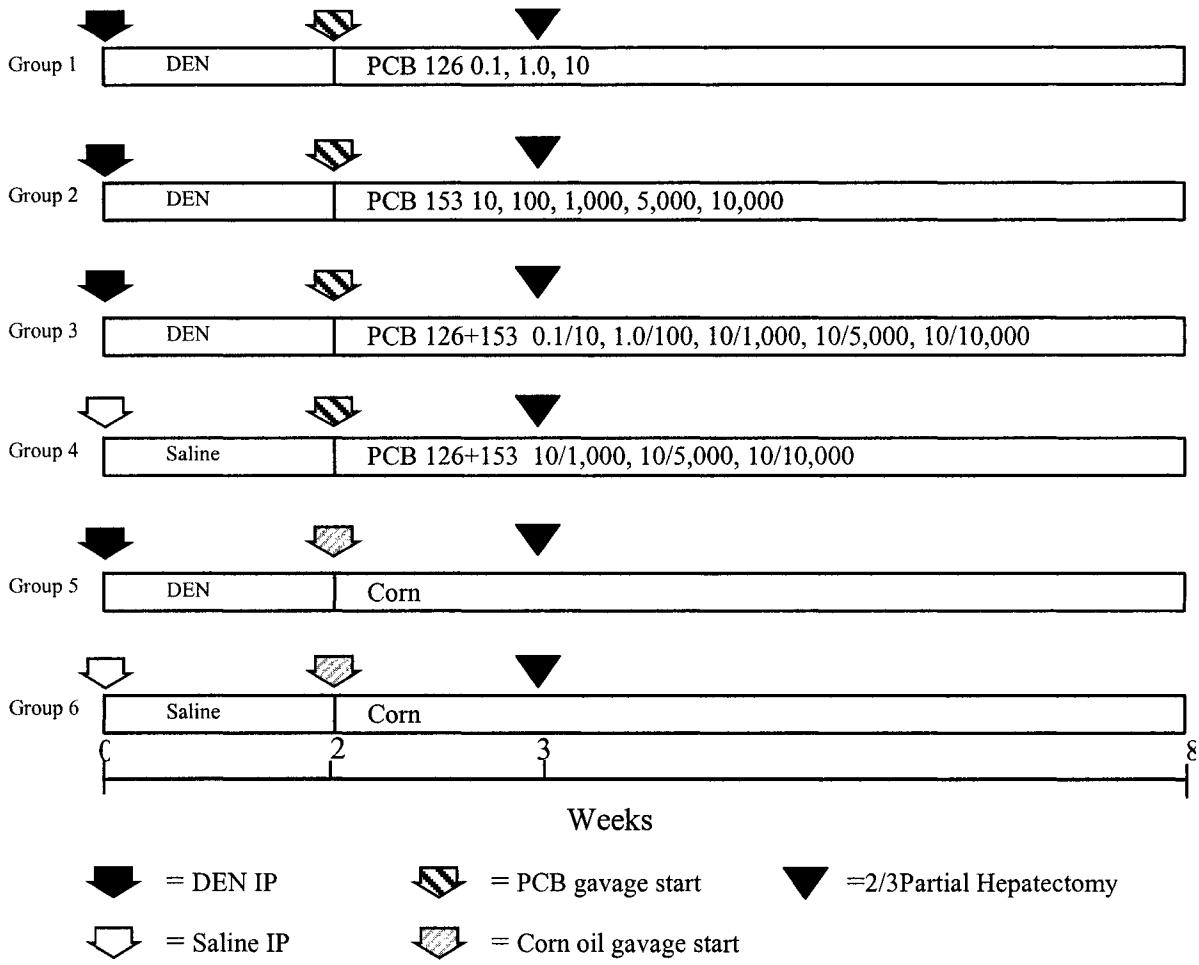

Figure 2.1. Experimental design for the initiation/promotion study with PCBs. Diethylnitrosamine (DEN) the initiation agent, was given via intraperitoneal injection (200 mg/kg) at day 0. PCB treatment three times a week via oral gavage began at week 2 and continued until completion of the study at week 8.

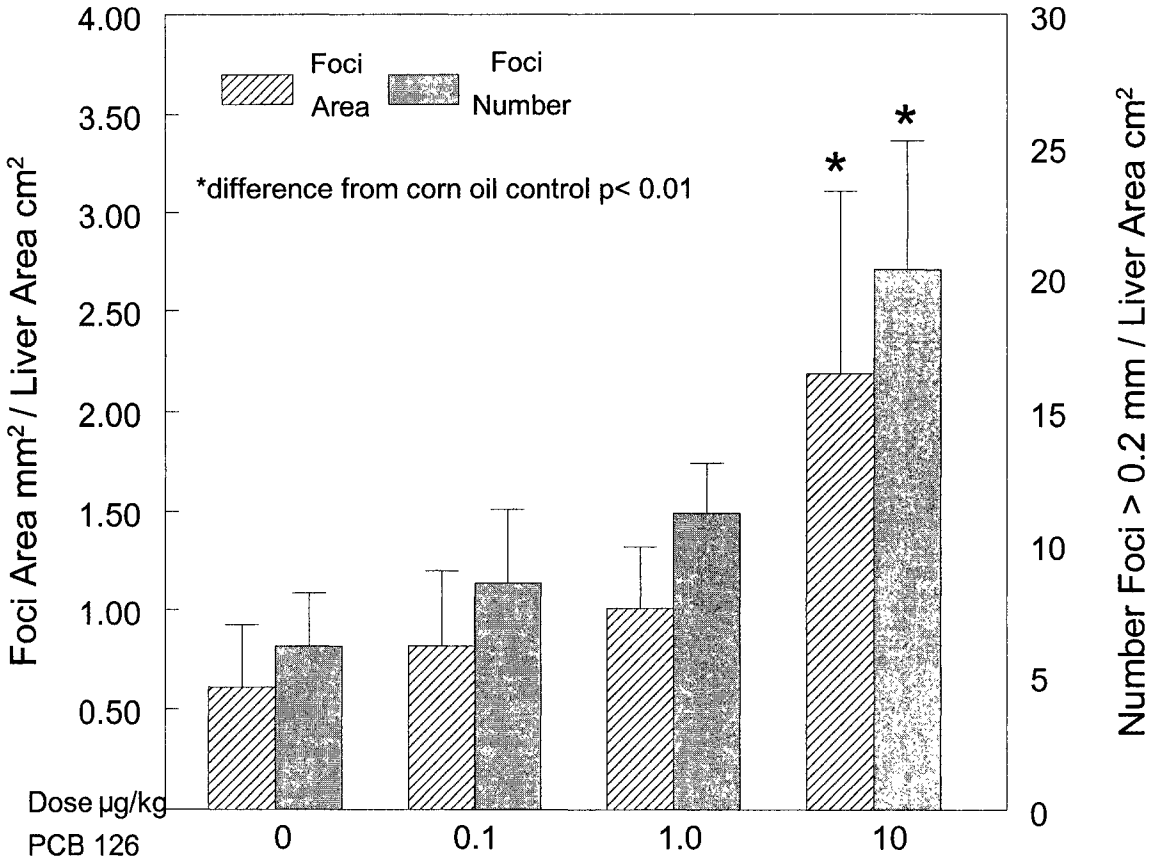

Figure 2.2. Area and number of GST-P positive foci (>0.2 mm diameter) in female F344 rats subjected to an initiation/promotion protocol using diethylnitrosamine (DEN) as an initiator and PCB 126 as a promoter agent.

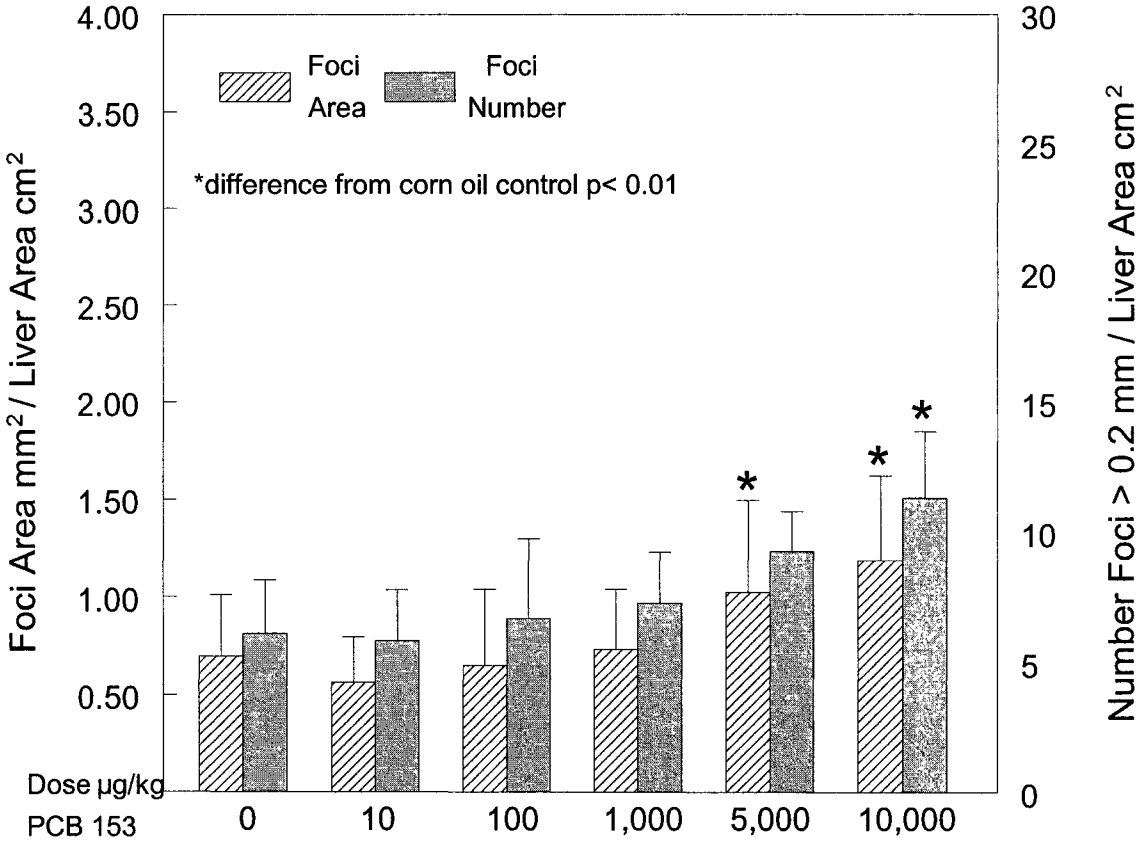

Figure 2.3. Area and number of GST-P positive foci (>0.2 mm diameter) in female F344 rats subjected to an initiation/promotion protocol using diethylnitrosamine (DEN) as an initiator and PCB 153 as a promoter agent.

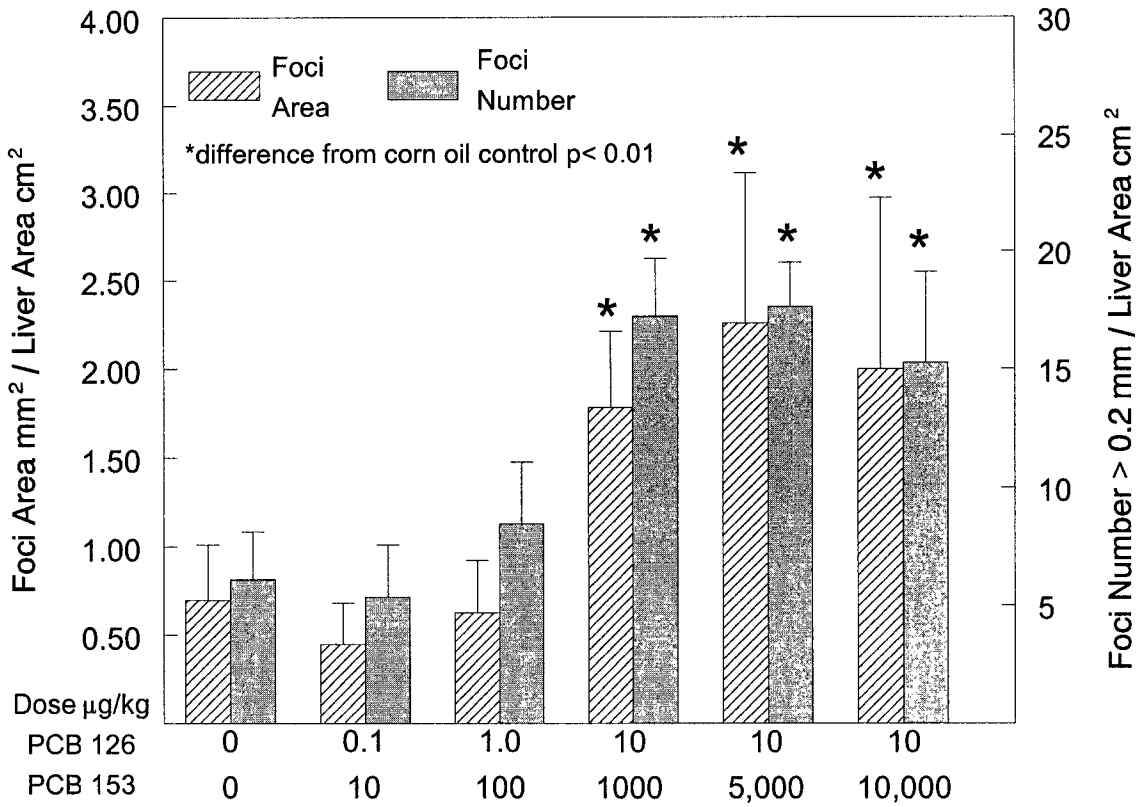

Figure 2.4. Area and number of GST-P positive foci (>0.2 mm diameter) in female F344 rats subjected to an initiation/promotion protocol using diethylnitrosamine (DEN) as an initiator and a mixture of PCB 126 and PCB 153 as a promoter.

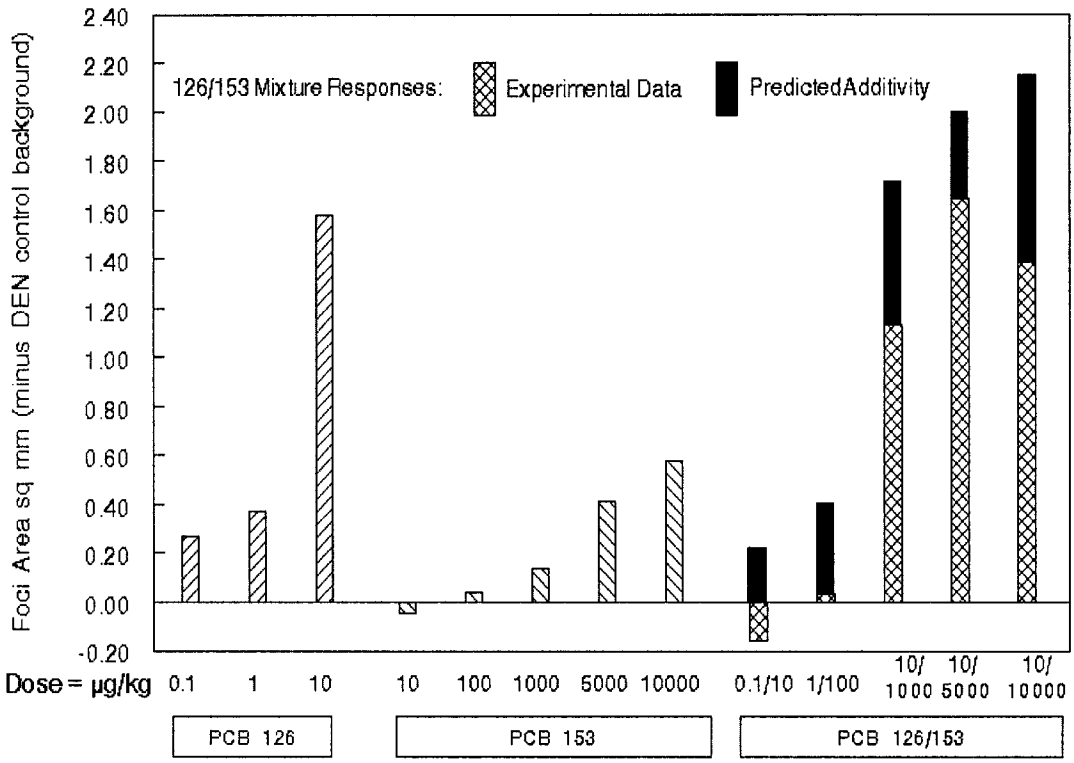

Figure 2.5. Total effect in each treatment group (area of GST-P positive foci with background DEN response subtracted) after treatment with PCB 126, 153, and PCB 126 and 153 combined.

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CHAPTER 3

Cell Proliferation, Apoptosis and Transforming Growth Factor Beta Receptor Expression in Rats Following Forty-Two Day Oral Exposure to PCB 126 (3,3',4,4',5-pentachlorobiphenyl) and PCB 153 (2,2',4,4',5,5'-hexachlorobiphenyl).

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ABSTRACT

Polychlorinated biphenyls (PCBs) are environmental pollutants and tumor promoters in the rodent liver. While the mechanisms of their promoting activities are not clear, one possibility is alterations in TGFβ signaling resulting in abnormalities of cell proliferation and apoptosis. In the present study, we evaluated the ability of two PCB congeners to modulate hepatic TGFβ II receptor expression as well as hepatocyte cell proliferation and apoptosis. Animals in this study received a single dose of diethylnitrosamine (200 mg/kg) followed by three times a week oral gavages of two PCBs, the planar PCB 126 (3,3',4,4',5-pentachlorobiphenyl) and the non-planar PCB 153 (2,2',4,4',5,5'-hexachlorobiphenyl). For PCB 126, the doses were 0.1, 1.0, and 10 μg/kg body weight. For PCB 153, the doses were 10, 100, 1,000, 5,000, and 10,000 μg/kg body weight. Combined PCB 126 and 153 exposures were; 0.1+10, 1+100, 10+1,000, 10+5,000, and 10+10,000 μg/kg, respectively. Treatment with individual and combined PCBs throughout the study did not significantly

alter cell proliferation compared to initiated and non-initiated controls. Individual and combined exposure of high doses of PCBs did not significantly alter apoptotic rates compared to controls. Individual and combined PCB treatment did not result in increases or decreases in TGFβ II receptor or TGFβ I receptor levels when compared to controls. This study shows that short-term oral administration of PCBs alone or in combination at these doses does not alter cell proliferation, apoptosis or induce changes in TGFβ II receptor levels.

BACKGROUND

Polychlorinated biphenyls (PCBs) are halogenated aromatic hydrocarbons that have been used widely in industry and are now recognized as worldwide environmental pollutants (Robertson and Hansen, 2001). The biological effects induced by PCBs are dependent on the molecular structure of individual congeners (McFarland and Clarke, 1989; Safe, 1994; Robertson and Hansen, 2001). Congeners lacking or having one chlorine substitution in the ortho position and an additional chlorine in the para position of the biphenyl ring have a more co-planar conformation. PCB congeners substituted in both para positions and at least two meta positions are structurally related to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and they elicit comparable biological effects including binding with the aryl hydrocarbon receptor (Ah-r), induction of the monooxygenase enzymes cytochrome P450 1A1 and 1A2 (CYP1A1 and CYP1A2), thymic involution, the induction of a wasting syndrome, increased mortality, and a variety of toxic effects in the liver including carcinogenesis (Robertson and Hansen, 2001). Introduction of two or more chlorines in an ortho position results in decreased co-planarity between the two phenyl rings due to stearic interactions. Such PCBs

congeners exhibit liver metabolic activity that is "phenobarbital-like." These compounds are not as potent as the planar congeners but, like phenobarbital (PB), can induce hepatic tumor promotion. PCBs of this less planar group induce CYP2B1 and CYP2B2 or both CYP1A1/2 and CYP2B1/2 (Safe, S. H. 1994). The induction of CYP2B1 by less planar PCBs congeners is putatively mediated by the nuclear receptor constitutive androstane (CAR) (Muangmoonchai *et al.*, 2001).

Human epidemiologic data on the carcinogenicity of PCBs have been equivocal. While no overall increases in cancer-related mortality could be correlated with occupational PCB exposure, some studies reported non-statistically significant increases of specific cancers, including the grouping of liver, gall bladder, and biliary tract cancers (Brown, 1987). Although the carcinogenicity of PCBs in humans has not been established, numerous studies have demonstrated the carcinogenicity of PCBs in rodents (Faroon *et al.*, 2001), as well as their potent tumor-promoting capabilities (Mayes *et al.*, 1998; Glauert *et al.*, 2001). Findings that both planar and non-planar PCB congeners can act as promoters of carcinogenesis individually have been reported by numerous investigators (Buchmann *et al.*, 1991a; Haag-Grönlund *et al.*, 1997; Dean, Jr. *et al.*, 1998; Cogliano, 1998; Haag-Grönlund *et al.*, 1998; Tharappel *et al.*, 2002). Additionally, liver medium term bioassay results obtained for individual PCBs are similar to those reported for other tumor promoters, including phenobarbital, TCDD, and other halogenated aromatic compounds (Whitlock, Jr., 1986; Silberhorn *et al.*, 1990; Sargent *et al.*, 1991; Buchmann *et al.*, 1991b; Whysner and Williams, 1996; Park *et al.*, 2001).

Although it is known that both co-planar and non-planar PCBs can act as promoters of carcinogenesis, particularly in the liver, their mechanism of action is not known. One mechanism by which PCBs may promote hepatic tumors is by acting directly on TGFβ signal transduction pathways in the liver. TGFβ inhibits hepatic cell proliferation, enhances differentiation, and induces apoptosis. Functional receptors are required for TGFβ-induced growth inhibition (Feng *et al.*, 1995). A wide variety of cancers including hepatocellular carcinomas in rodents and humans have demonstrated alterations in the TGFβ receptor signaling. Particularly, the type II receptor is most commonly altered (Rossmanith and Schulte-Hermann, 2001). Our laboratory has previously reported hepatic promotional activity following administration of PCB 126 and PCB 153 individually and combined at the doses used in this study (Dean, Jr. *et al.*, 2002). The current study differs in that no partial hepatectomy was performed. A necrogenic dose of DEN was administered which did provide a mitogenic stimulus but not at the level of a partial hepatectomy. Liver regeneration in rats after partial hepatectomy is extensive with the whole process lasting up to 3 weeks. After surgical removal of liver lobes, the remaining cells proliferate to make up for the mass of the removed tissue. Termination of the proliferative response is mediated through TGFβ signaling pathways with increased TGFβ and TGFβ receptors expression (Fausto, 2000). This time course study designed to determine the effect of PCBs on cell proliferation, apoptosis and TGFβ signaling pathways. The partial hepatectomy was not performed to eliminate its confounding effects on those parameters. This study was designed to evaluate the ability of two PCB congeners to modulate TGFβ II receptor expression as well as hepatocyte cell proliferation and apoptosis in rat liver.

MATERIALS AND METHODS

Chemicals

PCB 126 was purchased from AccuStandard (New Haven, CT), PCB 153 was purchased from Ultra Scientific (North Kingstown, RI), and diethylnitrosamine (DEN) was purchased from Sigma (St. Louis, MO). All other reagents were of analytical grade.

Animals and Treatment

Thirty-day-old, female F344 rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and allowed to acclimate to altitude (Fort Collins CO, 5000 feet) for four weeks. Following acclimation, rats were randomized by weight and divided into treatment groups. All animals were housed (three per cage) in polycarbonate cages with corncob bedding and stainless steel wire tops and were maintained at 25°C with 55% humidity with lighting maintained on a 12-hr light-12-hr dark cycle. Control and exposed animals were given food (Harland Tedkad NIH-07 diet; Madison, WI) and deionized water *ad lib*. Rats were observed for clinical state twice daily, and food and water consumption and body weight were evaluated three times weekly. This study was conducted in accordance with the National Institutes of Health guidelines for the care of laboratory animals. Animals were housed in facilities fully accredited by the Association for Accreditation of Laboratory Animal Care.

At the start of the study, rats received either a single intra peritoneal (IP) injection of DEN (200 mg/kg) dissolved in 0.9% saline or an injection of saline only. Fourteen days after

initial DEN/Saline treatment, oral gavage administration of PCBs dissolved in 1 ml of corn oil vehicle or corn oil alone was begun. Oral PCB/corn oil gavages were administered 3 times weekly through the remainder of the 8-week study. Several dose levels of the two PCBs in corn oil, either alone or in combination were used. The experimental design is summarized in Figure 3.1 and shows the treatment and euthanasia schedule of experimental animals. The low PCB doses were aimed at achieving levels roughly comparable to what has been found in human tissues. The high PCB doses (PCB 126 at 1.0 and 10 μg/kg and PCB 153 at 1000-10000 μg/kg) were based on reported studies with PCB promotion of liver foci (Bager, Y. *et al.* 1995; Hemming, H. *et al.* 1993; Hemming, H. *et al.* 1995). Groups 1 and 2 consisted of DEN-initiated single PCB treated animals. Group 3 contained DEN-initiated PCB combination treated animals. Group 4 contained non-DEN-initiated high dose individual and combined PCB treated animals. Groups 5 and 6 contained the DEN control and vehicle control animals respectively. To avoid confusion, all subsequent mention of time points (in days) in the text refers to the time after the beginning of PCB treatment.

Three days prior to euthanasia, osmotic minipumps (Alzet® model 2ML1, 10 ml/h; Alza Corporation, Palo Alto, CA) were filled with 5-bromo-2'-deoxyuridine (BrdU; 20 mg/ml) and surgically implanted subcutaneously over the dorsal midscapular region. Animals were anesthetized using Isofluorane (Anaquest, Madison, WI) and incisions closed using stainless-steel wound clips.

Animals were euthanized at 3, 5, 7, 14, 18 and 42 days following administration of PCBs by aortic exsanguinations following isoflourane anesthesia. Whole livers were removed and

tissue sections were taken from the liver lobes. A 1-cm section of the jejunum was also dissected to serve as a positive control for the cell proliferation and apoptotic studies. For immunohistochemistry, tissue sections were fixed in 10% neutral-buffered formalin, embedded in paraffin, serially sectioned at 5 μm, and mounted on positively charged microscope slides. For Western-blots, livers were removed, snap frozen with liquid nitrogen and stored at –80°C until proteins were analyzed.

Immunohistochemical Staining

Liver sections were deparaffinized in xylene and rehydrated by passage through an alcohol series. Endogenous peroxidase was quenched in 3% H_2O_2 for 5 min. The slides were rinsed with deionized (DI) water and placed in phosphate buffered solution (PBS) (pH 7.4; 2.7 mM KCl, 0.14 M NaCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4). For antigen retrieval for BrdU, liver sections were microwaved on high power to boiling in a citrate buffer (Biogenex Labs, San Ramon, CA). After boiling, sections were microwaved at a reduced power for 10 min. Standard avidin/biotin (ABC) protocols were followed using immunoperoxidase kits specific for the primary BrdU antibody (Biogenex Labs). Incubation for BrdU was done at 37°C for 1 hour. DNA apoptotic fragmentation was evaluated by optimizing the recommended method of a commercial apoptosis detection kit (ApopTag ™ *In Situ* Apoptosis Detection Kit: Peroxidase; Serologicals, Atlanta, GA). Liver sections were digested in 25 μg/ml Proteinase K (Roche Diagnostics Corp., Indianapolis, IN) for 25 min at room temperature, washed in PBS for 2 min, and followed by a 2 minute wash in DI water. Endogenous peroxidase was quenched using 3% H_2O_2 followed by washes in PBS and DI water. Approximately 100 μl of Equilibration Buffer containing digoxigenin-conjugated

nucleotides (supplied by kit) was applied to each section for about 5 minutes. Sections were incubated with 54µl of working-strength ApopTag® terminal transferase enzyme in a humidified chamber at room temperature for 140 min. Specimens were then placed in a slide dish containing pre-warmed working-strength Stop-Wash Buffer (supplied by kit) and incubated in a 37°C waterbath for 30 min, agitating the solution every 10 min. Slides were washed in PBS for 5 min and then in DI water for 3 min. For all slides a final incubation with 3-amino-9-ethyl carbazole (AEC; Biomeda, Foster City, CA) stained cells positive for BrdU and DNA fragmentation. Slides were then counterstained with hematoxylin for histologic evaluation.

Determination of Cell Proliferation and Apoptosis

Cell proliferation was evaluated by using a modified 5-bromo-2'-deoxyuridine (BrdU) osmotic pump delivery method. In brief, BrdU (at 20 mg/ml) was continuously administered via subcutaneous osmotic mini-pumps that were surgically implanted three days prior to sacrifice. BrdU-positive cells were evaluated by light microscopy for labeled hepatocyte nuclei using a random field technique in conjunction with CHRIS (Cytology/Histology Recognition Information System, Sverdrup Technology). An estimated 5-6 random fields of each liver section was used to determine the labeling index (LI) by examining at least 1000 nuclei for BrdU incorporation. The percentage of labeled nuclei out of the total nuclei counted (1000) was used as the LI. Apoptotic cells were evaluated by using 8-10 random fields of each liver section totaling at least 1,000 cells. Quantification of the apoptotic index only included densely stained cells that have morphologic characteristics of apoptosis (*e.g.*, pyknosis, condensed cell volume, apoptotic

bodies). The percentage of labeled nuclei out of the total nuclei counted (1000) was used as the LI.

Western Blotting

For Western-blot analysis of TGFβ receptors, samples were prepared by homogenizing approximately 0.5 g of frozen liver tissue in 1.5 ml of 10 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.1 % SDS, 0.5% sodium deoxycholate, 1% NP-40, and 100 mg/ml phenylmethylsulfonyl fluoride. Homogenized samples were incubated on ice for 30 min, followed by centrifugation at 15,000 X *g* for 20 min. The resulting supernatant was collected, and total protein concentration determined in the sample. Following preparation, 20 μg protein aliquots of the liver homogenates were denatured in boiling water bath for 10 minutes and separated on a 8% SDS/polyacrylamide gel at a constant 180 V for 50 minutes. The separated proteins were then transferred to a nitrocellulose membrane and blocked overnight at 4°C with TBST solution (10 mM Tris-Cl, pH 7.5, 100 mM NaCl, 0.1 % Tween 20) containing 5% nonfat dried milk (NFDM). Filters were then incubated with rabbit polyclonal antibodies against TGFβ I receptors (sc-398, Santa-Cruz, CA) or TGFβ II receptors (sc-400, Santa-Cruz, CA) in TBST solution with 2% NFDM for 1 h at room temperature. Following the addition of primary antibody, the filter was washed 3 times with TBST. Filters were then incubated with anti-rabbit IgG horseradish-peroxidase conjugate (Amersham, Arlington Heights, IL) for 1 hour at room temperature. Following incubation with the secondary antibody, filters were washed 3 times in TBST. The blots were then developed using enhanced chemiluminescence (ECL) detection kit (Amersham, Arlington Heights, IL).

Total Protein

Total protein in samples was determined using the Bicinchoninic acid (BCA) Protein Assay Reagent (Pierce, Rockford, IL) with bovine serum albumin as a standard.

Statistical Analysis

Comparisons between the treatment groups and its respective controls were made using a Student's t-test. All statistical tests were performed using the Minitab statistical software package, release 10.5 (Minitab, Inc., State College, PA). The results are expressed as means ± standard deviations (SD). Significance was defined as $p < 0.05$.

RESULTS

Body and liver weight

No adverse clinical effects were observed in any of the treatment groups during the experimental period. Body and liver weights were measured at necropsy. Grossly, livers from animals dosed with PCB 126 at 10 μg/kg body weight or PCB 153 at 5,000 or 10,000 μg/kg individually or combined had enlarged livers with an enhanced reticular pattern. No histologic lesions were noted upon examination of H&E-stained liver sections. Specifically, no altered hepatic foci of any histologic type were noted throughout the duration the study. Table 3.1 summarizes final body weight and liver weights at the end of forty-two days of treatment. None of the PCBs had any significant effect on final body weight when compared to DEN/Corn Oil-treated controls. Liver weights were increased ($p<0.01$) in the highest dose PCB 126 group (10 μg/kg), the two highest dose PCB 153 groups (5,000 and 10,000 μg/kg) and the two highest PCB 126/PCB 153 mixture groups (10/5,000 and

10/10,000 μg/kg). Relative liver weights (liver weight as % of body weight) demonstrated similar changes as absolute liver weights (data not shown).

Cell proliferation

Changes in hepatocellular proliferation following administration of PCBs are represented in figures 3.2-3.5. Non-initiated (saline) high dose administration of individual and combined PCBs (PCB 126 at 10 μg/kg, PCB 153 at 10,000 μg/kg and 126/ PCB 153 10/10,000 μg/kg) did not result in statistically significant increases when compared to saline/corn oil controls (figure 3.2). There was a general increase in cell proliferation in all saline treatment groups that peaked at day 5 and then gradually decreased over the remainder of the study period. Hepatic cell proliferation rates from DEN-initiated rats treated with increasing doses of PCB 126 (0.1, 1.0, and 10 μg/kg) and PCB 153 (10, 100, 1,000, 5,000, and 10,000 μg/kg) are represented in figures 3.3 and figure 3.4 respectively. Cell proliferation rates of the highest single and combined dose groups PCBs (PCB 126 at 10 μg/kg, PCB 153 at 10,000 μg/kg and 126/ PCB 153 10/10,000 μg/kg) are represented in figure 3.5. In DEN-initiated rats, there is a general trend of highest cell proliferation at day 3 that gradually decreases over the remainder of the 42-day treatment period. There are no statistical differences in cell proliferation between any of the individual or combined PCB-treated DEN-initiated groups at any dose when compared to DEN/Corn oil controls. Although not statistically significant, the PCB mixture treatment group maintained the highest cell proliferation rate. After saline treatment this was true from day 14 on (Figure 3.2) and after DEN treatment it was true from day 7 through the end of the study period (Figure 3.5).

Apoptosis

To investigate the apoptotic inducing ability of PCBs in the early stages of carcinogenesis, *in situ* labeling of nuclear DNA fragmentation (TUNEL method) was performed on liver samples following forty-two days of high dose administration of PCBs (see figure 3.6). PCB 126 was administered at 10 μg/kg body weight (DEN/126). For PCB 153, the dose was 10,000 μg/kg body weight (DEN/153). The combined PCB 126 and 153 exposure was 10+10,000 μg/kg body weight. Hepatocellular apoptotic rates of DEN initiated rats exposed combined PCBs for forty-two days did not result in statistically significant changes when compared to saline/corn oil and DEN/corn oil controls.

TGFβ Receptor Expression

Western blot analysis for TGFβ II receptor did not show any significant induction or reduction of protein following high dose administration of individual or combined PCBs at any time point when compared to DEN controls (See figure 3.7). Additionally, no increases or decreases in TGFβ I receptor were noted during the study (data not shown).

DISCUSSION

The induction of cancer by chemicals in experimental animals is documented to be a multi-step process in many tissues and can be divided into three phases, initiation, promotion, and progression. The process of initiation involves a genotoxic event that is irreversible when followed by a round of DNA synthesis that "fixes" the mutation and results in the formation of an initiated preneoplastic cell. The promotion stage involves the selective clonal expansion of the initiated cell through an increase in cell proliferation, often accompanied

by a decrease in apoptosis. The events during promotion are dose dependent and reversible upon removal of the tumor promotion stimulus. The third stage, progression, involves genetic events that result in changes from the preneoplastic to the neoplastic state or from the benign to malignant states. This stage is irreversible and involves chromosome instability with aberrations and changes in ploidy.

PCBs, like other nongenotoxic agents, are believed function through non-DNA reactive mechanisms impacting on the promotion stage of carcinogenesis. While the exact mechanisms of action of PCBs on the process of neoplastic cell formation have not been established, changes in gene expression and cell growth controls have been implicated in their mode of action. Therefore, understanding the control of cell proliferation and apoptosis is vital to understanding the mechanisms of action of PCBs. Numerous studies have demonstrated the hepatocarcinogenicity of PCBs in rodents (Faroon *et al.*, 2001), as well as their potent hepatic tumor-promoting capabilities (Mayes *et al.*, 1998) (Glauert *et al.*, 2001). Findings that both planar and non-planar PCB congeners can act as promoters of hepatocarcinogenesis have been reported by numerous investigators (Buchmann *et al.*, 1991a; Haag-Grönlund *et al.*, 1997; Dean, Jr. *et al.*, 1998; Cogliano, 1998; Haag-Grönlund *et al.*, 1998; Tharappel *et al.*, 2002). Results from PCB liver promotional studies utilizing medium-term bioassays are similar to those reported for other tumor promoters, including phenobarbital, TCDD, and other halogenated aromatic compounds (Whitlock, Jr., 1986; Silberhorn *et al.*, 1990; Sargent *et al.*, 1991; Buchmann *et al.*, 1991b; Whysner and Williams, 1996; Park *et al.*, 2001). In tumor promotion studies, young animals are treated with an initiator, *i.e.*, a compound that will alter DNA structure and increase the probability of mutations being fixed during cell replication. The promotional effect of increased cell

proliferation enhances the likelihood of fixing a mutation as well as the probability of expanding a clone of initiated cells. Cell division can be initiated by necrotizing doses of DEN, by partial hepatectomy, or by using young, rapidly growing neonatal animals. Animals are then treated with the promotor and the livers examined for the number and size of preneoplastic foci of altered hepatocytes (FAHs). Preneoplastic FAHs have been known as the earliest emerging distinct phenotypic parenchymal changes in chemical hepatocarcinogenesis in rodents (Bannasch, 1986). Focal preneoplastic lesions appear prior to the occurrence of neoplasms. Generally, there are far more of these preneoplastic lesions than the subsequently developing neoplasms, but there is a clear correlation between the number of preneoplastic lesions and hepatocellular carcinomas (Tsuda *et al.*, 2003). Thus, in the promotional stage of chemical carcinogenesis the induction of DNA synthesis and mitosis by nongenotoxic mechanisms allow for the selective clonal growth of previously initiated cells into recognizable preneoplastic foci.

A variety of growth factors can stimulate or inhibit traversal through the cell cycle thereby controlling cell proliferation. There are "checkpoints" at different points of the cycle (G_1/S and G_2/M) where the cell ensures that all necessary prerequisites for the next stage are met, including ensuring that genetic material is intact (Zhang *et al.*, 1995). Interactions of cyclin dependent kinases (cdk) and cyclins regulate the steps of this process. One growth factor, TGFβ, normally inhibits epithelial cell proliferation, promotes differentiation, which also reduces the capacity of cells to divide, and induces apoptosis. The growth inhibitory effects of TGFβ are frequently mediated through the control of cell cycle machinery, including induction of the cyclin-dependent kinase inhibitors that prevent the release of transcription factors required for G1-S phase transition. In hepatocytes, TGFβ-mediated growth inhibition

and apoptosis both occur (Oberhammer *et al.*, 1991). It is unknown how the decision between G1-arrest and cell death in response to TGFβ is regulated.

Human and animal hepatocarcinogenic studies have revealed derangements of the TGFβ signaling pathway at the level of the TGFβ activation, TGFβ receptor expression and function, as well as post-receptor signaling (Rossmanith and Schulte-Hermann, 2001). Cells that lack response to the growth inhibitory actions of TGFβ gain a growth advantage and can be selected for clonal expansion. TGFβ is also reported to induce hepatocyte apoptosis (Bursch *et al.*, 1992), so lack of TGFβ response can lead to longer survival of cells, another factor that can be involved in clonal expansion and neoplastic growth (Mills *et al.*, 1995).

No statistically significant increase in cell proliferation was observed in any of the PCB treatment groups when compared to the appropriate saline-treated or DEN-initiated controls. Cell proliferation was highest in all DEN-initiated groups at day three then gradually decreased over the remainder of the study period. All saline treated groups showed a non-significant rise in cell proliferation from day three to day five. Because there were no differences between PCB-treated and control animals, these results are most likely due to compensatory cell proliferation following the necrogenic dose of DEN in the initiated animals and could be attributed to growth-related changes in the saline treated animals. A temporal 1-2 week increase in cell proliferation, followed by a return to normal mitotic activity in the liver even though the compound is still being administered has been demonstrated with a number of known epigenetic tumor promoting agents following short term administration (Klaunig *et al.*, 2000). Despite this, the carcinogenicity of many hepatic

tumor promoting compounds is dependent on the continued chronic administration for tumorigenesis to proceed (Schulte-Hermann *et al.*, 1998).

Apoptotic rates determined by the TUNEL method did not detect any increases or decreases in rats treated 42 days with individual and combined doses of PCB 126 at 10 μg/kg and PCB 153 10,000 μg/kg body weight (Figure 3.6) TGFβ has known apoptotic inducing ability in hepatocytes. If alterations in this pathway were present a decrease in apoptosis would be expected to occur. To determine if alterations in expression of TGFβ II receptor protein is affected by PCB treatment, Western blot anaysis of liver homogenates was performed. Protein levels of TGFβ II receptor did not show any significant induction or reduction following high dose administration of individual or combined PCBs at days 7, 14, 28 and 42 compared to DEN controls (See figure 3.7). Additionally, no increases or decreases were noted at day 3 or day 5 (data not shown). The combined results of no significant increase or decrease in cell proliferation, apoptosis suggest that they are either not occuring or not detectable in this model by these methodologies.

The development of resistance to the growth inhibitory effects of TGFβ due to the lack of TGFβ receptor expression have been reported in a variety of human cancers, but it is believed to occur relatively late in the process of carcinogenesis (Hahm *et al.*, 2002). Our laboratory has previously reported hepatic promotional activity following administration of PCB 126 and PCB 153 individually and combined at the doses used in this study (Dean, Jr. *et al.*, 2002). The current study differs in that no partial hepatectomy was performed. A necrogenic dose of DEN was administered which did provide a mitogenic stimulus but not

at the level of a partial hepatectomy. Liver regeneration in rats after partial hepatectomy is extensive with the whole process lasting up to 3 weeks. After surgical removal of liver lobes, the remaining cells proliferate to make up for the mass of the removed tissue. Termination of the proliferative response is mediated through TGFβ signaling pathways with increased TGFβ and TGFβ receptor expression (Fausto, 2000). This time course study was designed to determine the effect of PCBs on cell proliferation, apoptosis and TGFβ signaling pathways. The partial hepatectomy was not performed to eliminate its confounding effects on those parameters. Although there are initiation/promotion protocols that do not require a partial hepatectomy, such protocols utilize neonatal or juvenile animals that have age-related increases in hepatocellular proliferation or adult animals that are administered the promoting agent for prolonged periods (26-40 weeks) (Bannasch *et al.*, 2003). Histologic examination of livers from this study did not reveal formation of altered hepatic foci of any histologic type, suggesting that promotional clonal expansion of initiated hepatocytes did not occur following forty-two days administration of PCBs without partial hepatectomy.

It is possible that changes in TGFβ receptor protein expression are small and locally confined to preneoplastic foci and, therefore, not detectable by gross biochemical methods evaluating the liver as a whole. As previously mentioned, the promotional stage of carcinogenesis is a foci-forming event with selective clonal expansion of previously initiated cells. In studies of regression of liver hyperplasia, animals are administered mitogens until hyperplastic nodules form. The mitogen then is withdrawn and hyperplastic nodules are examined for regression and mechanisms of cellular removal. Studies have shown when

biochemical methods evaluating whole liver are used such as Northern and Western blots, neither TGFβ mRNA or protein, nor TGFβ receptors are found to exhibit significant changes during growth or regression although they are known to be involved (Grasl-Kraupp *et al.*, 1998). Immunoblot methodologies utilize large samples of liver homogenates irrespective of individual nodule involvement and undoubtedly contain large quantities of normal tissue. Therefore, it is not surprising that a foci dependent event may not be detected by these procedures. However, changes in expression of TGFβ receptors were detected in apoptotic hepatocytes by immunohistochemistry (Bursch *et al.*, 1993) and in hepatocytes of liver hyperplasia regression studies by *in situ* hybridization (Oberhammer *et al.*, 1996). These results emphasize the importance of using methods evaluating changes at the local tissue level, *i.e.* within and outside of FAHs. Therefore, increased duration of chemical exposure in the absence of another promotional event like partial hepatectomy, which was the case in this study, it would be difficult to detect alterations in TGFβ signaling pathways because of the lack of FAHs. Thus, while this experiment did not support dysfunction in TGFβ pathways it also does not establish that they are not involved in PCB 126 or 153 carcinogenesis.

ACKNOWLEDGMENTS

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ES05949 and National Institute of Environmental Health Sciences Mentored Clinical Scientist Development Award, K08ES00314.

Table 3.1

Body and liver weights in diethylnitrosamine initiated rats treated with polychlorinated biphenyls 126 and 153 alone and in combination for 42 days.

Dose (μg/kg bw)		Body Weight (g) ± SD ✦	Liver Weight (g) ± SD ✦
DEN Corn Oil	0	178.2±10.5	5.51± 341
PCB 126	0.1	165.6±10.4	5.48± .399
	1.0	166.97±6.05	5.94±.241
	10	167.17±1.23	7.51±.262 *
PCB 153	1000	171.97±7.81	5.51±.313
	5000	174.87±9.75	7.60±.182 *
	10000	169.10±5.63	7.89±.257 *
PCB Mixture 126/153	0.1/10	179.47±7.92	6.14±.213
	1/100	157.3±11.3	5.98±.672
	10/1000	152.4±10.3	7.68±.918
	10/5000	153.27±3.43	9.09±.092 *
	10/10000	152.33±3.51	9.66±.665 †

* Significant difference ($p<0.01$) from initiated control group
† Significant difference ($p<0.001$) from initiated control group
✦ Weights at time of eight week sacrifice.
Data expressed as mean ± standard deviation

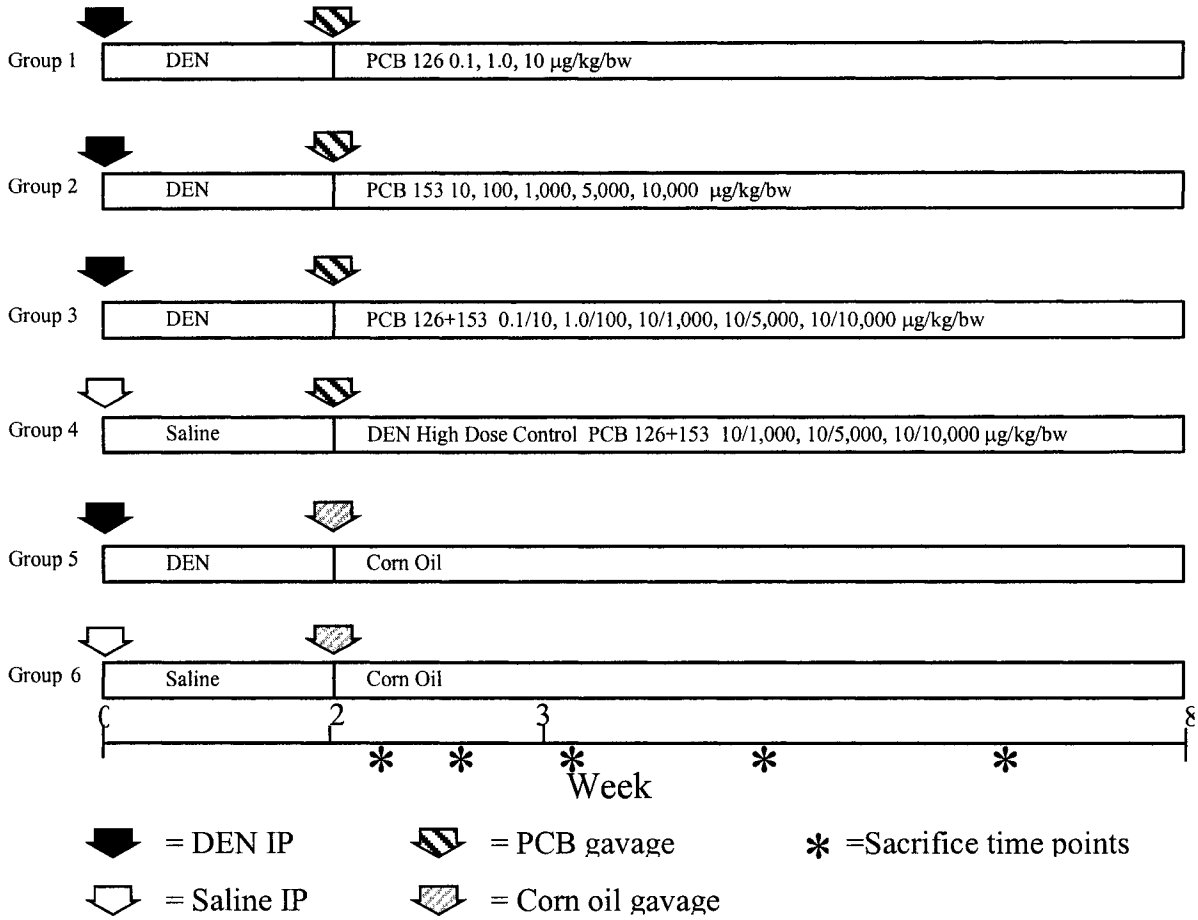

Figure 3.1 Experimental design for the PCB time course exposure. The assay time period was a total of 8 weeks in length. Injection of the initiator (diethylnitrosamine, 200 mg/kg ip) was performed at Week 0 and PCB treatment began at Week 2. *Animals were sacrificed at 3, 5, 7, 14, 28 and 42 days after administration of PCBs.

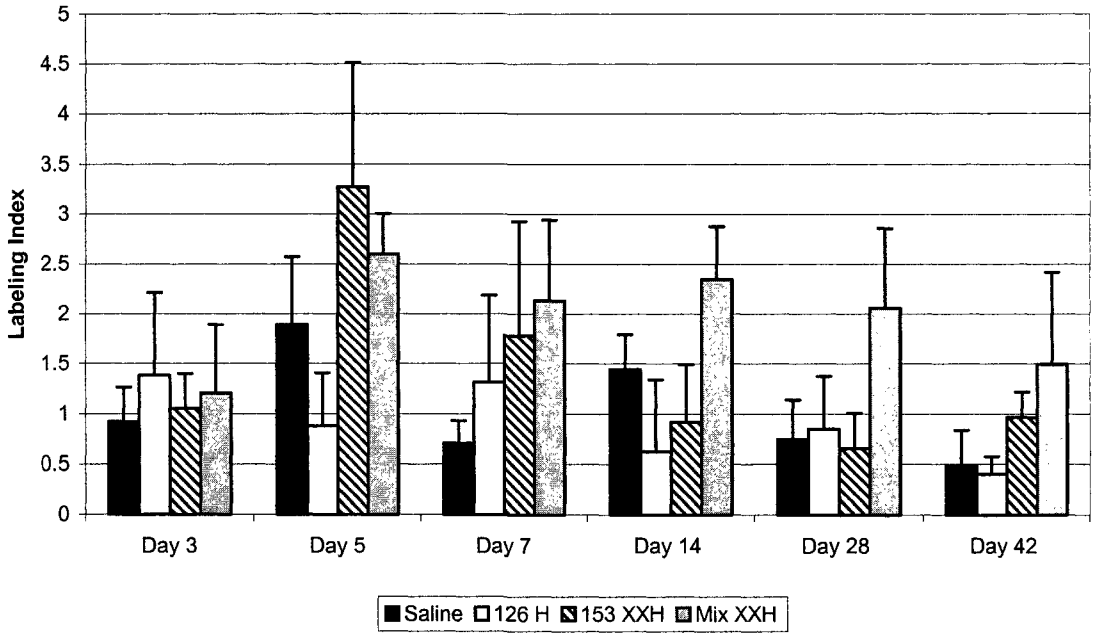

Figure 3.2 Cell proliferation in non-initiated saline/PCB-treated rats over a forty-two day period. For PCB 126, the dose was 10 μg/kg body weight (126h). For PCB 153, the dose was 10,000 μg/kg body weight (153 XXH). The combined PCB 126 and 153 exposure was 10+10,000 μg/kg body weight (Mix XXH), respectively.

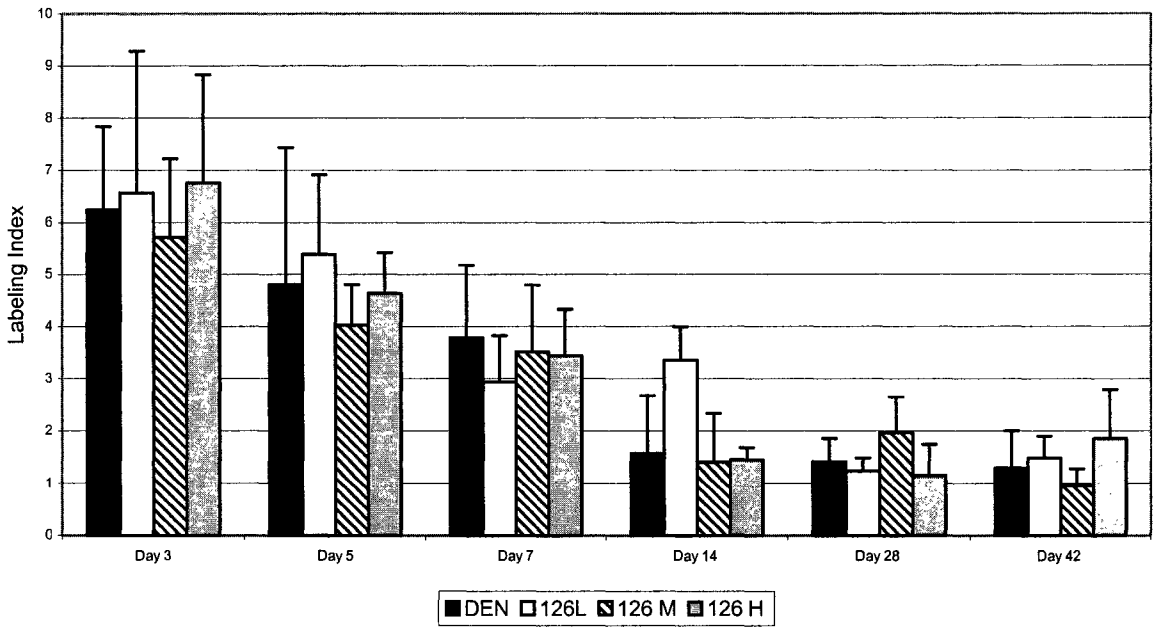

Figure 3.3 Cell proliferation in DEN-initiated PCB 126-treated rats over a forty-two day period. For PCB 126, the doses were 0.1 (126L), 1.0 (126M), and 10 (126H) μg/kg body weight.

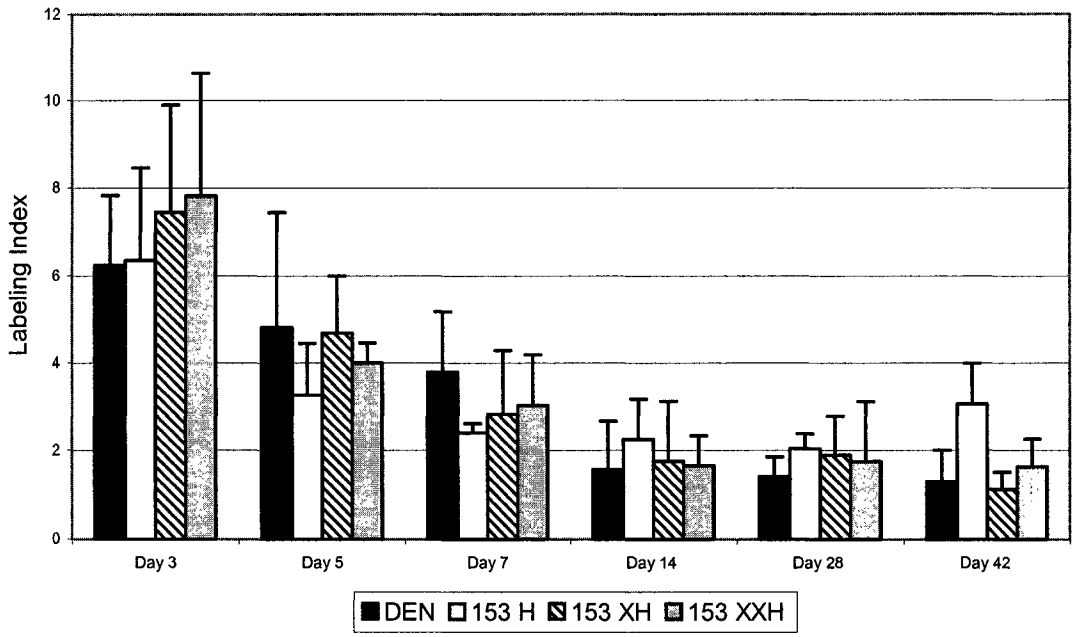

Figure 3.4 Cell proliferation in DEN-initiated PCB 153-treated rats over a forty-two day period. For PCB 153, the doses were 1,000 (153H), 5,000 (153XH), and 10,000 (153XXH) μg/kg body weight.

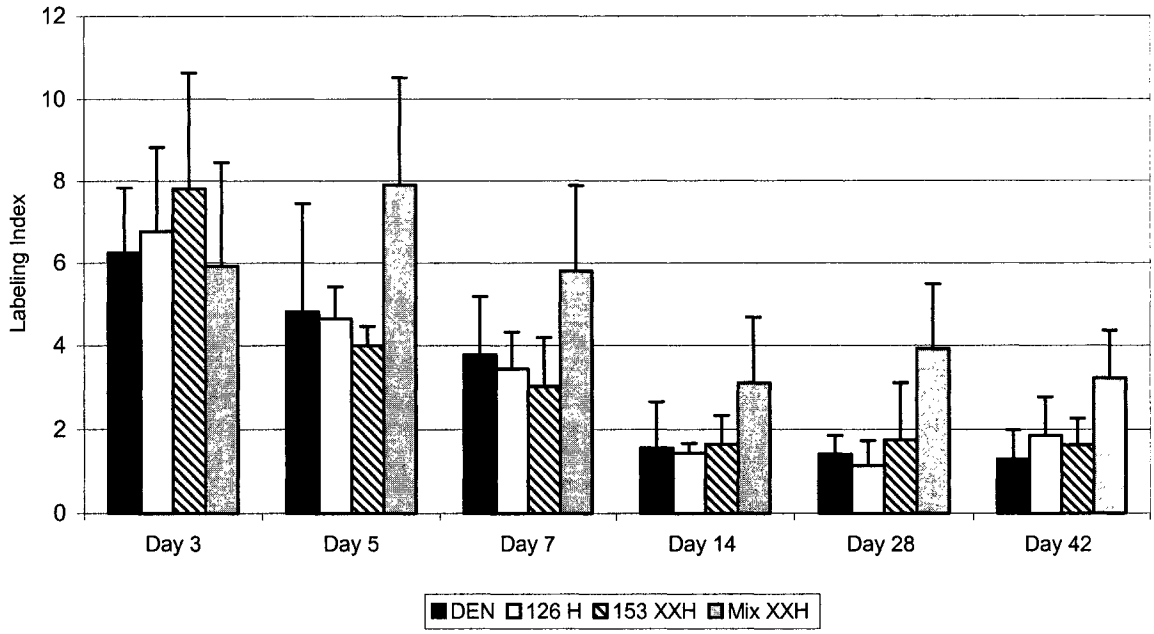

Figure 3.5 Cell proliferation in DEN-initiated PCB-treated rats over a forty-two day period. For PCB 126 the dose was 10 μg/kg body weight (126h). For PCB 153, the dose was 10,000 μg/kg body weight (153XXH). The combined PCB 126 and 153 exposure was 10+10,000 μg/kg body weight (Mix XXH), respectively.

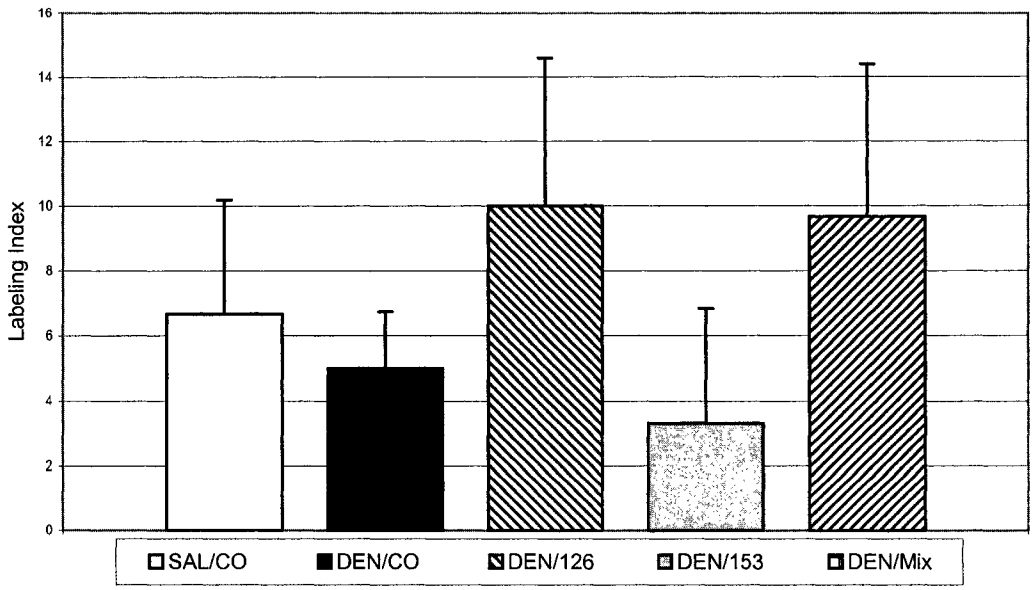

Figure 3.6 Apoptotic rates in rats treated for forty-two days with saline/corn oil, DEN/corn oil or DEN/PCBs. For PCB 126 the dose was 10 μg/kg body weight (DEN/126). For PCB 153, the dose was 10,000 μg/kg body weight (DEN/153). The combined PCB 126 and 153 exposure was 10+10,000 μg/kg body weight (DEN/Mix), respectively.

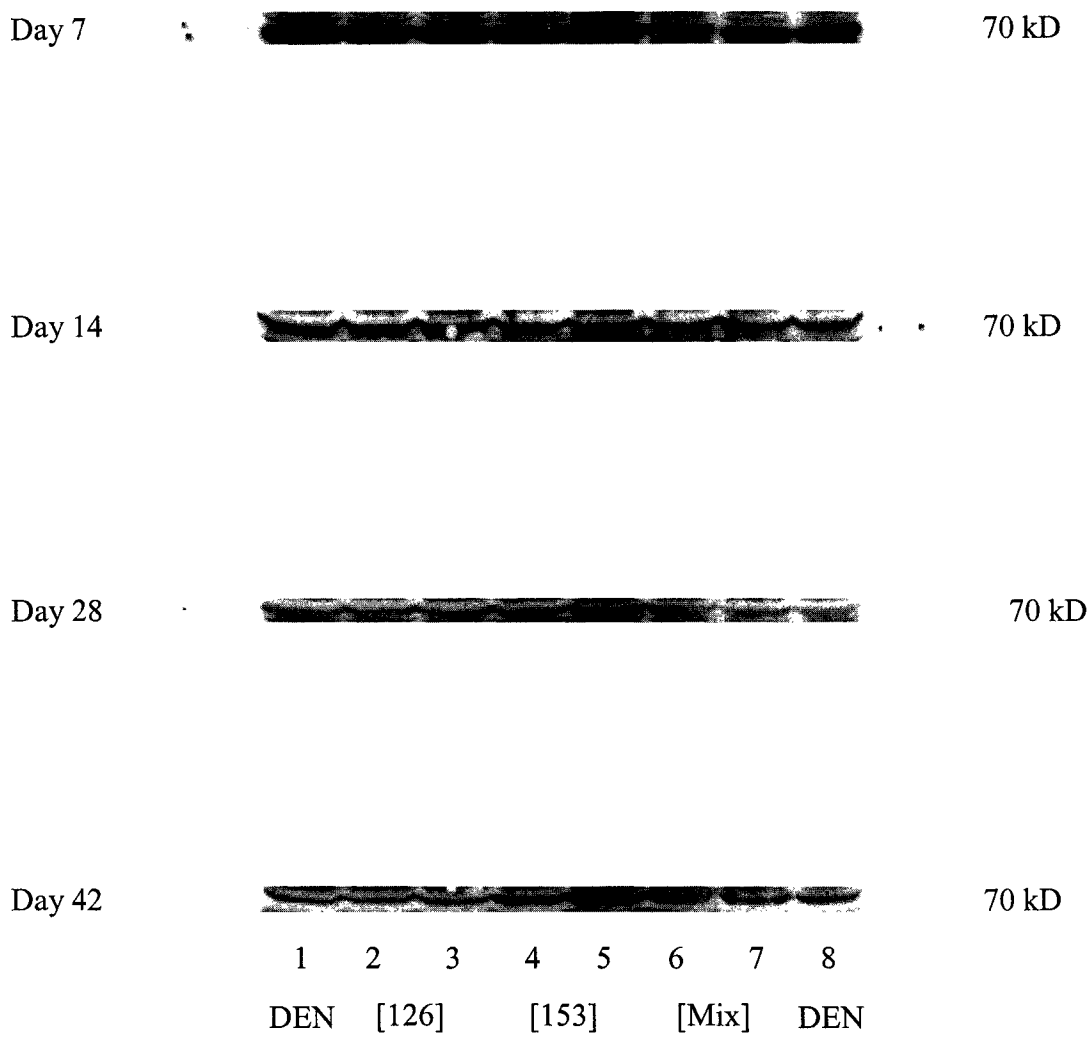

Figure 3.7. Western blot analysis of TGFβ II receptor expression in PCB-treated animals. Twenty micrograms of total protein was loaded. Lanes 1 & 8 were DEN control animals, lanes 2-3 were treated with DEN/PCB 126 at 10 μg/kg body. Lanes 4-5 were treated with DEN/PCB 153 at 10,000 μg/kg body. Lanes 6-7 were treated with DEN and a combination of PCBs 126 and 153 at 10+10,000 μg/kg.

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CHAPTER 4

Expression of Transforming Growth Factor Beta (TGFβ) and TGFβ Type II Receptor in Preneoplastic Hepatic Lesions Following Administration of PCB 126 (3,3',4,4',5-pentachlorobiphenyl) and PCB 153 (2,2',4,4',5,5'-hexachlorobiphenyl) in Rats

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ABSTRACT

Polychlorinated biphenyls (PCBs) are environmental pollutants and tumor promoters in the rat liver. Although the mechanisms of their promoting activities are not clear, one possible mechanism is the alterations in TGFβ signaling. Transforming growth factor (TGFβ) normally inhibits growth of epithelial cells and induces apoptosis in hepatocytes. The expression of TGFβ, which regulates cell proliferation, is closely associated with the expression level of TGFβ type II receptor and insensitivity to TGFβ has been regarded to be an important change in hepatic preneoplastic lesions as well as in hepatocellular carcinomas. This study investigates the immunohistochemical localization of TGFβ and TGFβ II receptor and their relationship with apoptosis and cell proliferation following individual and combined exposure to PCBs. Rats were exposed to PCB 126 at 10 μg/kg and PCB 153 at 10,000 μg/kg body weight using Ito's liver medium-term bioassay method. Compared with diethylnitrosamine (DEN) initiated controls, TGFβ-positive preneoplastic foci increased in

PCB 126 treated animals, and this correlated with a reduced TGFβ II receptor expressed in preneoplastic lesions. Treatment with PCB 153 and a mixture of PCB 126 with PCB 153 did not result in significant changes in TGFβ or TGFβ II receptor when compared to DEN-initiated controls. Our data suggest that the expressions of TGFβ and TGFβ II receptor are significantly altered during the promotion stage of hepatocarcinogenesis in the rat following treatment with PCB 126 and that these changes might contribute to the development and progression of preneoplastic lesions.

BACKGROUND

Polychlorinated biphenyls (PCBs) are halogenated hydrocarbons that prior to 1977 were widely used in industry. Their lipophilicity and stability, which made them applicable to their varied industrial applications, also allowed them to persist in nature, leading to their accumulation in the environment and biological systems (McFarland and Clarke, 1989; Safe, 1994; Robertson and Hansen, 2001). The use of PCBs is now banned in many countries and the environmental levels have been reported to be decreasing in temperate industrialized areas (Liem *et al.*, 2000), while they have increased in other areas (Faroon and Olsen, 2000). There is great concern for toxic effects of PCBs and other dioxin-like compounds since, despite the decline, human exposure is still close to or in excess of the recommended tolerably daily intake (Ahlborg *et al.*, 1992; Liem *et al.*, 2000).

The biological effects induced by PCBs are dependent on the molecular structure of individual congeners (McFarland and Clarke, 1989; Safe, 1994; Robertson and Hansen, 2001). Congeners lacking or having one chlorine substitution in the ortho position with an

additional chlorine in the para position of the biphenyl ring have a more planar conformation. PCB congeners substituted in both para positions and at least two meta positions are structurally related to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and they elicit comparable biological effects including binding with the aryl hydrocarbon receptor (Ah-r), induction of the monooxygenase enzymes cytochrome P450 1A1 and 1A2 (CYP1A1 and CYP1A2), thymic involution, the induction of a wasting syndrome, increased mortality, and a variety of toxic effects in the liver including carcinogenesis (Robertson and Hansen, 2001). Introduction of two or more chlorines in an ortho position results in decreased planarity between the two phenyl rings due to stearic interactions. Such PCBs congeners exhibit liver metabolic activity that is "phenobarbital-like." These compounds are not as potent promoters as the planar congeners but, like phenobarbital (PB), can induce hepatic tumor promotion. PCBs of this less planar group induce CYP2B1 and CYP2B2 or both CYP1A1/2 and CYP2B1/2 (Safe, S. H. 1994).

Human epidemiological data on the carcinogenicity of PCBs have been equivocal. While no overall increases in cancer-related mortality could be correlated with occupational PCB exposure, some studies reported non-statistically significant increases of specific cancers, including the grouping of liver, gall bladder, and biliary tract cancers (Brown, 1987; Gustavsson and Hogstedt, 1997; Bertazzi *et al.*, 2001). However, one analysis that combined results from 3 cohorts having different durations, levels of exposure, latencies, and follow-up did report statistically significant increases for a grouping of liver/biliary tract/gall bladder cancers (Nicholson WJ, 1994). Although the carcinogenicity of PCBs in humans has not been firmly established, numerous studies have demonstrated the

carcinogenicity of PCBs in rodents (Faroon *et al.*, 2001), as well as their potent tumor-promoting capabilities (Mayes *et al.*, 1998; Glauert *et al.*, 2001). Findings that both planar and non-planar PCB congeners can act as promoters of carcinogenesis individually have been reported by numerous investigators (Buchmann *et al.*, 1991; Haag-Grönlund *et al.*, 1997; Dean, Jr. *et al.*, 1998; Cogliano, 1998; Haag-Grönlund *et al.*, 1998; Tharappel *et al.*, 2002). Synergistic, antagonistic and additive hepatic promotional responses have all been reported following treatment with mixtures of planar and non-planar mixtures (Glauert *et al.*, 2001). Reports of synergistic hepatic tumor promotional activity are of concern considering humans are continually exposed through the environment to mixtures of planar and non-planar PCBs. The varied responses following PCB exposure emphasizes the importance understanding the mechanisms underlying their promotional activity so appropriate human risk assessments can be made.

Although it is known that both planar and non-planar PCBs can act as promoters of carcinogenesis, particularly in the liver, their mechanism of action is not understood. One mechanism by which PCBs may promote hepatic tumors is by acting directly on TGFβ signal transduction pathways. TGFβ inhibits hepatic cell proliferation, enhances differentiation, and induces apoptosis. Functional receptors are required for TGFβ-induced growth inhibition (Feng *et al.*, 1995) and some cancer cells that are not inhibited by TGFβ lack functional receptors (MacKay *et al.*, 1995). A wide variety of cancers including hepatocellular carcinomas in rodents and humans have demonstrated alterations in the TGFβ signaling pathways with altered expression and function of the TGFβ type II receptor being most commonly reported (Rossmanith and Schulte-Hermann, 2001).

Our laboratory has previously reported an increase in promotion of placental glutathione-S-transferase positive (GST-P+) preneoplastic liver cell foci following individual administration of the planar PCB 126 and non-planar PCB 153 (Dean, Jr. *et al.*, 2002). We also found that treatment with the mixture of PCB 126 and 153 resulted in antagonistic GST-P+ focus formation. This present study addresses whether TGFβ pathway signaling mechanisms might be involved in the formation of the preneoplastic hepatic lesions seen during PCB promotion in these rats.

MATERIALS AND METHODS

Chemicals

PCB 126 was purchased from AccuStandard (New Haven, CT), PCB 153 was purchased from Ultra Scientific (North Kingstown, RI), and diethylnitrosamine (DEN) was purchased from Sigma (St. Louis, MO). All other reagents were of analytical grade.

Animals

Thirty-day-old, female F344 rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and allowed to acclimate to altitude (Fort Collins, CO, 5000 feet) for four weeks. Following acclimation, rats were randomized by weight and divided into treatment groups. All animals were housed (three per cage) in polycarbonate cages with corncob bedding and stainless steel wire tops and were maintained at 25°C with 55% humidity with lighting maintained on a 12-hr light-12-hr dark cycle. Control and exposed animals were

given food (Harland Tedkad NIH-07 diet; Madison, WI) and deionized (DI) water *ad lib.* Rats were observed for clinical state twice daily, and food and water consumption and body weight were evaluated three times weekly. This study was conducted in accordance with the National Institutes of Health guidelines for the care of laboratory animals. Animals were housed in facilities fully accredited by the Association for Accreditation of Laboratory Animal Care.

Experimental Design

To evaluate promotional potential we used a medium-term bioassay for promoters of hepatocarcinogenesis (Ito, N. *et al.* 1989). This assay employs placental glutathione-S-transferase positive (GST-P+) liver cell foci as markers of preneoplasia in the rat after treatment with the known initiator diethylnitrosamine (DEN) followed by partial hepatectomy and exposure to a test chemical or chemical mixture. At the start of the study, rats received either a single intra peritoneal (IP) injection of DEN (200 mg/kg) dissolved in 0.9% saline or an injection of saline only. Fourteen days after initial DEN/Saline treatment, oral gavage administration of PCBs dissolved in 1 ml of corn oil vehicle or corn oil alone was begun. Several dose levels of the two PCBs in corn oil, either alone or in combination were used (Figure 4.1). The high PCB doses (PCB 126 at 10 μg/kg and PCB 153 at 10,000 μg/kg) were based on reported studies with PCB promotion of liver foci (Bager, Y. *et al.* 1995; Hemming, H. *et al.* 1993; Hemming, H. *et al.* 1995). Groups 1 and 2 consisted of DEN-initiated single PCB-treated animals. Group 3 contained DEN-initiated PCB combination-treated animals. Group 4 contained non-DEN-initiated high dose individual and combined PCB-treated animals. This group served to assay the potential of high dose

PCBs alone and in combination to induce preneoplastic foci without prior DEN exposure. Groups 5 and 6 contained the DEN control and vehicle control animals respectively. On day 21 after initial DEN/Saline treatment, a 2/3 partial hepatectomy was performed on all animals. Post-operative pain management consisted of Tylenol® and codeine (200 mg/kg and 60 mg/kg, respectively) administered in the drinking water for 72 hours. Gavages were administered 3 times weekly through the remainder of the 8-week study. Animals were euthanatized by aortic exsanguination following isoflourane anesthesia. Whole livers were removed and tissue sections were taken from the liver lobes. A 1-cm section of the jejunum was also collected to serve as a positive control for the cell proliferation and apoptosis studies. Tissue sections were fixed in 10% neutral-buffered formalin, embedded in paraffin, serially sectioned at 5 μm, and mounted on positively charged microscope slides. One section was stained with hematoxylin and eosin for histologic evaluation.

Immunohistochemical staining and liver foci analysis

A total of four liver sections were taken for examination from each animal; one each from the right posterior lobe and caudate lobe and two from the right anterior lobe. Tissue sections were fixed as above. For all immunohistochemical staining procedures liver sections were deparaffinized in xylene and rehydrated by passage through an alcohol series. Endogenous peroxidase was quenched in 3% H_2O_2 for 5 min. The slides were rinsed with DI water and placed in Phosphate Buffer Solution (PBS) (pH 7.4; 2.7 mM KCl, 0.14 M NaCl, 1.5 mM KH_2PO_4, 8.1 mM Na2HPO4). Individual procedures for antigen retrieval, antibody use, incubation times and tissue handling are listed below. For all procedures a final

incubation with the chromogen 3-amino-9-ethyl carbazole (AEC; Biomeda, Foster City, CA) was used. Slides were then counterstained with hematoxylin for histologic evaluation.

TGFβ and TGFβ II Receptor Staining

For immunohistochemical staining of TGFβ and TGFβ II receptor, sections were heated to boiling in a 600W microwave oven three times interspersed by five minute cooling periods in citrate buffer (Biogenex Labs, San Ramon, CA). Immunohistochemical stain was performed by the avidin-biotin peroxidase complex method using the anti-rabbit Vectastain ABC elite kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. To detect TGFβ and TGFβ II receptor, rabbit polyclonal antibodies against TGFβ1 (sc-146, Santa-Cruz, CA) and TGFβ II receptor (sc-400, Santa-Cruz, CA) were incubated at 4°C overnight at a dilution of 1:200 and 1:50, respectively.

PCNA Staining

Cell proliferation was evaluated by staining hepatocytes for proliferating cell nuclear antigen (PCNA) using mouse anti-PCNA monoclonal antibodies (DAKO Crop., Carpinteria, CA) followed by the avidin-biotin peroxidase complex method using the anti-mouse Vectastain ABC elite kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. For antigen retrieval liver sections were microwaved on high power until boiling in a 1:4 dilution of saturated lead thiocyanate solution (Shi *et al.*, 1991). After boiling, sections were microwaved at a reduced power for 10 minutes. Primary antibody was incubated in a humidified chamber at 37°C for 1 hour.

Apoptosis Staining

DNA apoptotic fragmentation was evaluated by optimizing the recommended method of a commercial apoptosis detection kit (ApopTag ™ *In Situ* Apoptosis Detection Kit: Peroxidase; Serologicals, Atlanta, GA). Liver sections were digested in 25 μg/ml Proteinase K (Roche Diagnostics Corp., Indianapolis, IN) for 25 min at room temperature, washed in PBS for 2 min, and followed by a 2-min wash in DI water. Approximately 100 μl of Equilibration Buffer containing digoxigenin-conjugated nucleotides (supplied by kit) was applied to each section for about 5 minutes. Sections were incubated with 54μl of working-strength ApopTag® terminal transferase enzyme in a humidified chamber at room temperature for 140 min. Specimens were then placed in a slide dish containing pre-warmed working-strength Stop-Wash Buffer (supplied by kit) and incubated in a 37°C water bath for 30 min, agitating the solution every 10 min. Slides were washed in PBS for 5 min and then in DI water for 3 min.

TGFβ1 and TGFβ II receptor foci analysis

Number and area of TGFβ and TGFβ II receptor foci were measured using an Olympus light microscope coupled with the Bioquant NOVA® for Windows® 98 (Version 5.00.8) computerized histomorphometry program (B and M Biometrics Inc., Nashville, TN). These measurements consisted of manually outlining the stained foci on the liver sections and allowing software to compute the enclosed area. Reference areas of liver sections were measured on a separate system using a Dage CCD72 MTI camera (Dage Corporation, Michigan City, IN) connected to a Bioquant® for Windows system (Version 2.6) (B and M Biometrics Inc., Nashville, TN). In this system, images of the liver sections were projected

on a computer terminal and automatically outlined and measured by the computer software. Results for the treatment and control groups were expressed as foci area in mm^2 of foci per area of liver sections examined in cm^2 (Kopp-Schneider, 2003).

Determination of Cellular Proliferation and Apoptosis

PCNA positive cells in which nuclei are homogeneously stained are indicative of the S phase of the cell cycle and were used to determine the cell proliferation rate in this study. Only TUNEL-positive cells that included densely stained cells with morphologic characteristics of apoptosis (*e.g.*, pyknosis, condensed cell volume, apoptotic bodies) were used to determine the apoptotic rate. Labeling index was evaluated by light microscopy for labeled hepatocyte nuclei using a random field technique by counting 1,000 nuclei per animal in both randomly selected preneoplastic lesions and in the surrounding liver parenchyma. In cases where the total number of hepatic nuclei was less than 1,000 in the available section, *e.g.*, where there were too few foci, the labeling index was based on a lower cell count.

Statistical Analysis

Areas of TGFβ and TGFβ II receptor foci were analyzed using the Kruskal-Wallis test. This analysis was followed by posthoc comparisons of the PCB treatment group means compared to DEN- corn-oil control via Dunn's multiple comparison procedure. Cell proliferation inside and outside foci for each treatment was analyzed using an unpaired t test with a two-tailed p value. All statistical tests were performed using the GraphPad Instat statistical

software package, version 3.0 (GraphPad Software, Inc., San Diego, CA). All results are expressed as means ± standard deviations (SD).

RESULTS

Histologic evaluation

No adverse clinical effects were observed in any of the treatment groups during the experimental period. Routine histologic screening identified mild to moderate biliary hyperplasia in approximately 1-2 percent of animals. This change was observed in all animal groups and occurred in a lobar to diffuse pattern. A similar change has routinely been observed in medium-term bioassays performed in our laboratory and has been attributed to some degree of biliary obstruction following partial hepatectomy.

Immunohistochemical staining for TGFβ 1 showed individual and small clusters of positive cells present within some preneoplastic foci. TGFβ expression was mainly located in the cytoplasm of hepatocytes in a diffuse to stippled pattern (Figure 4.2). Immunohistochemical staining for the TGFβ II receptor was reduced in preneoplastic lesions when compared to the adjacent normal liver parenchyma (Figure 4.3). Immunohistochemical staining for TGFβ and TGFβ II receptor on serial sections of the same preneoplastic lesions identified a subpopulation of foci that showed both increased staining for TGFβ and decreased staining for TGFβ II receptor compared to adjacent liver parenchyma (Figure 4.4). This effect occurred most frequently in PCB 126-treated animals. Results concerning number of GST-P-positive preneoplastic lesions and percentages of foci staining for increased TGFβ and decreased TGFβ II receptor are summarized in Table 4.1.

Quantitative morphometric evaluation of TGFβ1 and TGFβ II receptor foci

Analysis of TGFβ positive foci showed treatment with PCB 126 alone resulted in a significant ($p<0.01$) increase in area foci compared with corn oil controls (Figure 4.5). Treatment with PCB 153 alone did not increase area of TGFβ positive foci compared with DEN controls (Figure 4.5). Treatment with PCB 153 in combination with PCB 126 increased the area of TGFβ positive foci but this was not statistically significant compared to corn oil controls (Figure 4.5). Analysis of TGFβ II receptor foci with decreased staining showed treatment with PCB 126 alone resulted in an increase in area of negatively stained foci compared with corn oil controls ($p<0.05$) (Figure 4.6). Treatment of non-DEN-initiated rats with and without PCBs did not result in formation of preneoplastic foci showing GST-P positive, TGFβ positive or TGFβ II receptor negative foci (Group 4 and Group 5, data not shown).

Cellular Proliferation and Apoptosis

Results of hepatic apoptotic labeling inside and outside preneoplastic foci are shown in (Figure 4.7). No statistical increases or decreases in apoptosis were noted in any of the PCB treatment groups when compared to corn oil controls. Apoptotic rates are slightly but not statistically significantly elevated outside preneoplastic foci in PCB-treated animals compared to apoptotic rates inside foci. While, hepatic cell proliferation rates are statistically significantly increased inside preneoplastic foci compared the surrounding hepatic parenchyma in all treatment group including DEN/CO ($p<0.05$) (Figure 4.8), no

statistical increases or decrease in cell proliferation inside or outside foci are present in PCB treated animals when compared to corn oil controls.

DISCUSSION

Polychlorinated biphenyls (PCBs) are halogenated aromatic hydrocarbons that have been used widely in industry and are now recognized as worldwide environmental pollutants (Robertson and Hansen, 2001). Mixtures as well as purified congeners of PCBs have promoting activity in the liver and have been demonstrated to cause liver cancer in rodents (Silberhorn *et al.*,1990; Glauert *et al.*, 2001). Initiation/promotion protocols are frequently employed to study the promotional activity of potential carcinogens. In these studies, a single initiating dose of a chemical that damages DNA is followed by enhancement of cell replication (via partial hepatectomy or cytotoxicity) to fix that damage into the genome (initiation). Then partial hepatectomy and/or chronic exposure to a chemical produces clonal expansion of altered cells (promotion). In carcinogen-treated rats, single hepatocytes that express the placental form of glutathione-S-transferase (GST-P) represent putative initiated cells that have not yet been promoted (Dragan, Y. *et al.* 1995; Dragan, Y. P. *et al.* 1994a; Dragan, Y. P. *et al.* 1994c; Moore, M. A. *et al.* 1987; Tsuchida, S. and Sato, K. 1992). Foci of altered hepatocytes (FAH) which consist of hyperplastic enzyme-altered cells that stain for GST-P are considered to be preneoplastic in nature (Farber, E. and Sarma, D. S. R. 1987; Pitot, H. C. 1990) and are the most likely to progress to form true neoplasms in the rodent (Dragan, Y. P. *et al.* 1994b; Dragan, Y. P. *et al.* 1994a; Hasegawa, R. and Ito, N. 1994; Ogiso, T. *et al.* 1990). Although preneoplastic foci are reversible and most regress, some

foci continue to progress to adenomas and carcinomas (Grisham, J. W. 1997). Our laboratory has previously reported tumor-promoting ability in the rat liver following individual and combined exposure to PCB 126 and PCB 153 (Dean, Jr. *et al.*, 2002). Although it is known that both planar and non-planar PCBs can act during the promotional stage of carcinogenesis, particularly in the liver, their mechanism of action is not known. This study investigates the possible role of TGFβ and TGFβ II receptor in hepatic carcinogenesis and their relationship with apoptosis and cell proliferation following individual and combined exposure to PCB 126 and 153.

The TGFβ ligand and receptor system is an important tumor suppressor responsible for restraining epithelial cell proliferation in an autocrine and paracrine fashion (Markowitz and Roberts, 1996). It inhibits hepatocyte DNA synthesis and induces apoptosis. Many experiments have demonstrated dysregulations of TGFβ expression and signaling in liver carcinogenesis (Rossmanith and Schulte-Hermann, 2001). Normal expression of the TGFβ II receptor is necessary for TGFβ to exert its growth inhibitory effect. Interruption of this pathway may occur at multiple levels, including transcription, receptor mutation and signal transduction pathways.

The present study showed TGFβ II receptor protein expression was reduced in preneoplastic foci following administration of PCB 126 and this change coincided with a statistically significant increase in preneoplastic foci during the promotional stage of carcinogenesis (Dean, Jr. *et al.*, 2002). This finding is in agreement with other rodent chemical hepatocarcinogenicity studies that demonstrated that decreased expression TGFβ II receptor

resulted in increased formation of preneoplastic foci during promotion (Park *et al.*, 2001a) (Lim *et al.*, 1999). Transgenic rodent models which constitutively express a dominant negative (non-functional) TGFβ II receptor have also shown increased susceptibility to formation of preneoplastic and neoplastic lesions in the liver in initiation/promotion protocols (Kanzler *et al.*, 2001). In human hepatocarcinogenesis, TGFβ II receptor expression is reduced in liver cirrhosis, which might be considered a preneoplastic condition, as well as in human hepatocellular carcinoma (Bedossa *et al.*, 1995; Kiss *et al.*, 1997). Hepatocytes from cirrhotic livers exhibited a 50% reduction of TGFβ II receptor expression in comparison to normal hepatocytes. Accordingly, two fold higher concentrations of TGFβ were necessary to induce a 50% inhibition of DNA synthesis in these hepatocytes (Kiso *et al.*, 1994). These results all suggest that loss of normal tumor suppressive effects of a functional TGFβ signaling pathway may be involved in the promotional stages of carcinogenesis.

We also observed that PCB 126 significantly increased area of TGFβ positive foci. Normal and regenerating liver respond to TGFβ but, under normal circumstances they do not express it (Rossmanith and Schulte-Hermann, 2001). Non-parenchymal cells are the main source of hepatic TGFβ. However, expression of TGFβ is variable in livers with preneoplastic lesions and especially in rat livers from chemical carcinogenicity studies. Some studies report staining only in mesenchymal cells surrounding preneoplastic lesions or within neoplastic hepatocytes (Evarts *et al.*, 1990; Nakatsukasa *et al.*, 1991) while others report findings similar to ours where TGFβ is present in preneoplastic and neoplastic hepatocytes in human and rats (Lim *et al.*, 1999; Abou-Shady *et al.*, 1999; Park *et al.*, 2001a).

Animals treated with PCB 126 had the highest percentage of preneoplastic foci with decreased TGFβ II receptor expression and an increase in TGFβ expression (Table 4.1). This coincided with the strongest promotional effect as seen in GST-P positive focus induction (Dean, Jr. *et al.*, 2002). Cancer cells can secrete larger amounts of TGFβ than their normal counterparts and the association of TGFβ secretion with cancer is strongest in the most advanced stages of tumor progression (Blobe *et al.*, 2000).

Inhibition of TGFβ signaling would select for preneoplastic foci that have increased cell proliferation and/or reduced apoptosis compared to surrounding hepatocytes. Our study did find increased area of preneoplastic foci with reduced expression of TGFβ II receptor. There was a consistent increase in cell proliferation within foci in rats treated with PCBs, but this was not statistically significantly different between PCB 126, PCB 153 or combined exposure groups. While apoptosis was slightly greater outside than inside foci, this was not statistically significant.

A potential reason for this observed lack of significant difference in apoptosis could be the functional redundancy with respect to the growth inhibitory mechanisms of TGFβ in the liver. Under physiological circumstances, other TGFβ-related or unrelated cytokines may be involved. For example activin, a member of the TGFβ superfamily which acts via different receptors, is a potent inhibitor of hepatocellular growth (Russell *et al.*, 1999) and may be responsible for tissue homeostasis in the liver in case of disruption of TGFβ signaling pathway. Further, apoptosis is an infrequent event and was only evaluated at one time point

in this study. Although the TUNEL method is widely used to detect apoptosis, it is not a full measure of that process (Levin *et al.*, 1999). Although TGFβ can induce hepatocellular apoptosis *in vitro* and *in vivo,* the exact mechanism by which TGFβ mediates apoptosis is not completely understood. The release of cytochrome C, activation of caspase-8 and caspase-3 have been shown for liver epithelial cells (Oliver and Roberts, 2002). It is possible that differences in apoptotic rates may be detectable if a more specific method of analysis such as activated caspase is employed.

Lastly, resistance to TGFβ-mediated growth inhibition is reported to be a late event in tumor progression (Haddow *et al.*, 1991), and it has been shown for a variety of human tumors or tumor cell lines including hepatocytes. An *in vivo* time course study showed TGFβ II receptor transcript and protein expression reduced with time, particularly during later steps of the promotion (Park *et al.*, 2001a). *In vitro* treatment of numerous hepatocyte cell lines with TGFβ only partially suppressed the transformed phenotype in highly transformed and tumor cell lines representing late stages in progression, while it induced apoptosis in weakly or moderately transformed cell lines representing early stages in progression (Reeder and Isom, 1996). These results suggests that alterations of TGFβ pathways are progressive and the sensitivity of hepatocytes to TGFβ, while not as prominent during the early steps of the promotion in the rat, may become evident during the late steps of the promotion allowing cancerous cells to circumvent the apoptotic effect of TGFβ. Preneoplastic foci in rodents from numerous studies have varying sensitivity to the anti-proliferative effects of TGFβ (Wollenberg *et al.*, 1987; Stenius, 1993; Park *et al.*, 2001). This varied development of TGFβ-resistant phenotypes in these published studies is likely

dependent on the different initiators, promoters and protocol durations used to produce preneoplastic foci. In our study, there was increased cell proliferation in preneoplastic foci although no differences in apoptosis inside and outside foci was found. However, cell proliferation and apoptosis were not different between DEN control and DEN-PCB-treated groups. The finding that TGFβ II receptor is significantly reduced in some foci is suggestive that these foci may be insensitive to TGFβ signaling.

We suggest that alterations in the TGFβ signaling pathway may be a possible mechanism of tumor promotion by planar PCBs. Negative selection is the condition under which the growth regulatory environment in an organ suppresses division rates of normal cells and encourages growth and clonal expansion of altered cells (Jirtle *et al.*, 1991). Although, this negative selection mechanism was originally proposed to explain promotional effects of phenobarbital, it has been suggested it may be broadly applicable to a diverse group of liver tumor promoters, including planar PCBs (Andersen *et al.*, 1995). In this mechanism, a mitogenic stimulus is counteracted by elaboration of mitosuppressant growth factors. Specific preneoplastic cells acquire a change that renders them insensitive to the mitoinhibitory environment deriving a growth advantage compared to normal cells. Colonies of these cells expand and form foci that may eventually progress to tumors. For PCB 126 exposure there was decreased transforming growth TGFβ II receptor expression in 7.4% of foci, more than that of DEN/control, PCB 153 or the PCB 126/153 mixture. This finding coincided with the strongest promotional effect in our study (Dean, Jr. *et al.*, 2002). Treatment during promotion would select for cells with decreased TGFβ II receptor

expression that are unresponsive to mitoinhibitory and apoptotic effects of TGFβ resulting in a growth advantage.

Investigation with longer duration exposure to PCB 126 utilizing an initiation/promotion/progression model with multiple sacrifice time points is currently being conducted in our laboratory. It will be of interest to follow foci with altered TGFβ signaling pathways over a longer time period and to determine if preneoplastic foci that develop resistance to the tumor suppressive effects of increased TGFβ subsequently are those that progress to become TGFα-positive, a putative reflection of progression (Kaufman *et al.*, 1992; Moser *et al.*, 1997). This study might also reveal a clearer indication of patterns of cell proliferation and apoptosis in such foci.

Treatment with the non-planar PCB 153 at 10,000 μg/kg alone did result in statistically significant increase in GST-P positive foci in our promotional study (Figure 2.3, this dissertation) however, this did not result in significant increases in foci with TGFβ positive or TGFβ II receptor reduced staining foci (Figures 4.5 and 4.6) compared to DEN controls. Although from our current study we cannot conclude that PCB 126 and PCB 153 appear to be functioning as promoters by the same mechanism, results from our previous study (Dean, Jr. *et al.*, 2002) demonstrate that planar and non-planar PCBs do have interactive effects. PCBs exist as mixtures in the environment and individuals are exposed to a wide variation of individual of PCB congeners. Elucidation of congener specific mechanisms will allow for more precise risk assessments in the future.

In summary, results from this study demonstrated an increase in preneoplastic lesions in the liver that have reduced expression of TGFβ II receptors and enhanced TGFβ expression following PCB 126 administration. These results suggest that the planar PCB 126 and other strong Ah-r agonists may cause significant alterations in TGFβ signaling pathways during the promotional stage of hepatocarcinogenesis. This finding to our knowledge is the first report suggesting that altered TGFβ signals may be involved in mechanisms of PCB hepatic tumor promotion.

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Table 4.1

Numbers of GST-P positive preneoplastic foci per treatment group and percentages of immunohistochemically stained foci with either reduced TGFβ II receptor staining, increased TGFβ staining or both decreased TGFβ II receptor staining plus increased TGFβ staining.

Number of GSTP + Foci per Treatment Group		Foci with Decreased TGFβ II Receptor Staining Number (%)	Foci with Increased TGFβ Staining Number (%)	Foci with Both Decreased TGFβ II Receptor and Increased TGFβ Staining Number (%)
DEN	406	11 (2.7)	2 (0.5)	0 (0.00)
PCB 126	1121	52 (4.6)	31 (2.7)	2/27 (7.4)
PCB 153	614	12 (1.9)	2 (0.3)	0 (0.00)
PCB Mix	1451	20 (1.3)	21 (1.45)	1/20 (5.0)

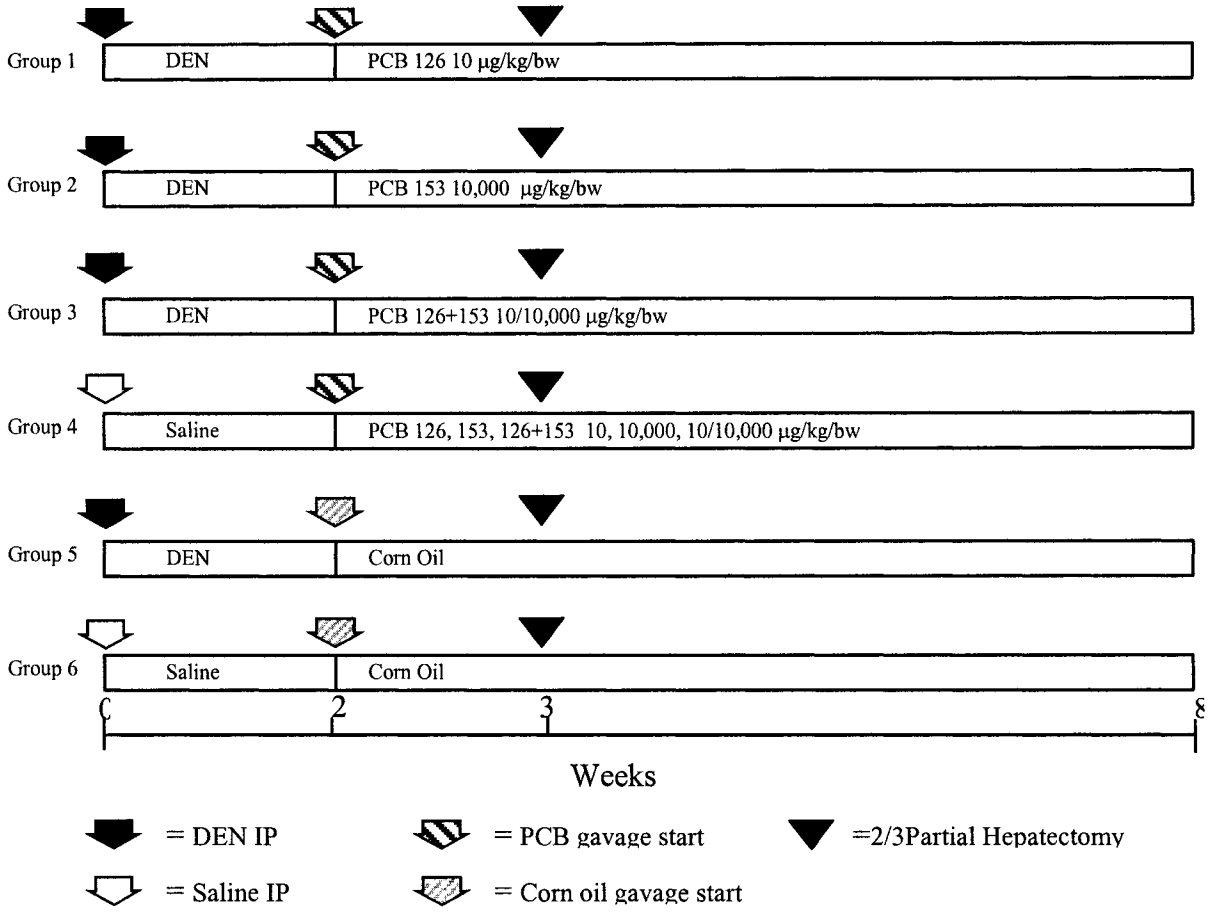

Figure 4.1. Experimental design and PCB dosages for the medium-term liver bioassay. Diethylnitrosamine (DEN) the initiation agent was given via intraperitoneal injection (200 mg/kg) at day 0. PCB treatment three times a week via oral gavage began at week 2 and continued until completion of the study at week 8.

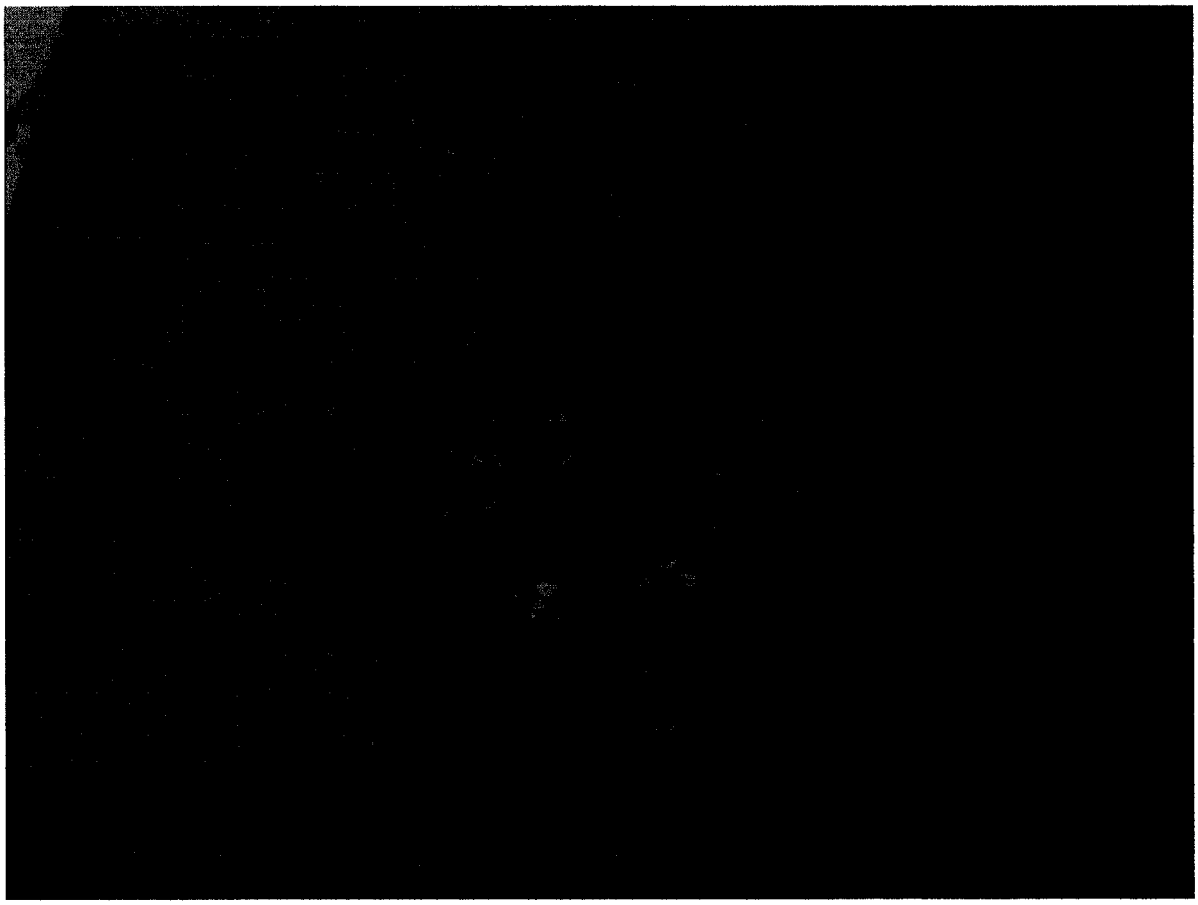

Figure 4.2 Immunohistochemical staining for TGFβ II in rat treated with PCB 126 dosed at 10 μg/kg body weight utilizing a medium-term eight-week bioassay for promotion. Individual and clusters of hepatocytes stain positive. The cytoplasm is diffusely stained with a stippled pattern. Magnification 200X.

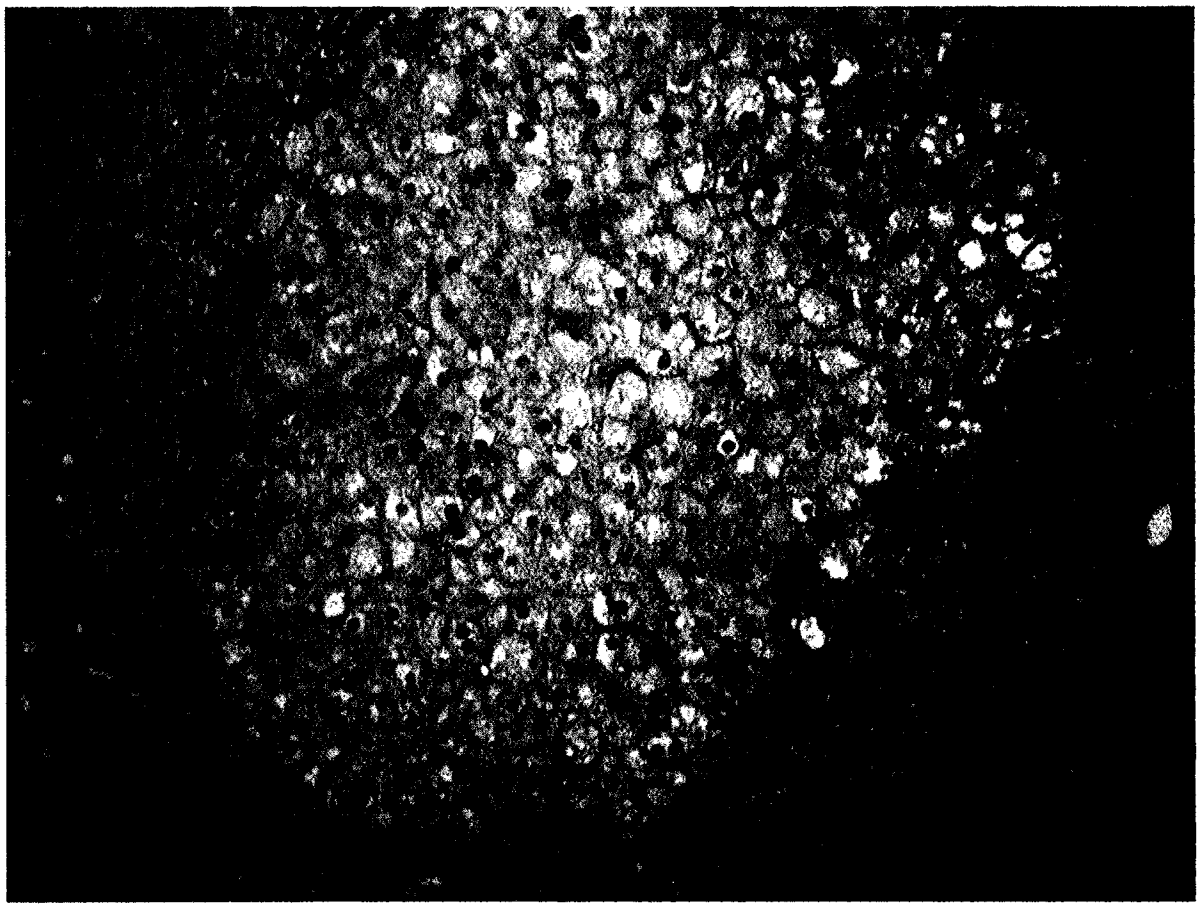

Figure 4.3 Immunohistochemical staining for TGFβ II receptor in rat treated with PCB 126 dosed at 10 μg/kg body weight utilizing a medium-term eight-week bioassay for promotion. There is a marked diffuse decrease of receptor expression within the focus of altered hepatocytes compared to normal surrounding hepatocytes. Magnification 200X.

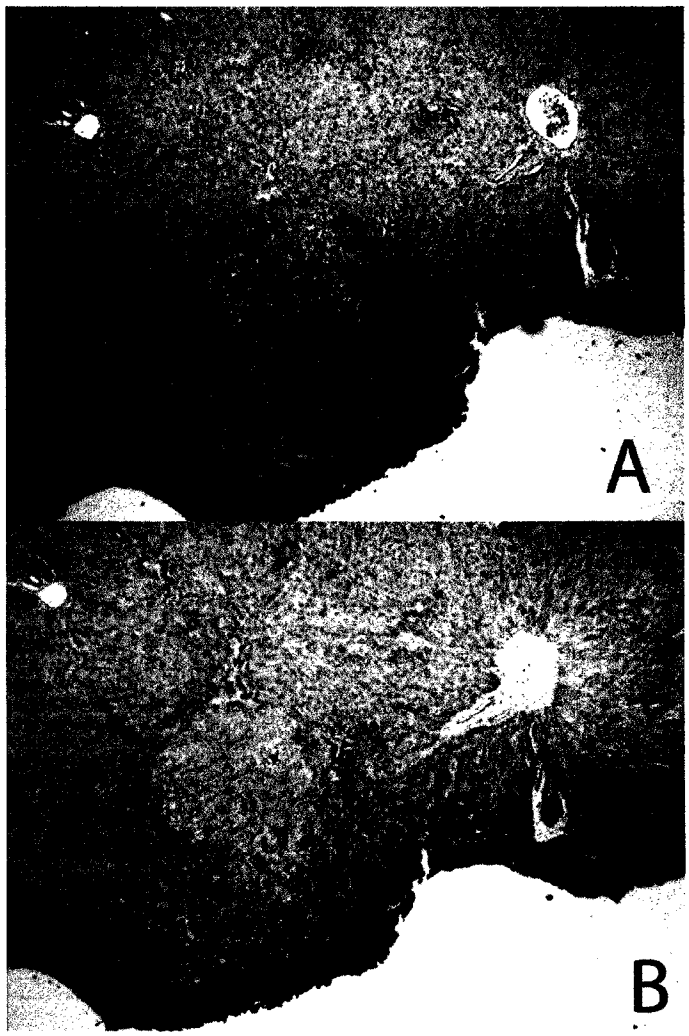

Figure 4.4 Immunohistochemical staining for TGFβ and TGFβ II receptor in rat treated with PCB 126 at 10 μg/kg body weight utilizing a medium-term eight-week bioassay for promotion. A) There is increased TGFβ staining of individual and clusters of hepatocytes within the focus of altered hepatocytes. B) This serial section demonstrates that cells of the same preneoplastic focus that were positive for TGFβ also have decreased TGFβ II receptor expression. Magnification 100X.

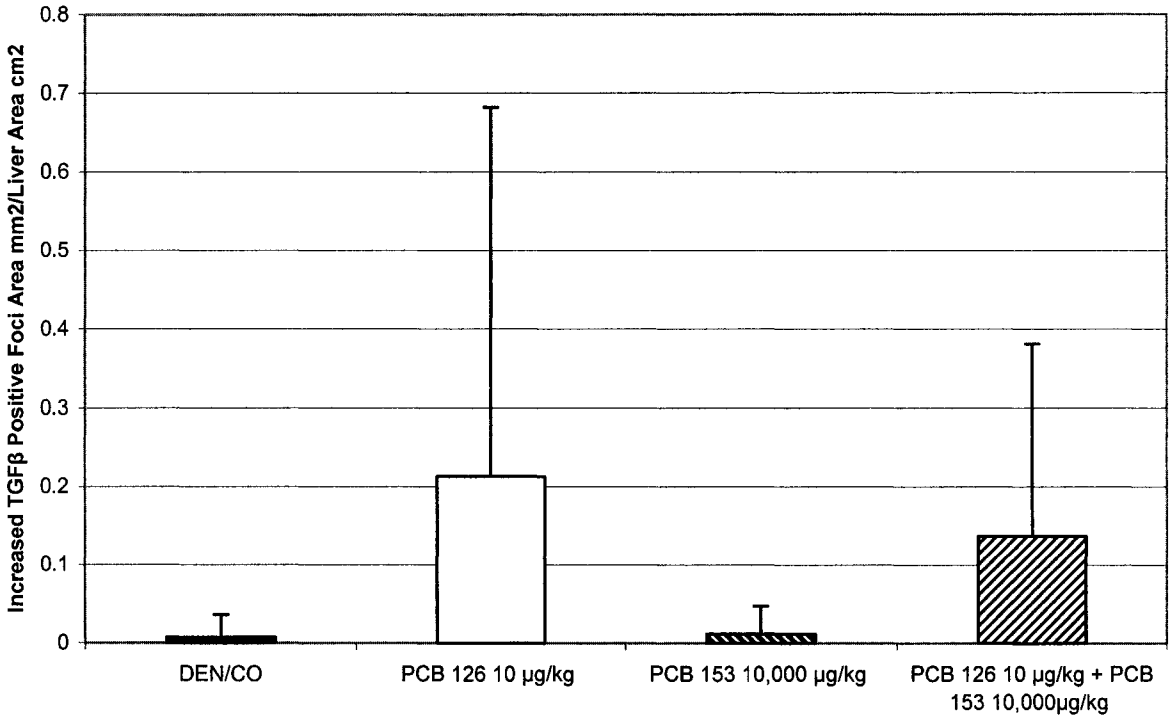

Figure 4.5 Area of foci with increased TGFβ staining (foci Area mm2/liver Area cm2) in female F344 rats subjected to an initiation/promotion protocol using diethylnitrosamine (DEN) as an initiator and PCBs 126 and 153 alone and in combination as the promoters. Values shown are Means ± SD. By the Kruskal-Wallis Test, the variation among column medians is significantly greater than expected by chance ($p<0.001$). Using Dunn's Multiple Comparison Test, the difference between DEN/CO and DEN/PCB 126 mixture were significant ($p<0.01$). The differences between DEN/CO and PCB 153 alone or PCB 153/126 mixture were not significant ($p>0.05$).

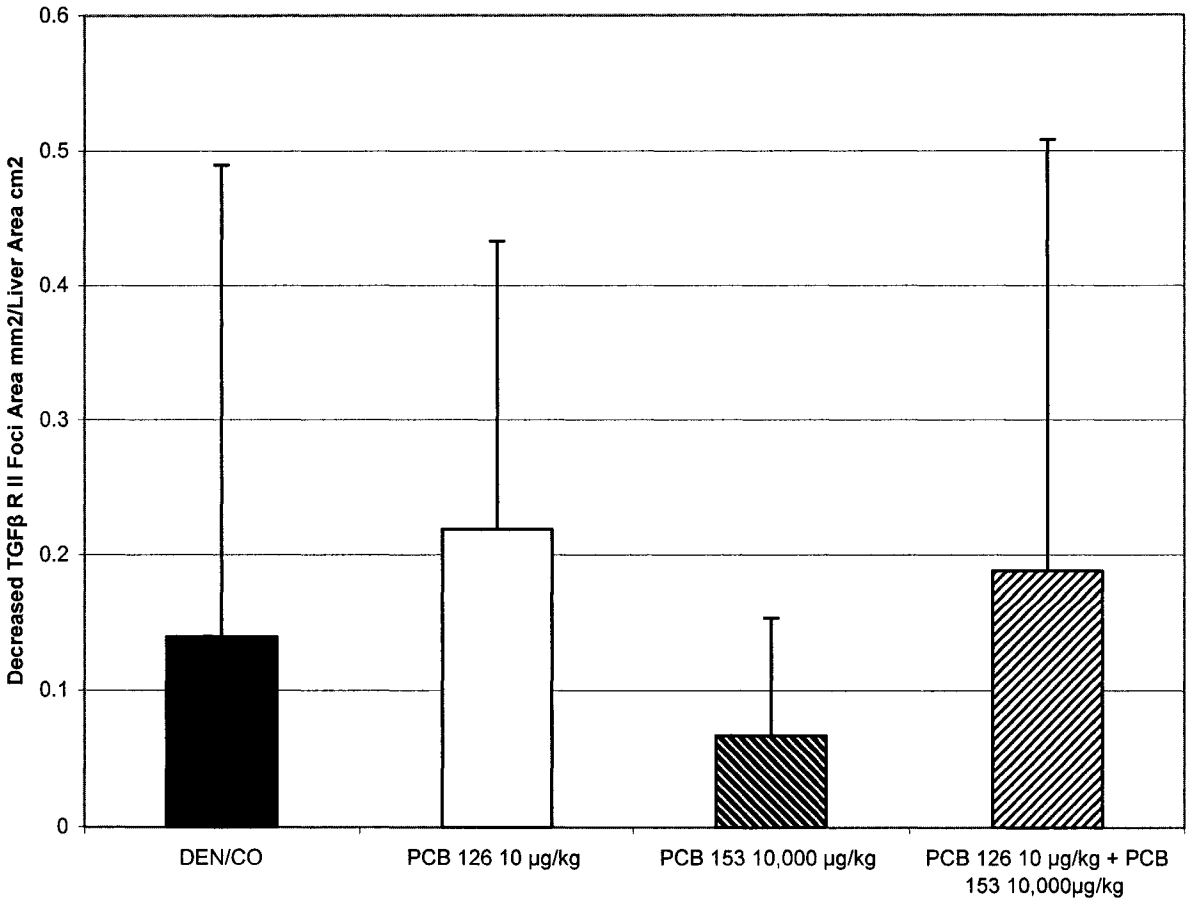

Figure 4.6 Area of foci with reduced TGFβ II receptor (foci Area mm2/liver Area cm2) in female F344 rats subjected to an initiation/promotion protocol using diethylnitrosamine (DEN) as an initiator and PCBs 126 and 153 alone and in combination as the promoters. Values shown are Means ± SD. By the Kruskal-Wallis Test, the variation among column medians is not quite significantly greater than expected by chance ($p<0.067$). Using Dunn's Multiple Comparison Test, the difference between DEN/CO and DEN/PCB 126 mixture were significant ($p<0.05$). The differences between DEN/CO and PCB 153 alone or PCB 153/126 mixture were not significant ($p>0.05$).

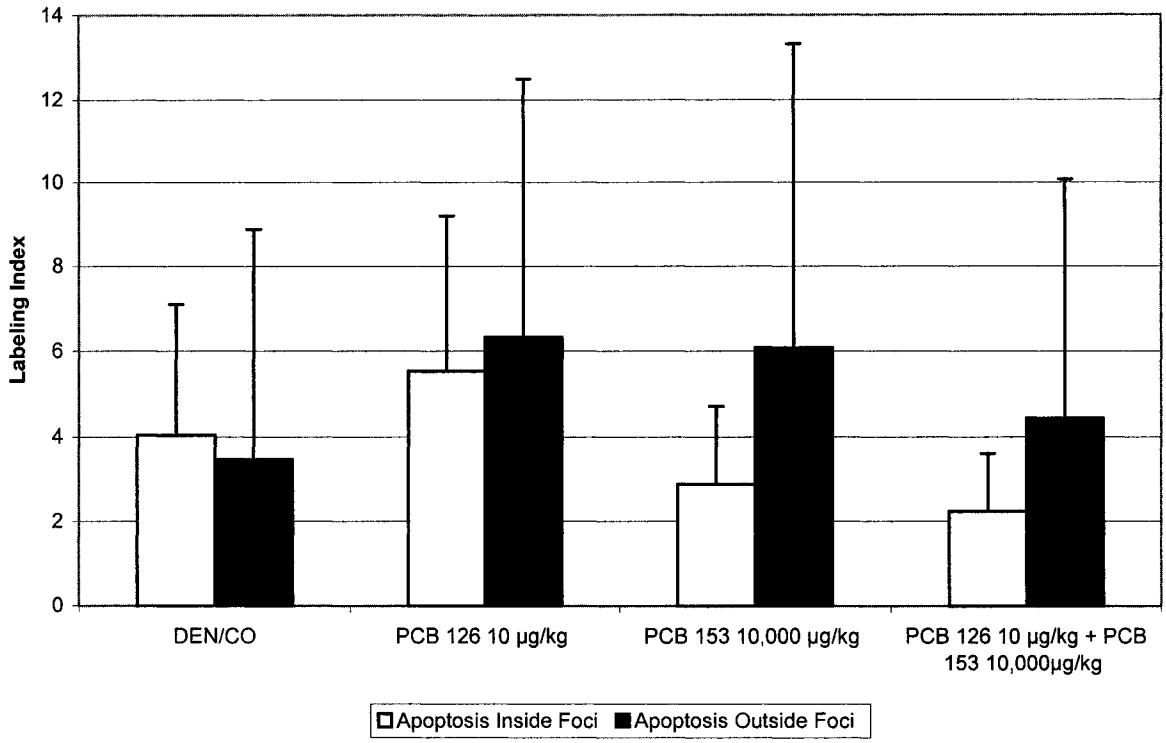

Figure 4.7 TUNEL-positive apoptotic cells inside and outside preneoplastic foci of altered hepatocytes. Values shown are Means ± SD from measurement of randomly selected separate preneoplastic lesions and adjacent normal hepatic parenchyma. By the Kruskal-Wallis Test, the variation among column medians is not significantly greater than expected by chance ($p<0.05$). Using Dunn's Multiple Comparison Test, no significant differences were present in any PCB treatment groups when compared to DEN/CO controls. Using the unpaired t test no statistically significant differences in apoptosis inside and outside foci were identified for any treatment group.

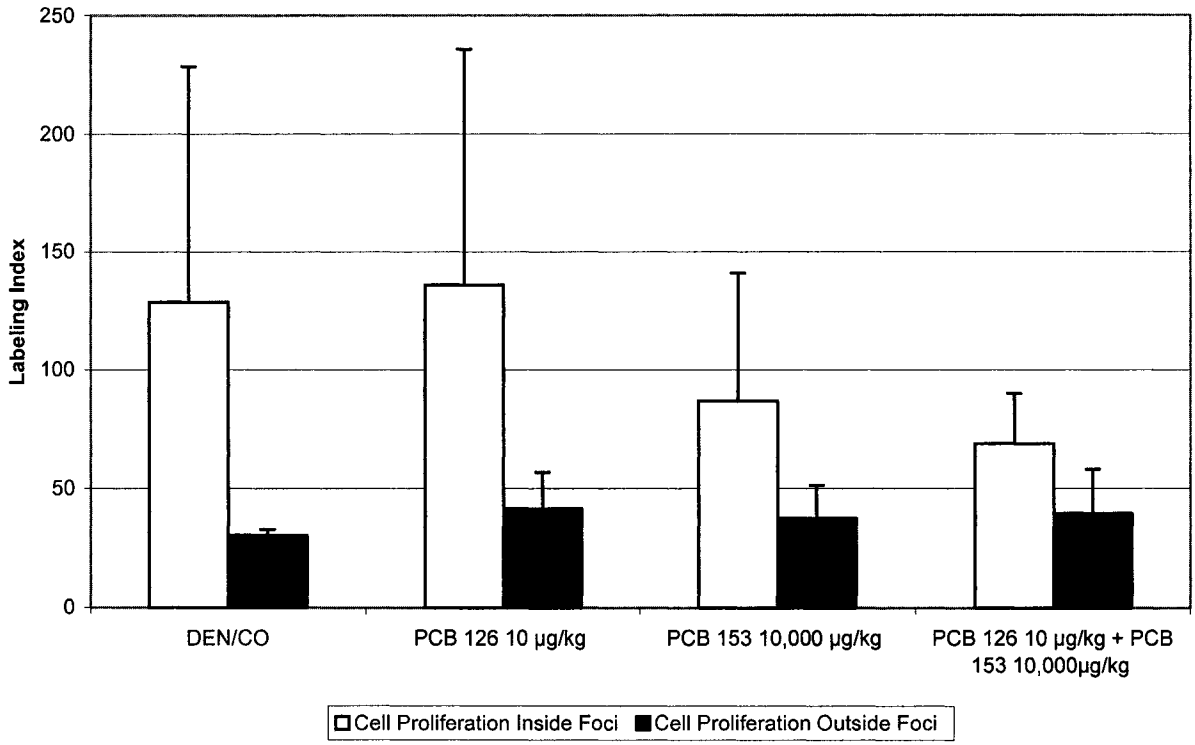

Figure 4.8 Cell proliferation inside and outside preneoplastic hepatic foci as measured by PCNA S phase staining. Values shown are Means ± SD from measurement of randomly selected separate preneoplastic lesions and adjacent normal hepatic parenchyma. By the Kruskal-Wallis Test, the variation among column medians is not significantly greater than expected by chance ($p<0.05$). Using Dunn's Multiple Comparison Test, no significant differences in cell proliferation were present in of any PCB treatment groups when compared to DEN/CO controls. Using the unpaired t test statistically significant differences in cell proliferation inside and outside foci were present for all treatment groups $p<0.05$.

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CONCLUSION AND FUTURE DIRECTIONS

Polychlorinated biphenyls (PCBs) are environmental pollutants and tumor promoters in the rat liver (Glauert *et al.*, 2001). PCBs are persistent environmental contaminants and are present as mixtures of congeners with differing physical and biological characteristics (Robertson and Hansen, 2001). Their impact on biological systems may be due to interactions between themselves and other classes of chemical pollutants thereby making them good candidates for the study of chemical mixtures. Many PCB congeners are known tumor promoters in the rodent liver and readily demonstrate hepatocarcinogenicity (Faroon *et al.*, 2001). Based on abundant rodent data the Environmental Protection Agency and National Toxicology Program have concluded that PCBs are reasonably anticipated to be carcinogenic in humans.

Synergistic, antagonistic and additive hepatic promotional responses have all been reported following treatment with mixtures of planar and non-planar PCB (Sargent *et al.*, 1991; Bager *et al.*, 1995; Berberian *et al.*, 1995; Haag-Grönlund *et al.*, 1998; Dean, Jr. *et al.*, 2002; Tharappel *et al.*, 2002). Despite this, there are few relevant data on such interactive biological effects and risk estimation is based on data for individual PCB congeners. The toxic equivalency factor (TEF) concept is used to facilitate the risk assessment of PCBs and related substances and the potency of the most toxic dioxin, 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), is used as a standard and set to one, and

other dioxin-like chemicals are assigned TEFs that are fractions thereof (Safe, 1994; Whysner *et al.*, 1996; Giesy and Kannan, 1998; van den Berg *et al.*, 1998). The total toxic equivalency (TEQ) of a mixture is the sum of the individual potencies. Several assumptions are made when utilizing the TEF scheme. First, all interactions are presumed to be additive in nature. Second, TEFs are assigned based on a chemical's affinity to bind to the aryl hydrocarbon receptor (Ah-r), which non-planar PCB congers are unable to do. Therefore, the current system does not include the potential impact of non-planar PCB congeners even though they are known promoters.

Our laboratory at the Center for Environmental Toxicology and Technology has long-standing interest in the effects of environmental chemicals on health. A number of studies over the past decade have evaluated environmental chemical mixtures including such chemicals as lead, arsenic, tetrachlorobenzene, trichloroethylene, vinyl chloride, carbon tetrachloride and PCBs among others (Constan *et al.*, 1996; El-Masri *et al.*, 1996; Thomas *et al.*, 1998; Pott *et al.*, 1998; Pott *et al.*, 2000). Part of the impetus for the studies reported here were the results from a paper which reported synergistic hepatic tumor promoting activity following exposure to a mixture of the planar PCB 126 (3,3',4,4',5-pentachlorobiphenyl) and the non-planar PCB 153 (2,2',4,4',5,5'-hexachlorobiphenyl) (Bager *et al.*, 1995). Understanding the interactions between PCB 126 and PCB 153 is important due to the potent hepatotoxicity of PCB 126 and the ubiquitous nature of PCB 153. If synergism were occurring between these congeners current risk assessments would markedly underestimate the risk.

We designed experiments to evaluate the interactive promoting effect of PCB 126 and 153, spanning a dose range which included high doses known to induce preneoplasia and low doses that were consistent with what might be seen from environmental exposures (McFarland and Clarke, 1989; Bager *et al.*, 1995)

The biological effects induced by PCBs are dependent on the molecular structure of individual congeners. PCBs that are substituted in both *para* and at least two *meta* positions, but not in any of the *ortho* positions, may form a planar configuration similar to TCDD. These non-*ortho* substituted PCBs appear to have TCDD-like activity and induce a variety of reproductive, immunologic and hepatic toxic effects (Safe, 1994). Planar PCBs and other halogenated hydrocarbons such as TCDD produce their biological effects through a receptor-mediated response, binding to the cytosolic Ah-r. This is followed by changes in gene expression (Safe, 1994; Robertson *et al.*, 2000). Like TCDD, planar PCB congeners induce CYP1A1 and CYP1A2. Substitution of two or more chlorines in an ortho position results in decreased planarity between the two phenyl rings due to stearic interactions. Such PCBs congeners exhibit liver metabolic activity that is "phenobarbital-like." These compounds are not as potent as the planar congeners but, like phenobarbital (PB), can induce hepatic tumor promotion. PCBs of this less planar group induce CYP2B1 and CYP2B2 or both CYP1A1/2 and CYP2B1/2 (Safe *et al.*, 1985; Safe, 1994). The induction of CYP2B1 by phenobarbital and, putatively, by non-planar phenobarbital-like PCB congeners is mediated by the nuclear receptor constitutive androstane (CAR) (Muangmoonchai *et al.*, 2001; Oliver and Roberts, 2002).

We hypothesized that lack of response to transforming growth factor beta (TGFβ) inhibition of cell proliferation and apoptosis may be a common mechanism of promotion, contributing to the synergism. TGFβ inhibits hepatic cell proliferation, enhances differentiation, and induces apoptosis in hepatocytes. The expression of TGFβ, which regulates cell proliferation, is closely associated with the expression level of TGFβ type II receptor and insensitivity to TGFβ has been regarded to be an important change in hepatic preneoplastic lesions as well as in hepatocellular carcinomas (Rossmanith and Schulte-Hermann, 2001). While the mechanisms of PCB promotion are unclear, we hypothesized that the individual planar and non-planar PCB congeners may act like other chemicals with similar physical characteristics and metabolic effects, *i.e.*, the planar PCB 126 would have potent TCDD-like activity, while the non-planar PCB 153 would have strong phenobarbital (PB)-like metabolic activity. TCDD treatment can interfere with TGFβ receptor configuration leading to a truncated non-functional receptor (Personal communication, Dr. F. Matsamura, University of California, Davis, 1996; this information has not been published and, at the present time, no mention of alterations in TGFβ receptors following exposure to TCDD can be found in the literature). Tumor cells promoted by PB have reduced TGFβ receptor expression and reduced TGFβ activation from its latent form (Jirtle *et al.*, 1994). Therefore, one might explain the co-planar/non-planar PCB synergism if PCB 126 induced a nonfunctional TGFβ receptor, in conjunction with putative reduced TGFβ receptor expression and TGFβ activation.

Promotion of altered hepatic foci was evaluated utilizing Ito's medium-term eight-week bioassay for promoters of hepatocarcinogenesis (Ito *et al.*, 1988). Results from our

promotional study found antagonism of placental glutathione S-transferase (GST-P) preneoplastic foci formation between these two PCB congeners. This finding was surprising because the high doses of PCBs used in our study and the Bager study were comparable. In our study, the PCB 126 doses were 0.1, 1.0, and 10 μg/kg body weight. For PCB 153, the doses were 10, 100, 1,000, 5,000, and 10,000 μg/kg body weight. Combined PCB 126 and 153 exposures were; 0.1+10, 1+100, 10+1,000, 10+5,000, and 10+10,000 μg/kg, respectively. In the Bager study, the PCB 126 the doses were 1.0, 3.16 and 10 μg/kg body weight. For PCB 153, the dose was 5,000 μg/kg body weight. Combined PCB 126 and 153 exposures were; 1.0 + 5,000, 3.16 + 5,000 and 10 + 5,000 μg/kg, respectively. The Bager study differed from our medium-term bioassay. In the Bager study Sprague-Dawley rats were subjected to partial hepatectomy on the first day of the study followed by DEN administration (30mg/kg) 24 hours later. All PCB administration was subcutaneous and started five weeks after partial hepatectomy with a loading dose that was five times the maintenance dose. PCB where then administered once a week for the remainder of the 25 week study. These differences between the two studies potentially contributed to our conflicting promotional results. An important difference between the studies was the marker of preneoplastic foci. In the Bager, study γ–glutamyltranspeptide (GGT) was used while GST-P was used in our study. The same laboratory published a second study investigating the interactive effects of PCB 126 and PCB 153 and this time antagonistic promotional activity was reported (Haag-Grönlund *et al.*, 1998). In this second study, the PCB 126 the doses were 0.13, 0.93 and 6.6 μg/kg/week. For PCB 153, the doses were 220, 1,556, 11,003 μg/kg/week. Combined PCB 126 and 153 exposures were; 0.13 + 220, .93 + 1,560 and 6.6 + 11,003 μg/kg/week,

respectively. An important difference in the second study was that GST-P was the marker of preneoplasia. This suggests that results from initiation/promotion protocols studying the same chemical that used different preneoplastic markers may not be totally comparable.

A possible mechanism for the antagonistic promotional response we observed for the PCB mixture is competitive binding of the Ah-r. A study that exposed mice to mixture of TCDD and high doses of PCB 153 (750 to 1000 μmol/kg) showed with competitive binding assays that PCB 153 competitively displaced TCDD from the Ah-r receptor (Biegel *et al.*, 1989). Likewise, the high concentrations of PCB 153 administered in our study, which became further elevated in the liver when co-administered with PCB 126, could compete with the dioxin-like PCB 126, which is a much stronger promoter, for the Ah-r binding, resulting in antagonism. Experiments using similar competitive binding assays could answer this question.

Another possible mechanisms of antagonistic interactions are dispositional interactions (van der Kolk *et al.*, 1992; De Jongh *et al.*, 1995; van Birgelen *et al.*, 1996) whereby the chemical concentration in the target organ is changed by co-exposure, which occurs when two chemicals produce opposite effects on the same physiological function. PCB 153 concentration can be increased in liver by a high dose of TCDD (van Birgelen, A. *et al.* 1996). PCB 153 is lipophilic and, since TCDD appears to increase PCB 153 levels in the liver, it has been suggested that this is related to increased fat in liver cells due to toxicity of the TCDD (van Birgelen, A. *et al.* 1996). This is unlikely in our study, since we saw

no histologic evidence of hepatic toxicity or visible fat increase after exposure to individual congeners or mixtures. However our promotional study did have statistically significant increase in PCB 153 liver levels after combined PCB exposure.

The induction of CYP2B1 by phenobarbital is mediated by the nuclear receptor constitutive androstane (Muangmoonchai *et al.*, 2001; Oliver and Roberts, 2002) It is currently believed that all induction of CYP2B1 is mediated through the CAR receptor. It is possible that the statistically significant increase in PCB 153 levels after combined PCB 126/153 exposure is a receptor mediated event caused by PCB 126 induction of the CAR receptor. Western blot analysis from PCB 126 treated animals could be performed to confirm such induction.

We conducted a time course study to examine the effect of PCB 126 and 153 after DEN initiation but in the absence of partial hepatectomy. We hypothesized that the PCB administration would result in changes in cell proliferation and apoptosis related to altered TGFβ signaling. In our study, we could not demonstrate statistically significant alterations in cell proliferation, apoptosis, or TGFβ signaling in DEN/PCB-treated rat livers at any time point through 42 days. The necrogenic dose of DEN (200 mg/kg i.p.), while being a mitogenic stimulus, was not a sufficient stimulus to drive the formation of preneoplastic foci in an eight-week assay without partial hepatectomy. A time course study using the Solt and Farber initiation/promotion protocol which included partial hepatectomy was successful in detecting increases in TGFβ and decreases of TGFβ II receptor in preneoplastic foci by immunohistochemistry (Park *et al.*, 2001). These

changes were detectable as early as 10 days after partial hepatectomy and progressed within foci throughout the duration of the 56-day study. Northern blot analysis detected alterations in expression of TGFβ and TGFβ II receptor in preneoplastic foci however, not until days 42 and 56 (Park *et al.*, 2001). These results demonstrate that biochemical assays that use whole tissue homogenates are effective at detecting changes in TGFβ receptor expression in livers when a sufficient foci volume of preneoplastic foci is present. In retrospect, incorporation of partial hepatectomy into our time course study would have resulted in significant AHF formation and might have given us more relevant data on TGFβ signaling if not on cell proliferation and apoptosis. Partial hepatectomy stimulates compensatory cell proliferation and, when this is complete, there are increases in TGFβ and TGFβ II receptor, which may last up to three weeks. We avoided partial hepatectomy in our design because it is known to alter all these factors by it self and we were interested in the pure PCB effect.

Alterations in TGFβ signaling are involved in the promotional and progressional stages of carcinogenesis, therefore, future time course studies should be conducted longer than eight weeks to better assess this process. Currently in our laboratory, a 24-week initiation/promotion/progression study is being conducted with PCB 126 treatment at 10 μg/kg. Immunohistochemical and biochemical methods outlined in our studies reported here should be effective at detecting TGFβ signaling changes in this longer term experiment.

Results from the immunohistochemical studies of preneoplastic foci in PCB 126-treated rats from our initiation/promotion study are of great interest. We found that TGFβ - positive preneoplastic foci increased in PCB 126-treated animals and this correlated with a reduced TGFβ II receptor expression in preneoplastic lesions. Foci with this reduced TGFβ II receptor phenotype may become insensitive to the TGFβ mitoinhibitory signals allowing them to clonally expand forming preneoplastic foci. Our finding of reduced expression of TGFβ II receptor in preneoplastic foci by immunohistochemistry suggests one of the follow events is occurring: (1) transcription of receptor mRNA is reduced (2) the receptor is truncated and not detectable by our antibody or (3) there is defective processing of the receptor with mislocalization. Experiments that could be performed to address decreased mRNA are Northern Blot analysis (dependent on foci area) or *in situ* hybridization with examination for changes inside and outside foci. To address functionality or localization, monoclonal antibodies that are specific to the external ligand-binding domain, as well the internal kinase domains, are available and could be used. Likewise, *in situ* hybridization to those functional domains with the appropriate probes could also be employed. Alternatively, immunohistochemical analysis of downstream proteins induced by TGFβ II receptor binding such as CDK inhibitors p15, p21, and p27 could be analyzed inside and outside of foci. Laser capture microdissection could also be used to excise foci and DNA from microdissected cells could be amplified for functional exons by polymerase chain reaction and analyzed for alterations by DNA sequencing.

We demonstrated immunohistochemically, that expression of TGFβ is increased in some preneoplastic livers lesions. Neoplastic cells can secrete larger amounts of TGFβ than their normal counterparts (Blobe *et al.*, 2000) and humans with hepatocellular carcinoma have increased levels in their plasma (Ito *et al.*, 1991). If preneoplastic foci with increased expression of TGFβ are secretory, as could be the case for some foci in our study, they might induce apoptosis and arrest growth of adjacent normal hepatic parenchyma in a paracrine fashion providing an additional growth advantage to the clonally expanding TGFβ-resistant hepatocytes. Therefore, future studies could include measuring circulating levels of TGFβ to determine if there is increase in plasma as seen in some human hepatocellular carcinoma patients (Ito *et al.*, 1991). Lack of increase plasma levels, however, would not rule out the presence of a localized paracrine response.

Although, the majority of receptor-related disruptions in TGFβ signaling pathways are due to alterations of type II TGFβ receptor, expression of the TGFβ I receptor is necessary for TGFβ to exert its growth inhibitory effect. Therefore, alterations if TGFB I receptor should also be evaluated.

In conclusion, results from this study has direct bearing on the TEF concept for risk assessment of PCB mixtures. Antagonism between planar and non-planar PCBs in environmental mixtures would result in less toxic effects than expected based on calculated TEFs. Risk assessment calculations based on exposure to such mixtures would be overestimated. Planar PCBs like PCB 153, which are not assigned official TEFs

because they are assumed to act primarily via other routes than the Ah receptor-mediated pathway, can reduce the promotional effects caused by planar PCBs and, probably, other TCDD-like substances. Since PCB153 and other non-planar PCBs are abundant in environmental samples (Fischer *et al.*, 1998; Hansen, 1998; van den Berg *et al.*, 1998) and are the most prominent congeners in human tissues (van der Kolk *et al.*, 1992), there is a need for special consideration of these substances in risk assessment of PCB mixtures. Additionally, results from these studies suggest that planar PCBs may cause significant alterations in TGFβ signaling pathways during the promotional stage of rodent hepatocarcinogenesis. Although additional examination is required to confirm this suggested mechanism, answering these questions will further improve our understanding of interactions of PCB mixtures and ultimately improve human risk assessment regarding their exposure.

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