DISSERTATION

POLYCHLORINATED BIPHENYLS AND ARSENIC IN HEPATOCARCINOGENESIS

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JON TODD PAINTER ENTITLED "POLYCHLORINATED BIPHENYLS AND ARSENIC IN HEPATOCARCINOGENESIS" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

POLYCHLORINATED BIPHENYLS AND ARSENIC IN HEPATOCARCINOGENESIS

Polyhalogenated aromatic hydrocarbons such as polychlorinated biphenyls (PCBs) and dioxins are worldwide environmental pollutants and potential human carcinogens. These chemicals may be found together and with other chemicals such as arsenic (As), a known human carcinogen, in hazardous waste sites. The carcinogenic mechanisms of these substances remain unclear and there have been few studies examining such mixtures. The aim of our studies was to examine these compounds as individual chemicals and in mixtures, in order to better understand their roles in carcinogenesis.

We measured the activity of cytochrome P-450 1A1 (CYP1A1), a metabolizing enzyme induced in the liver after exposure to TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin), PeCDF (2,3,4,7,8-pentachlorodibenzofuran), the coplanar PCB 126 (3,3',4,4',5-pentachlorobiphenyl) and the noncoplanar PCB 153 (2,2',4,4',5,5'-hexachlorobiphenyl), as individual chemicals and in mixtures. We found supporting evidence for the additive toxic equivalency factor (TEF) approach to risk assessment for dioxin-like, aryl hydrocarbon receptor (AhR)-binding chemicals but found a greater-than-additive increase in CYP1A1 activity in mixtures of the AhR-binding PCB 126 and the non-binding PCB 153. We found evidence that increases in 4-

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hydroxy estrogen catechol metabolites induced by the dioxin-like chemicals may help explain the higher sensitivity of female rats to hepatocellular neoplasia.

The second study explored the interactions between PCB 126 and As in hepatocarcinogenesis using a 24-week initiation-promotion-progression model in the rat. We evaluated promotion of preneoplastic foci using the marker glutathione-S-transferase placental form (GST-P) and progression using the markers transforming growth factor-alpha (TGFα) and transforming growth factor-beta receptor II (TGFβII-r). Exposure to PCB 126 resulted in increased relative area and number of GST-P+ and TGFα+ foci. Arsenic as a single agent had no significant effect on foci size or number compared to controls. Arsenic combined with PCB 126 resulted in decreased relative area of GST-P+ and TGFα+ hepatic foci compared with PCB 126 alone. This effect was more prominent earlier in the study at 16 weeks than later at 24 weeks, suggesting a time-dependent effect of arsenic as an anti-promoting and/or progressing agent. A subset of large preneoplastic foci was GST-P+, TGFα+, and TGFβII-r negative, suggesting the altered hepatocytes in these foci possess a more advanced phenotype.

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DEDICATION

This dissertation is dedicated to my mother Betty P. Ammon for her unwavering support and assistance through all my academic years, to my sister Kim Painter who never ceases to raise my spirits with stories of her adventures, and to my fiancé Kim Kiker who has been the source of my recent inspiration.

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CHAPTER 1

GENERAL INTRODUCTION

I. Basic Carcinogenesis

Cancer is the leading cause of death in the United States, recently surpassing cardiovascular disease, and it accounts for 23% of all mortality (American Cancer Society 2005; Jemal *et al.* 2004). It affects everyone, from newborns to the elderly, almost all species of animals, and even plants. Any cell type capable of division can become neoplastic, and malignancies can arise in any organ of the body. Cancer research has expanded rapidly over the last several decades, and we have made great strides in diagnosis, treatment, and determining pathogenesis.

While recent advances in the genetics of cancer are elucidating our understanding of the basic mechanisms involved in carcinogenesis, this understanding has confirmed the wide diversity of the underlying nature of the disease. For this reason, it is unlikely that there will ever be a single easy "cure" for cancer. Once diagnosed, it must be treated based on the type and biologic behavior of the tumor, while targeting precise treatment to the specific characteristics of the patient. The treatment of disseminated breast cancer in an elderly woman, leukemia in a child, a colonic polyp, and Stage III colon cancer must be different and will likely have very different outcomes. For these reasons, we

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ultimately must give as much attention to the prevention of cancer as to the diagnosis and treatment of this important disease.

Fundamentally, cancer is a disorder of cell growth and behavior (Kumar *et al.* 2005), manifested in profound changes in the host's own cells and tissues. The types of cancer are as varied as the causes of cancer. We now know of countless causes of cancer, ranging from inherited mutations in the genome to excessive ultraviolet radiation in sunlight, to any of the hundreds of chemicals found in tobacco smoke. It can be caused by numerous infectious agents, from viruses to bacteria to nematodes and trematodes. Both healing wounds and livers of alcoholics can become neoplastic. While cancer incidence in general increases with age, there are numerous types that are prevalent in children.

Despite this variability in the epidemiology and outward manifestations of cancer, there are numerous fundamental characteristics shared by all neoplasms, most of which occur at the molecular level. As neoplasms progress from benign to malignant, specific genotypic and phenotypic changes can be found. Following is a brief accounting of some of the important factors and traits.

The Cell Cycle

The majority of the cells in the body exist in a quiescent state, performing their specific functions but not dividing to form new cells. Under certain physiologic conditions, cells replicate their DNA and divide, undergoing a sequence of steps that are part of the cell cycle. There exist numerous checks and balances in the normal state to prevent uninhibited cell division and these defenses are inactive or ineffective in the neoplastic state.

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The cell cycle is divided into four distinct stages: G_1, S, G_2, and M, with quiescent cells considered to be in G_0. In between each of the four stages are transitions or checkpoints, with those at G_1/S and G_2/M considered the most important (Schafer 1998). Once cells reach the S phase, they can continue to divide independent of growth factors (Kumar *et al.* 2005). Each transition is facilitated by two groups of proteins known as cyclins and cyclin-dependent kinases (CDKs). CDKs are constitutively present but must be activated by binding with cyclins, only formed at certain times during the process. CDKs drive the cell cycle by phosphorylating target proteins which allow for entry into the subsequent stage of the cycle (Ekholm and Reed 2000). These activator proteins are regulated by CDK-inhibitors (Kumar *et al.* 2005).

The retinoblastoma protein (pRb) acts as the first main checkpoint in the cell cycle, regulating movement through the G_1/S transition of the cell cycle. In its active, hypophosphorylated state, pRb sequesters and blocks the action of the E2F transcription factors that control the genes responsible for moving cells from the G_1 to the S phase (Weinberg 1995). When pRb is phosphorylated via normal cell proliferation signals or its pathway is disrupted in some way, it is inactivated and E2F is released to allow progression into the S phase of the cell cycle.

The protein p53 is referred to as the "guardian of the genome," preventing the division of genetically damaged cells. It is a DNA binding protein that mostly acts to control transcription of several other genes, notably the CDK inhibitor *p21* and *GADD45* (growth arrest and DNA damage). The major functions of p53 are cell-cycle arrest at G_1 and initiation of apoptosis in response to DNA damage (Kumar *et al.* 2005). p53 has been found to play a role in transforming growth factor beta (TGFβ)-induced growth

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arrest, acting through the common Smad2 pathway (Cordenonsi *et al.* 2003) (see TGFβ section below). p53 upregulates the BAX pro-apoptotic protein to cause the death of the cell if the DNA damage cannot be repaired. Furthermore, p53 has been found to increase levels of thrombospondin-1, a potent angiogenesis inhibitor, therefore restricting tumor growth and metastasis by restricting blood supply (Dameron *et al.* 1994).

Hallmarks of Malignant Transformation

There are six classically recognized alterations necessary for malignant transformation and all six appear to be shared by all types of cancer. These alterations do not necessarily occur in a particular order and certain mutations may allow for more than one alteration to occur at a given point in the process. These "hallmarks of cancer" are self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg 2000). In most cases, these represent the hijacking and manipulation of normal cell and tissue processes resulting in unregulated growth.

Normal cells require growth signals in order to move from the quiescent G_0 phase into the active stages of the cell cycle. Neoplastic cells have evaded this requirement, acquiring self-sufficiency in growth signals. This usually occurs via the transformation of protooncogenes, physiologic regulators of cell proliferation and differentiation, into oncogenes which promote autogenous cell growth in the absence of normal mitogenic signals (Kumar *et al.* 2005). Oncogenes act by mimicking normal growth signaling by altering one of three mechanisms: extracellular growth signals (such as transforming growth factor alpha, TGFα), transcellular transducers of those signals (such as *RAS*), or intracellular pathways that convert signals into a growth response (Hanahan and

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Weinberg 2000). Many cancer cells acquire autocrine stimulation, a positive feedback loop whereby the cells themselves produce growth factors such as TGFα to which they are responsive (Kaufmann *et al.* 1997).

Under physiologic conditions, normal cells will cease replication and division when under the influence of growth-inhibitory signals, such as TGFβ. Cells may be forced back into the quiescent G_0 state or they may be stimulated to terminally differentiate and forever lose their ability to enter the cell cycle (Hanahan and Weinberg 2000). Most antiproliferative signals act via the Rb pathway, directing pRb to remain in the hypophosphorylated state, binding the E2F transcription factor. Neoplastic cells are refractory to these antiproliferative signals, either through downregulation of receptors or the formation of dysfunctional receptors. In order to evade terminal differentiation, neoplastic cells may upregulate an oncogene, such as *c-Myc*, to increase production of the growth-stimulatory transcription factor c-Myc (Foley and Eisenman 1999).

In order for a neoplasm to grow, it must have a way to increase cell proliferation while decreasing cell loss. The ability to evade apoptosis allows the neoplastic cell population to avoid loss via programmed cell death. To accomplish this, any one of the various steps along the apoptotic pathway must be disrupted. Apoptotic sensors such as the tumor suppressor p53 or Bax protein, or apoptotic effectors such as the caspases, may be affected (Hanahan and Weinberg 2000).

Once a neoplastic cell population has achieved the first three capabilities described above, it must still evade the intrinsic intracellular limiting process known as senescence. The DNA of all cells contains telomeres, genetic repeated sequences on the end of chromosomes. These telomeres shorten by a specific amount with each cell cycle

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and, after a certain number of divisions, the cell can no longer divide. This process works independently from the cell-to-cell signaling pathways described above (Hanahan and Weinberg 2000). Neoplastic cells escape senescence by the overexpression of the enzyme telomerase (Bryan and Cech 1999), which restores telomeres to their original length. Telomerase is normally only expressed in cells during development and in certain populations of stem cells that must maintain replicative potential.

All tissues, including neoplastic ones, require a blood supply in order to receive oxygen and nutrients. Without this blood supply, a neoplastic mass is unable to grow beyond a size where simple diffusion is sufficient (approximately 2mm in diameter) (Kumar *et al.* 2005). Therefore, neoplastic cells must stimulate the growth of blood vessels to support the growing tumor. Tumor angiogenesis is accomplished by the secretion of factors that result in the formation of new capillaries, along with the inhibition of angiogenic inhibitors. Vascular endothelial growth factor (VEGF) is representative of the pro-angiogenic factors, while thrombospondin-1 typifies the inhibitors (Hanahan and Weinberg 2000). Once the balance has been tipped toward angiogenesis, more rapid neoplastic growth can occur.

The presence of tumor blood vessels also allows for the spread of the neoplastic cells to distant sites, a process known as metastasis. This, along with local tumor invasion, is the most concrete evidence of malignant neoplasia. Most human cancer deaths are not caused by the primary tumor, but by distant metastases in vital organs (Sporn 1996). The two types of proteins that anchor normal cells and early neoplastic cells are cadherins (cell-to-cell) and integrins (cell-to-matrix) (Bogenrieder and Herlyn 2003). Both of these must be altered for metastasis to occur. Once neoplastic cells are

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released from the primary tumor, they must be able to colonize and invade new tissues. Colonization is achieved through the upregulation of tissue-specific integrins on the neoplastic cell surface. Increased production of extracellular proteases such as matrix metalloproteinase (MMP) and the decreased production of protease inhibitors break down surrounding tissue structure, which allows malignant cells to invade more readily (Chambers and Matrisian 1997). Certain MMPs produced by neoplastic cells are able to activate latent transforming growth factor-beta (TGFβ), resulting in apoptosis of surrounding normal parenchyma and more rapid spread of the malignancy (Derynck *et al.* 2001).

Hepatocellular Neoplasia

Hepatocellular carcinoma comprises 90% of all human primary liver cancers. Known etiologies include hepatitis B and C viruses, aflatoxin exposure, and causes of cirrhosis such as chronic alcoholism. The evidence for PCBs as a cause of human liver cancer is suggestive, but not certain, in the cases of hepatocellular and gall bladder carcinomas and currently PCBs are classified as probable (Group 2A) human carcinogens (Charlier *et al.* 2004; Golden *et al.* 2003; Kimbrough 1995; Siemiatycki *et al.* 2004). While some studies have shown an association between arsenic exposure and the development of human hepatocellular carcinoma (Centeno *et al.* 2002; Chen *et al.* 1986; Chiu *et al.* 2004), others have not (Guo 2003), so the evidence is equivocal. Although the incidence of hepatocellular carcinoma in North America is 2-4 cases per one hundred thousand in population, in southeast Asia the incidence is as high as 150 per one hundred thousand. Oval cells, the bipotential liver stem cells capable of differentiation into both hepatocytes and biliary epithelium, are believed to give rise to hepatocellular carcinoma

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(Dumble *et al.* 2002). Benign hepatocellular adenomas also occur, often as a consequence of oral contraceptive steroid use (MacSween *et al.* 2002).

Although hepatocytes are the main cell type in the liver, there are other cells in this organ, all of which can become neoplastic. Both benign and malignant tumors of the biliary epithelia occur, and cholangiocarcinoma is associated with liver fluke infestation but not cirrhosis (MacSween *et al.* 2002). Angiosarcoma, or hemangiosarcoma, is a malignant neoplasm of the blood vessel endothelium and may be seen in the liver as a consequence of vinyl chloride or arsenic (As) exposure (Popper *et al.* 1978). Other uncommon primary liver tumors include hepatoblastoma, hemangioma, lymphangioma, and lymphoma (MacSween *et al.* 2002).

In addition, the liver is one of the main sites of metastatic cancer, as a large proportion of visceral blood passes through this organ. Therefore, any neoplastic cells in the blood may lodge in the liver sinusoids and, if their receptor profile is appropriate, can seed out and form a metastasis. Lung, mammary gland, and gastrointestinal tract are the most common sites of primary tumors that metastasize to the liver (MacSween *et al.* 2002). Metastatic cancer in the liver is more common than any of the primary liver tumors.

While, as noted above, human liver cancer incidence varies widely throughout the world, the liver is commonly used as a model for the general study of carcinogenesis because it is a target for many xenobiotic agents due to its major role in metabolism and detoxification.

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Oxidative Injury

Cells generate energy via mitochondrial respiration, producing free radical reactive oxygen species (ROS) as byproducts (Kumar *et al.* 2005). Free radicals are electrophilic molecules containing one or more unpaired electrons in their outer orbits (Klaassen 2001). Under normal conditions, cellular defenses such as antioxidants remove ROS and prevent injury. Oxidative stress occurs when these defenses are inadequate or overwhelmed. ROS are produced in the mitochondria and spread throughout the cell, damaging cellular nucleic acids (single-stranded breaks in DNA), lipids (cell membrane peroxidation), and proteins (fragmentation and cross-linkages). Oxidative stress and injury most often ends in the death of the cell, but it can also lead to non-lethal changes in DNA that may shift the affected cell to a neoplastic phenotype (Dreher and Junod 1996; Janssen *et al.* 1993).

The primary ROS which may damage the cell are superoxide anion ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical ($OH^{\bullet}$) which is produced from H_2O_2 through the Fenton reaction (Klaassen 2001) (see Figure 1.1). $OH^{\bullet}$ is believed to be the primary ROS involved in direct attack on DNA (Kitchin and Ahmad 2003). Redox cycling occurs when existing free radicals continuously react with oxygen, producing multiple superoxide anions. Several cellular enzymes are important in the removal of ROS. These include catalase and glutathione peroxidase, which convert H_2O_2 to water, and superoxide dismutases, which convert $O_2^{\bullet}$ to H_2O_2 (Haschek *et al.* 2002; Klaassen 2001; Kumar *et al.* 2005). The Phase II detoxification enzyme family glutathione-S-transferase (GST) enzymes, including glutathione peroxidase and glutathione reductase, are particularly effective at eliminating electrophilic ROS by

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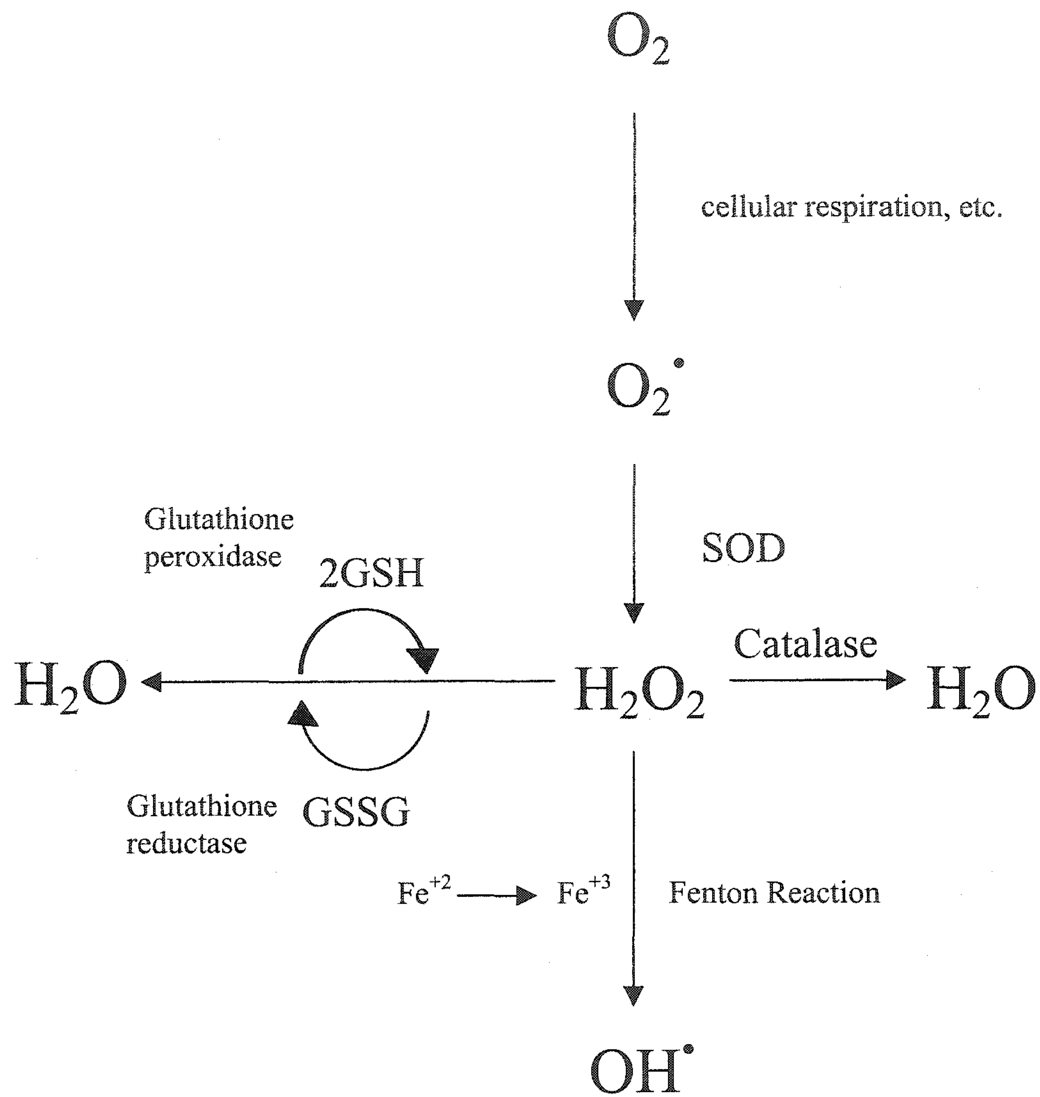

Figure 1.1. Pathways of oxidant injury & enzymatic defenses. O_2 = oxygen. $O_2^{\bullet}$ = superoxide anion. H_2O_2 = hydrogen peroxide. H_2O = water. $OH^{\bullet}$ = hydroxyl radical. Fe = iron. SOD = superoxide dismutase. GSH = reduced glutathione. GSSG = oxidized glutathione.

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glutathione conjugation (Strange *et al.* 2001). Although the primary purpose of these enzymes is ROS removal, under certain conditions, increased amounts of ROS or more toxic ROS can be generated. For instance, when a particular enzyme such as catalase is inadequate, H_2O_2 will persist and damage the cell. If the downstream GST enzymes are also inadequate, H_2O_2 can be converted to the more toxic $OH^{\bullet}$. When $O_2^{\bullet}$ and H_2O_2 are present together in the presence of cellular transition metals such as iron, $OH^{\bullet}$ may be produced via the Fenton reaction (Kitchin and Ahmad 2003). Due to its extremely short half-life, $OH^{\bullet}$ reacts with tissues before detoxification is possible.

One of the products of DNA damage by ROS is the formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) that can be detected in cells and tissues and serves as a useful marker of oxidative stress. 8-OHdG has been shown to lead to base mispairing mutations when the affected DNA replicates (Clayson *et al.* 1994). Increased levels of 8-OHdG have been found after exposure of laboratory animals to numerous compounds, notably arsenic (An *et al.* 2004; Hei and Filipic 2004; Kitchin and Ahmad 2003) and diethylnitrosamine (Nakae *et al.* 1997), implicating oxidative stress as a potential mode of carcinogenesis for these chemicals. The Phase I xenobiotic biotransformation enzymes in the cytochrome P-450 monooxygenase family (CYPs) are known to produce ROS as a consequence of their detoxification processes. This is one mechanism by which CYP-inducing polycyclic aromatic hydrocarbon chemicals such as dioxin or PCBs may act as carcinogens (Nebert *et al.* 2004). Certain PCBs have been shown to increase the DNA binding activity of the oxidative stress-responsive transcription factors AP-1 and NFκB (Tharappel *et al.* 2002).

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Nitric oxide (NO) is a chemical mediator produced by many cell types. NO can act as a free radical and can be converted to reactive peroxynitrite anion as well as other reactive nitrogen metabolites (Klaassen 2001; Kumar *et al.* 2005) that can damage the cell. NO acts to cleave cellular DNA by endonuclease activation and it can also promote apoptosis by mediating accumulation of the tumor suppressor p53 (Brüne *et al.* 1995).

Arsenic has been shown to produce ROS during its *in vivo* metabolism (An *et al.* 2004; Barchowsky *et al.* 1999; Huang *et al.* 2004). ROS induced by the exposure of mice with dimethylarsinic acid (DMA), a metabolite of As, have been shown to participate in lung and skin carcinogenesis (An *et al.* 2004). Arsenite (AsIII) appears to increase $OH^{\bullet}$ production, and both animal models and human samples show increased lipid peroxidation secondary to As exposure (Hei and Filipic 2004). Samples of As-related skin cancer and As-exposed normal skin demonstrate increased levels of 8-oxodG when compared to samples of As-unrelated skin cancer and normal skin (An *et al.* 2004; Matsui *et al.* 1999).

Transforming Growth Factor-α

Cell proliferation is an important mechanism in chemical carcinogenesis (Williams 1997). A variety of growth factors can stimulate or inhibit passage through the cell cycle. Acquisition of autocrine growth involving the growth factor TGFα characterizes altered hepatocytes during the early stages of carcinogenesis in rats and humans (Grisham 1997). TGFα binds to the epidermal growth factor (EGF) receptor and is a potent growth factor for hepatocytes (Burr *et al.* 1996) and microvascular endothelial cells. Although hepatic expression of TGFα is low in normal liver, expression increases from preneoplastic foci to adenomas to carcinomas, suggesting that it is important in

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neoplastic progression (Burr *et al.* 1996; Dragan *et al.* 1995; Grisham 1997). Expression of TGFα correlates well with areas of progression in chemical carcinogenesis protocols and these areas are most likely to progress to tumors (Burr *et al.* 1996; Dragan *et al.* 1995; Grisham 1997; Steinmetz and Klaunig 1996). Preneoplastic hepatic foci in a rat study that expressed TGFα grew more rapidly than TGFα negative foci, and almost all the tumors studied were TGFα positive (Hufnagl *et al.* 2001).

TGFα also has been reported to accelerate hepatocarcinogenesis by reducing apoptosis during transformation (Christensen *et al.* 1999; Santoni-Rugiu *et al.* 1998). In colon cancer cells, TGFα has been shown to regulate cell adhesion function via the EGF receptor-mediated expression of integrin alpha(2) (Sawhney *et al.* 2004). TGFα mRNA has been used as a prognostic indicator in hepatocellular carcinoma, with low levels indicating a more differentiated tumor and better prognosis (Daveau *et al.* 2003). Experimental anti-cancer agents that inhibit hepatocarcinogenesis do so in part through the suppression of TGFα expression and cell proliferation (Kagawa *et al.* 2004).

Transforming Growth Factor-β

TGFβ normally inhibits epithelial cell proliferation, at least in part by up-regulation of the CDK-inhibitors p21 and p27 (Kumar *et al.* 2005; Park *et al.* 2001), and promotes differentiation. TGFβ also stimulates wound healing via stimulation of fibroblast proliferation (Govinden and Bhoola 2003), and it inhibits lymphopoiesis and myelopoiesis. TGFβ acts to prevent the phosphorylation that inactivates pRb (Hanahan and Weinberg 2000) and induces hepatocyte and oval cell apoptosis (Mills *et al.* 1995; Park *et al.* 2001). The full transcriptional activation of p21 by TGFβ requires p53, and p53-deficient cells have an impaired response to TGFβ (Cordenonsi *et al.* 2003).

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Experimental data provide *in vivo* evidence of the tumor suppressor activity of TGFβ during chemical hepatocarcinogenesis (Kanzler *et al.* 2001).

Smads are a small family of structurally related proteins that function in TGFβ receptor signaling pathways (Derynck *et al.* 2001). Smad2 and Smad3 are phosphorylated by TGFβ receptor I (TGFβI-r, see below), form heterodimeric complexes with Smad4, and translocate to the nucleus to activate transcription of target genes (Derynck *et al.* 2001). While most Smads are involved in transcription, certain members of the family (Smads 6 and 7) act to inhibit transcription, providing a negative feedback loop (Derynck *et al.* 2001). p53 can physically interact *in vivo* with Smad2 in a TGFβ-dependent fashion (Cordenonsi *et al.* 2003).

TGFβ1 is the most abundant and important form in the liver and is synthesized by Ito cells and hepatocytes (Oberhammer *et al.* 1996; Rossmanith and Schulte-Hermann 2001; Roth *et al.* 1997). The cytokine is released in a protein-bound, latent form, and the complex cannot bind with its specific receptors until it is bound to the mannose-6-phosphatase/insulin-like growth factor II (M6P/IGFII) receptor on the hepatocyte and is activated by plasmin cleavage (Grisham 1997; Roth *et al.* 1997). Once activated, TGFβ1 binds to three TGFβ cell surface receptor proteins (types I, II, and III) with high affinity. TGFβ1 binds to the type II receptor which then recruits type I receptor into the complex and causes phosphorylation and cytoplasmic kinase activation of the type I receptor. The type III receptor is proposed to be involved with regulating access to I and II. The expression of TGFβ1 is closely associated with the expression level of TGFβII-r (Park *et al.* 2001). TGFβ1 has profound effects on the expression of genes coding for G_1 cyclins and associated kinases. This prevents the formation of active cyclin-cyclin dependent

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kinase complexes, inhibiting the cell cycle and regulating cell proliferation (Hemming *et al.* 1993; Mills *et al.* 1995).

TGFβ1 has been shown to be important in neoplastic cell proliferation. Impairment of negative growth regulation can occur through reduced expression of TGFβI or II receptors and/or M6P/IGFII receptor, both of which characterize preneoplastic hepatocytes in both rats and humans (Mills *et al.* 1998; Rossmanith and Schulte-Hermann 2001). In combination, these changes can markedly impair inhibitory action of TGFβ1 on hepatocarcinoma cells (Grisham 1997). This lack of response to TGFβ inhibition can allow for selective clonal expansion of preneoplastic and neoplastic cells. A number of human tumor cells are reported to be resistant to the inhibitory effects of TGFβ, including cancers of the liver, colon, breast, bladder, stomach, thyroid, kidney, prostate, and lung (Matoba *et al.* 1998). This has been associated with reduced expression and/or mutations of either type I or II receptors (Matoba *et al.* 1998).

Genetically altered mice heterozygous for the deletion of the TGFβII-r gene have increased susceptibility to hepatocellular carcinogenesis (Im *et al.* 2001). In contrast to wild-type mice, which have two normal *TGFβII-r* alleles, heterozygotes require only one "hit" to disrupt the TGFβ tumor suppression pathway. This is similar to human childhood retinoblastoma or Li-Fraumeni syndrome, in which the inheritance of one mutant allele in the *Rb* or *p53* genes (respectively) predisposes those affected to certain types of malignancies (Kumar *et al.* 2005).

There is experimental evidence that expression of TGFβ1 and TGFβII-r is altered during the promotion stage of hepatocarcinogenesis in the rat, contributing to the development and progression of preneoplastic lesions (Park *et al.* 2001). Overexpression

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of the cell cycle protein cyclin D1 may also play a role in the resistance of some hepatocellular carcinoma cells to TGFβ1 (Jong *et al.* 2002). Furthermore, the surrounding normal hepatic parenchyma remains susceptible to TGFβ secreted by preneoplastic cells, so surrounding cells will likely undergo apoptosis, allowing for more rapid expansion of the resistant altered cells (Park *et al.* 2001).

The known liver tumor promoter phenobarbital inhibits TGFβ-induced apoptosis (Christensen *et al.* 1998) via the downregulation of the TGFβ receptors I, II, and III (Reisenbichler *et al.* 1994) as well as the M6P/IGFII receptor (Jirtle *et al.* 1994). Apoptosis-inducing substances such as interferon-α have been found to act via increased production of TGFβ1 (Alvarez *et al.* 2004).

II. Chemical Carcinogenesis

Since the 18th century and the experiments of Sir Percival Pott showing scrotal skin cancer in chimney sweeps as a result of chronic exposure to soot (Shindell and Goldberg 1981), we have known of a link between chemical exposure and carcinogenesis. Today, hundreds of chemicals have been shown to cause cancer in animals, with a significant number of those also shown to have carcinogenic effects in humans. Implicated chemicals may act on their own as direct carcinogens or may be metabolized in the body from a procarcinogenic form to an active chemical. These compounds often interact with other environmental agents such as radiation or tumorigenic viruses to exert their ultimate effects. Furthermore, only rarely is an individual exposed to a single chemical. Since chemicals most often occur in complex

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mixtures, exposure is often to multiple chemicals potentially acting as multiple types of carcinogens.

Carcinogenesis as a multistep process

There is a multistage nature of carcinogenesis, with the process broken down into three, somewhat arbitrary, stages. While most if not all carcinogenic chemicals follow this general pattern, the number of steps spent in each stage is quite variable and clear distinctions between stages are difficult to elucidate. Different chemicals can act in any of the stages. Many chemicals are thought to act in either the first (initiation) or second (promotion) stages, with a combination and continuation of exposures contributing to the third (progression) stage. Carcinogenic agents contributing to all three stages of the process are referred to as "complete carcinogens." Genetic mutations are important in the initiation and progression stages, whereas cell proliferation is most important in the promotion stage.

Initiation. The first stage of chemical carcinogenesis is known as initiation. Initiating agents cause direct damage to DNA, resulting in a heritable, irreversible change. Initiating agents are diverse and cause their damage through a variety of mechanisms. Some act directly on the cellular DNA, while others – the majority – enter the body or cell as procarcinogens and are then activated by the metabolizing enzymes of the cell. These are also known as indirect carcinogens. The metabolizing enzymes are often members of the cytochrome P-450 family of mono-oxygenases. These are countered by detoxifying enzymes such as glutathione-S-transferase, so the carcinogenic potential of indirect initiating agents also depends on a balance within the cell's own metabolic processes. Most initiators act as highly reactive electrophiles, having electron-

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deficient atoms that react with nucleophilic (electron-rich) sites in the cell, such as DNA (Kumar *et al.* 2005).

The number of cells affected and amount of genetic damage is dependent on the dose of the chemical exposure, with lower dose resulting in less damage. Chemicals vary widely in their ability to induce DNA damage, and minimal exposure to certain chemicals might be considerably more damaging than a large exposure to a less potent substance. In theory, any exposure may suffice to cause a mutation that will ultimately lead to a neoplasm. It is important to note that the vast majority of chemically-induced mutations will result in either DNA repair or, if the mutation cannot be repaired, death of the cell via apoptosis. These functions are performed by cellular controls such as the p53 protein, as mentioned previously. Thus, even if a mutation results in an initiated cell, most will not survive these innate cellular defenses. Organisms and their cells are constantly defending against DNA damage, both from endogenous and exogenous agents. Only when the cell's reparative capabilities are overwhelmed or inadequate, and the damage is not so severe as to kill the cell outright, are the conditions met for that initiated cell to survive.

An initiated cell that goes no further will merely live out its existence, eventually die, and be removed from its tissue by normal organ maintenance processes. However, should an initiated cell enter the cell cycle and divide, producing two viable cells with the same DNA mutation, that mutation now becomes "fixed." With further proliferation, the resultant group of cells, each containing the same mutation, is known as a clone. The clonality of neoplastic cells is a hallmark of carcinogenesis and this clonality will persist

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to some degree through all the stages of carcinogenesis and is often present as a diagnostic marker of overt neoplasms.

Promotion. Initiated cells would go no further and would eventually be eliminated by the body were it not for the exposure to a promoter. Chemical or other agents of promotion act by enhancing the proliferation of initiated cells, often resulting in an identifiable lesion called a "preneoplastic focus." Unlike initiation, there is no requirement for damage to DNA, and this stage of the process is reversible. In fact, most clonal proliferations and preneoplastic foci at this stage do regress, although some may go on to benign or malignant neoplasms (Grisham 1997). Prolonged exposure to the promoting agent is generally required. If the promoter is removed before cellular growth autonomy is achieved, cell proliferation will cease and, eventually, the preneoplastic foci would be eliminated. Promoters also may act by decreasing apoptosis of initiated cells. Promotion, therefore, is a balance between cellular proliferation and death.

Altered cells in these clonally expanded foci have an adaptive growth advantage over surrounding normal cells. While the peripheral normal cells are still under the control of physiologic constraints on growth, such as the lack of production of growth factors (e.g. TGFα) and responsiveness to growth inhibitors (e.g. TGFβ), the preneoplastic cells no longer respond to one or both of these restrictions. Furthermore, the altered cells may still produce growth restrictive substances such as TGFβ, but may not respond to them, leading to disruption in feedback control and further TGFβ production. Since the surrounding normal cells do respond to TGFβ, they will be induced to undergo apoptosis, allowing a more rapid growth of the abnormal focus. This competitive growth advantage may allow for rapid expansion of preneoplastic cells,

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facilitating their entry into the progression stage (and continued expansion throughout progression).

Progression. The third and final stage of carcinogenesis is progression. At this point, the combination of initiator and promoter has resulted in the clonal expansion to form an identifiable lesion or focus. Continued cellular proliferation allows for the accumulation of additional new alterations in genomic structure. Preneoplastic or neoplastic cells in progression are characterized by increasing karyotypic instability with evolution of phenotypically different populations leading to malignancy (Maronpot 1991; Williams 1997). Widespread irreversible changes in DNA occur, and these are often large-scale alterations such as deletions, aneuploidy, chromosomal breaks, or sister chromatid exchanges. It is during progression that many of the mutations leading to the hallmarks of malignancy occur, such as loss of p53 tumor suppressor control. The end result of this final stage of carcinogenesis will be an overt neoplasm that has achieved autonomous growth and evaded the tumor suppression mechanisms of the body.

III. TCDD/PeCDF

TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) is a polycyclic aromatic hydrocarbon (PAH) organochlorine classified as a definite (Group 1) human carcinogen (Siemiatycki *et al.* 2004), based on limited evidence in humans but extensive evidence in laboratory animals and mechanistic studies. This classification has been somewhat controversial since its original adoption (IARC Working Group 1997), but subsequent research has been supportive (Steenland *et al.* 2004). Dioxins are formed as waste products of combustion processes, especially from municipal incinerators (Tuppurainen

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et al. 2003). TCDD is highly lipophilic and persists in adipose tissue, allowing for bioaccumulation up the food chain. Non-carcinogenic effects of TCDD include immunosuppression, chloracne (severe dermal lesions) and teratogenicity (Kociba and Schwetz 1982; Smialowicz *et al.* 2004). Dioxin is also a known endocrine disruptor, altering multiple endocrine systems in affected animals (Birnbaum and Fenton 2003).

TCDD and dioxin-like agents such as 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) produce their toxic and carcinogenic effects via binding to the intracellular aryl hydrocarbon receptor (AhR) (Safe 1994). The resulting complex dimerizes with the AhR nuclear translocator protein (ARNT), allowing entry into the nucleus. This heterodimer then binds to a responsive DNA domain called the dioxin response element (DRE), enhancing transcription of downstream metabolism genes such as the heme-containing cytochrome P-450 monooxygenase enzymes CYP1A1 and 1A2 (Bunger *et al.* 2003; Safe 1994). These are Phase I xenobiotic biotransformation enzymes, found in highest concentration within hepatic microsomes (smooth endoplasmic reticulum) (Klaassen 2001). CYP1 enzymes are responsible for both activating and detoxifying PAHs via chemical binding of the AhR (Nebert *et al.* 2004). Constitutive binding of the AhR has been experimentally shown to facilitate the formation of liver tumors in mice, acting as a promoter (Moennikes *et al.* 2004).

TCDD is a potent liver carcinogen in rodents and risk assessment of dioxins is based on induction of liver tumors in rats, although there are wide differences in effect between strains (Viluksela *et al.* 2000). There is a marked female-specific hepatocarcinogenicity that is likely due to the influence of 17β-estradiol (E2), a hormone found in increased levels in females. Male rats exposed to TCDD and exogenous E2

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exhibited increased number and volume fraction of preneoplastic liver foci compared to males given TCDD alone (Wyde *et al.* 2002). TCDD has also been found to elicit cutaneous tumors in susceptible mice, both with dermal and oral exposure (Wyde *et al.* 2004).

Some research suggests that TCDD causes activation of the growth factor signal transduction pathway through an AhR-mediated process which, in turn, induces a mitogenic response (Matsumura 1994; Whysner and Williams 1996). Dioxin acts as a promoter, binding the AhR and stimulation cell proliferation. This effect affects the preneoplastic cells of altered hepatic foci but does not stimulate proliferation of normal hepatocytes (Buchmann *et al.* 1994).

To aid in risk assessment for persistent environmental contaminants including the dioxins and polychlorinated biphenyls (PCBs), toxic equivalency factors (TEFs) have used the common initial mechanism of Ah receptor binding. A TEF for a chemical indicates an order of magnitude of the toxicity of a dioxin-like compound expressed as a fractional equivalent of TCDD (Birnbaum 1994; Finley *et al.* 2003; Toyoshiba *et al.* 2004), the most toxic member of the group. This estimation allows for a certain amount of variability and uncertainty in risk assessment (Finley *et al.* 2003). A basic assumption of this classification with regard to mixtures is that the effect of the combined chemicals is equivalent to the added effects of the individual chemicals. This is important in that most of these chemicals are found not as solitary agents but in complex mixtures with both related and unrelated compounds.

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IV. Polychlorinated Biphenyls

Introduction/Epidemiology

Polychlorinated biphenyls (PCBs) are among the most frequently reported chemicals at hazardous waste disposal sites (EPA 2005) and are fifth on the Agency for Toxic Substances and Disease Registry (ATSDR)/Environmental Protection Agency (EPA) Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) Priority List of hazardous substances (ATSDR 2003). PCBs are halogenated aromatic hydrocarbons that have been used widely in industry and are worldwide environmental pollutants.

Historically, PCBs were first used in 1929 and eventually gained widespread use in dielectric fluids for transformers, heat transfer fluids, lubricants, and flame-retardants. Evidence of toxicity was first recognized in the late 1960's and PCBs were banned in the United States by the 1976 Toxic Substances Control Act. PCBs degrade slowly in the environment, are metabolized slowly, and bioaccumulate up the food chain (Kimbrough 1995; Wölfle 1997). Thus, PCBs continue to be important worldwide environmental pollutants (Kimbrough 1995; Wölfle 1997) and they exist as complex mixtures with other polychlorinated chemicals, including dioxins (Vanden Heuvel and Lucier 1993), as well as other classes of chemicals.

Chemical Structure

The biological effects and classification of PCB chlorinated isomers (congeners, 209 in number) are based on their molecular structure (Safe 1994). Depending on the position of their outlying chlorine atoms, congeners assume a coplanar or noncoplanar configuration (see Figure 1.2). Coplanar PCBs such as PCB 126 (3,3',4,4',5-

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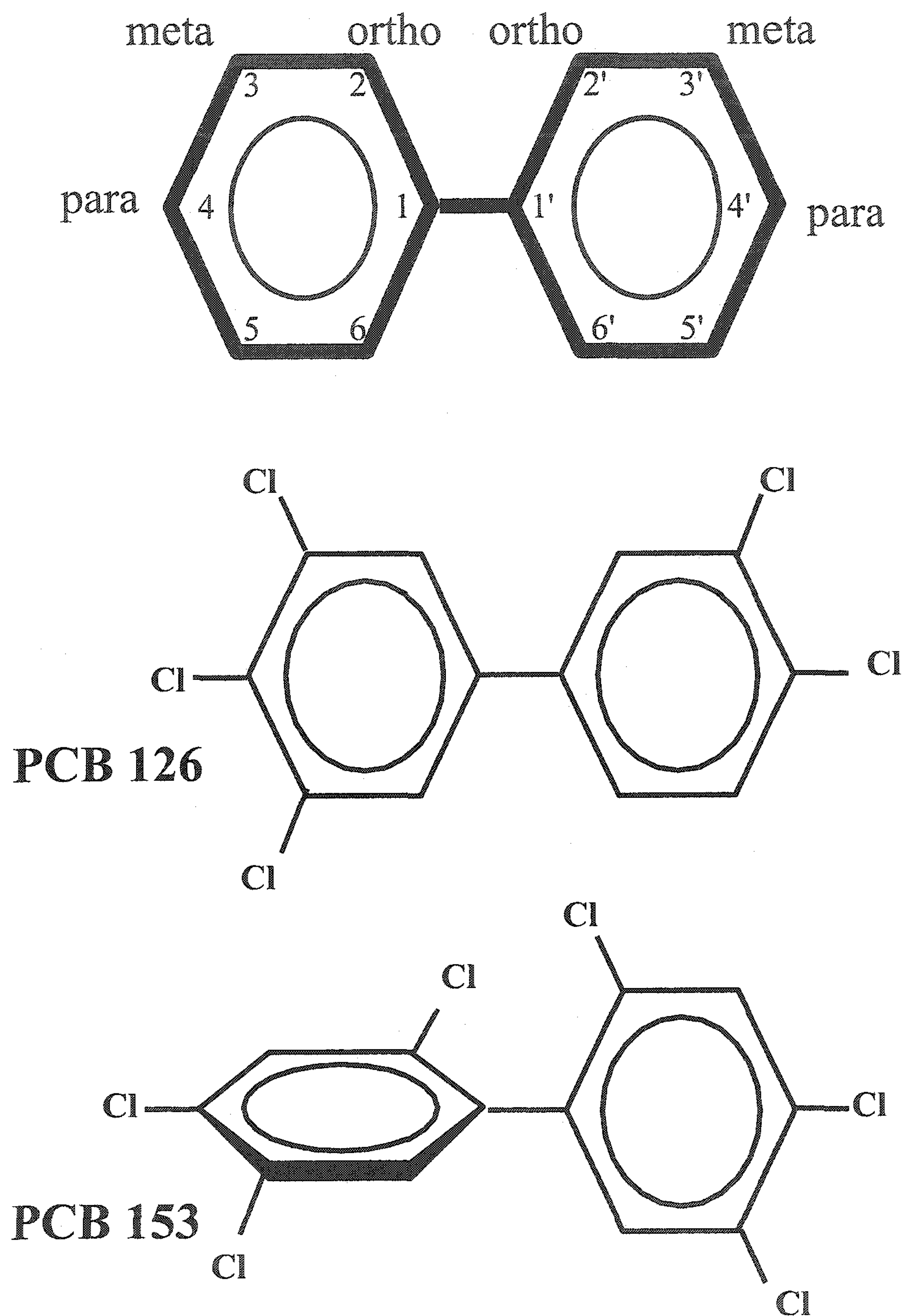

Figure 1.2. Generalized structure of polychlorinated biphenyl (top), the coplanar congener PCB 126 (3,4,5,3',4'-pentachlorobiphenyl, middle), and the non-coplanar congener PCB 153 (2,4,5,2',4',5'-hexachlorobiphenyl, bottom).

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pentachlorobiphenyl) are also known as dioxin-like PCBs. They lack or have one chlorine atom in the ortho position of the biphenyl rings, allowing for the coplanar configuration. This class of PCBs binds the cytosolic Ah receptor and elicits biological effects similar to the more potent TCDD, such as induction of the cytochrome P-450 enzymes 1A1 and 1A2. The noncoplanar PCBs such as PCB 153 (2,2',4,4',5,5'-hexachlorobiphenyl) have two or more chlorines in an ortho position, decreasing planarity between the two phenyl rings due to stearic interactions. Noncoplanar PCBs do not bind the Ah receptor. They elicit phenobarbital-like effects such as induction of cytochrome P-450 2B enzymes and hepatic neoplasia in rodents, by binding the constitutive androstane receptor (CAR) (Muangmoonchai *et al.* 2001). Past studies have found synergistic effects of mixtures of coplanar and noncoplanar PCBs (Bager *et al.* 1995) as well as antagonistic effects (Dean, Jr. *et al.* 2002; Haag-Grönlund *et al.* 1998).

PCBs in the environment exist as mixtures of congeners with different physical and biological characteristics that can interact with each other and with other pollutants, causing toxicity. Despite this, there are relatively few data on such interactive biological effects. Consequently, risk estimation is currently based on toxicologic data on individual PCBs, mainly the dioxin-like congeners (Giesy and Kannan 1998; Safe 1994). The dioxin-like PCB 126 is considered the most toxic congener, having the closest molecular structure to TCDD. While there has been extensive research on dioxins and dioxin-like PCB congeners, the actions of the nondioxin-like PCBs are less well studied, as are potential interactions between dioxin-like and nondioxin-like congeners. It has been stressed that without consideration of the toxicity of the nondioxin-like PCBs, current risk assessment for the PCBs is incomplete (van den Berg *et al.* 1998).

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Biological Effects

PCBs are known to cause adverse effects in both humans and other animals. In humans, these effects include chloracne, body weight loss, impaired immune function, short-term neurological alterations, and diverse hepatic effects such as hepatomegaly and elevated liver enzymes (Birnbaum 1994; Kimbrough 1995; Safe 1994). Analyses of human breast milk, blood, and adipose tissue demonstrate that probably all individuals are exposed to PCBs to some degree (Kimbrough 1995; McFarland and Clarke 1989). Laboratory animal studies demonstrate PCB-induced thymic involution, cachexia, increased mortality, and a variety of hepatic effects such as hepatomegaly and hepatotoxicity (Safe 1990; van Birgelen *et al.* 1996). While some of the effects of the coplanar and noncoplanar PCBs can be different, there can be similar effects, such as carcinogenesis.

The carcinogenic effects of PCBs have been well studied in laboratory animals, where the liver is the target organ. In most animal studies, individual PCB congeners and mixtures are reported to be nongenotoxic (Safe 1994; Silberhorn *et al.* 1990). Studies of coplanar and noncoplanar PCBs have shown that, after initiation with a variety of carcinogens, they can promote hepatocellular neoplasms, including carcinomas (Mayes *et al.* 1998; Moore *et al.* 1994; Silberhorn *et al.* 1990). There is a distinct sex-related difference in liver tumor susceptibility, with females showing a much greater response (Safe 1994). In humans, however, the evidence is less certain. PCBs are classified as probable (Group 2A) human carcinogens, with suggestive evidence of causation with liver, biliary tract, and breast cancers (Charlier *et al.* 2004; Demers *et al.* 2002; Golden *et al.* 2003; Kimbrough 1995; Siemiatycki *et al.* 2004).

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V. Arsenic

Introduction/Epidemiology

The metalloid arsenic (As) is a common groundwater contaminant and is found worldwide, with highest concentrations in SE Asia, notably India, Bangladesh and Taiwan. It is also common in the USA, especially in southwestern states, and As is among the most frequently reported chemicals at hazardous waste disposal sites (EPA 2005). It is first on the ATSDR/EPA CERCLA Priority List of hazardous substances (ATSDR 2003). Arsenic is classified as a definite (Group 1) human carcinogen, with strong evidence of causation with skin and lung cancers (Siemiatycki *et al.* 2004).

Exposure to As occurs in industrial settings via inhalation of vapors at smelters, during fossil fuel combustion, and during pesticide production (Huang *et al.* 2004). Environmental exposures usually occur through ingestion of contaminated food and especially water, where natural or manmade sources of As leach into nearby groundwater. As is well-absorbed through the gastrointestinal tract and, once absorbed, 95% is bound to the globin portion of hemoglobin in red blood cells and widely distributed throughout the body (Graeme and Pollack, Jr. 1998; Haschek *et al.* 2002; Pott *et al.* 2001). Historically, As was used in medicinal preparations for the treatment of leukemia, psoriasis, and asthma (Graeme and Pollack, Jr. 1998). Pharmacological use of As in the USA is now limited to veterinary medicine to treat certain blood-borne parasitic diseases and in humans for the treatment of certain discrete cell malignancies (Haschek *et al.* 2002).

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Chemistry

Arsenic is found both as inorganic and organic forms. Its inorganic forms are the trivalent arsenite, or arsenic trioxide (AsIII), and the pentavalent arsenate (AsV). Contaminated groundwater typically contains AsIII, while contaminated surface water contains AsV (Basu *et al.* 2001). The trivalent arsenite is considered the more toxic of the inorganic forms, in part due to its increased solubility (Aposhian *et al.* 2004; Haschek *et al.* 2002), and reduction of AsV to AsIII is likely an important pathway in As toxicity and carcinogenesis (Kitchin and Ahmad 2003).

Organic arsenic is represented by dimethylarsinic acid (DMA) and methylarsonic acid (MMA), the major metabolites after exposure to inorganic As in both humans and rodents (Kenyon and Hughes 2001). DMA and MMA form from the *in vivo* methylation of As, allowing for eventual elimination from the body. Methylation by the enzymes arsenic methyltransferase and monomethylarsonic acid reductase has traditionally been thought of as a detoxification process, since DMA is less acutely toxic than As (Aposhian *et al.* 2004; Kenyon and Hughes 2001; Pott *et al.* 2001). DMA causes specific single strand breaks in DNA, however, and is possibly the source of much of the carcinogenic effects of As. If this is the case, then DMA might be considered a complete carcinogen (Kenyon and Hughes 2001). Furthermore, reactive DMA and MMA intermediates can be formed during the reaction and may interact with DNA (Vahter 2002). DMA is also found naturally in the environment and is used commercially as an herbicide.

A particularly toxic form of As is the gas arsine, produced in the semiconductor industry when arsenic-containing acidic solutions are exposed to other metals. The binding of arsine to hemoglobin in the erythrocyte results in rapid depletion of the phase

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II detoxification enzyme glutathione and severe intravascular hemolysis (Graeme and Pollack, Jr. 1998; Haschek *et al.* 2002).

Biological Effects

Arsenic acts by binding to sulfhydryl groups on enzymes and other cellular proteins (Osweiler *et al.* 1985). Arsenic is a known human carcinogen and there is ample evidence of causation with regard to skin, liver, lung and urinary bladder cancers (Basu *et al.* 2001; Centeno *et al.* 2002; Chiu *et al.* 2004; Guo *et al.* 2004; Haschek *et al.* 2002; Tchounwou *et al.* 2004; Yoshida *et al.* 2004). In humans, skin has been the main target of As carcinogenicity due to the predilection of As for sulfhydryl-rich keratin (Haschek *et al.* 2002). Arsenic also is a known cause of hepatic angiosarcoma, a malignant neoplasm of the blood vessel endothelium (Popper *et al.* 1978) and low levels of arsenite stimulate endothelial cell proliferation in cell culture (Soucy *et al.* 2003). Humans may be more sensitive to As toxicity than other species because As methylation in humans is less efficient (Huang *et al.* 2004).

Although As is a proven human carcinogen, it has been difficult to demonstrate the carcinogenicity of inorganic As in experimental animal studies. The organic metabolite DMA has been found to cause multi-systemic cancers in numerous laboratory animal studies, notably urinary bladder, kidney, liver and thyroid gland (Kenyon and Hughes 2001). DMA is also a potent clastogen, inducing double strand breaks and cross-linking of cellular DNA (Wanibuchi *et al.* 2004). DMA acts in several experimental protocols in rodents as an initiator and promoter as well (Wang *et al.* 2002; Wanibuchi *et al.* 2004), suggesting a role for oxidative injury (see below). A novel transplacental model in mice has been developed, in which dams are exposed to inorganic arsenite in

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drinking water for a brief period during gestation. Their progeny develop dose-dependent multi-system neoplasms in the liver (hepatocellular carcinoma), lung, adrenal gland, and ovary after reaching adulthood, demonstrating that inorganic As can act as a complete carcinogen in mice (Liu *et al.* 2004; Waalkes *et al.* 2004; Waalkes *et al.* 2003).

The methods of action of As carcinogenesis are unclear. The strongest experimental evidence suggests production of chromosomal abnormalities, induction of oxidative stress, and activation of selected signal transduction pathways (Huang *et al.* 2004; Kitchin and Ahmad 2003). Other potential carcinogenic pathways are enhancement of cell proliferation and suppression of p53 (Kitchin 2001; Pott *et al.* 2001).

Arsenic is thought to act in the promotion and/or progression stages of carcinogenesis (Pott *et al.* 2001). It is a known clastogen, causing large-scale breaks and multilocus deletions in DNA, as well as sister chromatid exchanges. It has not been found to cause point mutations, as one would expect from an initiating agent. Arsenic has been shown to inhibit DNA repair by binding DNA ligase (Lynn *et al.* 1997; Tran *et al.* 2002). Other potential genetic mechanisms for As carcinogenesis include altered DNA methylation, as both hypo- and hypermethylation can lead to neoplastic transformation (Chen *et al.* 2004; Kitchin 2001). It is also thought to act as a "co-carcinogen" whereby its interactions with other environmental carcinogens result in a synergistic response.

Tissues affected most severely by As toxicity are those rich in oxidative enzymes, such as liver, lung, skin, endothelium, gastrointestinal tract (Haschek *et al.* 2002; Osweiler *et al.* 1985). Many of the acute (see below) and chronic effects of As, including cancer, are likely a result of the production of reactive oxygen and reactive nitrogen

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metabolites (Hei and Filipic 2004; Shi *et al.* 2004) such as the dimethylarsinic peroxyl radical (Yamanaka and Okada 1994). Livers from rats exposed to DMA in a medium-term bioassay (Ito test) showed increased preneoplastic lesions with increased CYP content and hydroxyl radical ($OH^{\bullet}$) formation, hallmarks of oxidative injury (Nishikawa *et al.* 2002). Increased amounts of 8-OHdG, a marker of oxidative stress, were found in human tissue samples of As-related skin cancer when compared to As-unrelated skin cancer (An *et al.* 2004). Cultured cells co-exposed to arsenite and dimethyl sulfoxide (DMSO), an oxygen radical scavenger, exhibited reduced mutagenic activity, suggesting the involvement of ROS in the mutagenic action of arsenite (Hei *et al.* 1998; Pott *et al.* 2001).

Arsenic appears to modulate gene expression by activating certain signal transduction pathways (Huang *et al.* 2004). Arsenite has been found to stimulate the expression of the proto-oncogenes *c-fos* and *c-jun*, and to increase the activity of the related transcription factor AP-1 (Cavigelli *et al.* 1996). Arsenite and arsenate exposure increase the activity of the transcription factor NFκB, a mitogen that has been implicated in several malignancies (Baldwin, Jr. 1996; Huang *et al.* 2004).

Chronic ingestion of arsenic via contaminated drinking water is the cause of human blackfoot disease (BFD), a peripheral vascular disease endemic to Taiwan (Chiu *et al.* 2004; Lin *et al.* 1998). It is characterized by clinical signs of numbness of extremities, followed by lameness and progressive cutaneous ulceration. These progress to gangrene, requiring amputation of the affected limb. Other non-neoplastic effects of chronic As exposure include hyperkeratosis of the hands and feet, formation of Mee's lines (semilunar white bands on nails), pigmentation of exposed skin, conjunctivitis,

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anorexia, cachexia, diabetes, cirrhosis, and dementia (Centeno *et al.* 2002; Haschek *et al.* 2002; Osweiler *et al.* 1985; Tchounwou *et al.* 2004).

Acute and peracute effects of arsenic intoxication seen after ingestion of large amounts of As include respiratory failure, asystole and death – for this reason, As was a popular assassination poison in the 1800's (Graeme and Pollack, Jr. 1998). Acute to subacute effects of As ingestion include necrohemorrhagic gastroenteritis, resulting in vomiting, diarrhea, and colic. This is most commonly seen in domestic animals, either through ingestion of herbicide-contaminated plant material or inappropriate use of pharmaceutical compounds containing As (Osweiler *et al.* 1985). Acute toxicity in humans is now rarely seen.

Paradoxically, AsIII is also used as a treatment for several cancers, including acute promyelocytic leukemia and lymphoma (Bode and Dong 2002). Arsenic acts via the induction of apoptosis in tumor cells (Zhu *et al.* 2003). Experimentally, the combination of arsenite and the polyunsaturated fatty acid docosahexaenoic acid induces apoptosis and increased intracellular lipid peroxidation, selectively toxic for malignant solid tumor cells, via an ROS-dependent mechanism (Baumgartner *et al.* 2004). Arsenite treatment also induces GADD45, a protein normally induced by the tumor suppressor p53, that allows for cell cycle arrest and DNA repair at the G_2/M checkpoint (Huang *et al.* 2004), potentially slowing or halting the growth of susceptible neoplasms.

VI. Interactive Effects of Chemical Mixtures

Chemical contamination of groundwater is a particular problem in the areas around the 40,000 hazardous waste disposal sites nationally (De Rosa *et al.* 1996). Most

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chemical exposures to humans are not to single chemicals, but rather to mixtures of chemicals by a variety of routes, including drinking water. There has been increasing recognition of the importance of and concerns over the health risks that may be associated with chemical mixture exposure (Calabrese 1995; De Rosa *et al.* 1996). Survey data on groundwater from hazardous waste sites in all regions of the U.S. (De Rosa *et al.* 1996) have revealed a large number of toxic chemicals in a variety of combinations, yet there is relatively little information available on the toxicity and carcinogenicity of mixtures of such agents.

There have been a limited number of carcinogenesis studies examining mixtures of coplanar (126) and noncoplanar (153) PCBs. One group found a greater-than-additive (synergistic) effect when rats were initially given a complete carcinogen followed by partial removal of the liver and twenty weeks of combination PCB treatment, using gamma-glutamyltranspeptidase(GGT)-positive hepatic foci as preneoplastic markers (Bager *et al.* 1995). Two subsequent experiments found antagonistic effects with this combination of PCBs and somewhat similar treatment protocols, with both using placental glutathione-S-transferase (GST-P) as a marker of preneoplasia (Dean, Jr. *et al.* 2002; Haag-Grönlund *et al.* 1998). Studies examining the combination of chlorinated hydrocarbons and toxic metals are uncommon and these tend to be of an epidemiological nature (Bordajandi *et al.* 2004; Miao *et al.* 2000; Skaare *et al.* 1990). Only one citation has been found specifically examining PCBs and arsenic in a controlled setting: a study to determine acute toxicity of As, PCBs, and mixtures to Lake Michigan cisco fish fry (Passino and Kramer 1980). It was found that the mixture of As and PCBs was more acutely toxic than either compound alone.

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VII. Research Objectives

Since environmental pollutants are most likely to be encountered as multiple chemical exposures, especially near hazardous waste disposal sites, the most realistic assessment of toxicological effects should be based on the toxicological interactions in chemical mixtures. Polychlorinated biphenyls (PCBs) are a major component of hazardous wastes and, among themselves, represent mixtures of congeners that have differing actions and potential risks, including carcinogenesis. PCBs are usually found as mixtures with other chemicals that may pose similar risks. Arsenic is also a major environmental contaminant.

The overall goal of the research presented herein was to better characterize the interactive effects of PCBs as agents of carcinogenesis, both in mixtures of PCB congeners (Chapter 2) and in mixtures with arsenic (Chapter 3). The overall hypothesis for this research is that the effects of mixtures of toxic chemicals commonly found at hazardous waste sites cannot necessarily be predicted from the actions of the individual chemicals.

For Chapter 2, "Interactions of Dioxin-Like Chemicals Based on CYP1A1 induction," the purpose of the experiment was to investigate the carcinogenic roles various polychlorinated aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), dioxin-like coplanar PCB congeners and phenobarbital-like noncoplanar PCB congeners might play using oxidative injury and the production of reactive oxygen species as a mechanism. The toxic equivalency factor (TEF) concept is the basis by which risk assessments for mixtures of

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dioxin-like agents are performed, and this assumes additivity of the toxic effects of the individual chemicals.

Hypothesis 1. A mixture of Ah-receptor agonists such as TCDD, PeCDF and PCB 126 will show an additive interaction with respect to oxidative metabolism and induction of cytochrome P450 1A1 enzyme.

Hypothesis 1a. The addition of non-AhR-binding compounds such as noncoplanar PCBs to mixtures containing dioxin-like chemicals will result in a non-additive interaction.

PCB 126 was used as the model compound for the coplanar congeners and PCB 153 as the model compound for the noncoplanar congeners. Since the induction of cytochrome P450 1A1 is a known effect of aryl hydrocarbon receptor binding by dioxin-like compounds and can be measured using the EROD assay, this method was chosen to perform the experiments. We also aimed to investigate the gender differences in carcinogenic potencies by examining the oxidative induction of reactive catechol estrogen metabolites.

Hypothesis 2. Induction of catechol estrogen metabolites in female rats may help explain gender differences in TCDD and PCB hepatocarcinogenesis.

For Chapter 3, "Interactions of PCB 126 and Arsenic in Hepatocarcinogenesis," the purpose of the experiment was to examine the carcinogenesis of both chlorinated hydrocarbons and arsenic, individually and in mixtures, using PCB 126 as the representative chlorinated hydrocarbon.

Hypothesis 3. The general hypothesis is that interactions between a mixture of PCB 126 and arsenic will result in nonadditive effects on hepatic promotion and progression.

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Hypothesis 3a. Arsenic will demonstrate negative/antagonistic effects in the promotion stage of carcinogenesis and will have positive/pro-carcinogenic effects in the progression stage.

Hypothesis 3b. PCB 126 will show promotional effects but will have no effect on the progression stage.

To examine these Chapter 3 hypotheses, we used a modification of the Ito medium-term bioassay system for the determination of liver carcinogens (Ito *et al.* 1989). The production of glutathione-s-transferase (placental form) positive foci of altered hepatocytes was used as the marker of the promotion stage of carcinogenesis, and the production of transforming growth factor-alpha (TGFα) positive foci was used as the marker of the progression stage of carcinogenesis. We also explored the role of TGFα and transforming growth factor-beta (TGFβ) and its receptors in the disturbance of cell proliferation and apoptosis.

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CHAPTER 2

Interactions of Dioxin-Like Chemicals Based on CYP1A1 Induction

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Introduction

Polychlorinated aromatic hydrocarbons (PAHs) such as dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD]), dioxin-like agents (2,3,4,7,8-pentachlorodibenzofuran [PeCDF]) and certain polychlorinated biphenyls (PCBs) produce their toxic and carcinogenic effects via binding to the intracellular aryl hydrocarbon receptor (AhR). The resulting complex dimerizes with the AhR nuclear translocator protein (ARNT), allowing entry into the nucleus. This heterodimer then binds to a responsive DNA domain called the dioxin response element (DRE), enhancing transcription of downstream metabolism genes such as the heme-containing cytochrome P-450 monooxygenase (CYP) enzymes 1A1 and 1A2 (Burke *et al.* 1994; Safe 1994). CYP1 enzymes are responsible for both activating and detoxifying PAHs via chemical binding of the AhR (Nebert *et al.* 2004). The noncoplanar PCB 153 does not bind the AhR and it causes transcription of the CYP2B enzyme (see below) (Safe 1992).

CYP enzymes can be measured directly in tissues using a Western immunoblot technique or by measuring the activity of the individual enzymes to catalyze specific reactions. The advantage of the activity measurement is that it only accounts for active

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protein. Activity of CYP1A1, the enzyme of interest in this study, is via measurement of the ability of liver microsomes (isolated smooth endoplasmic reticulum, containing multiple CYPs) to catalyze the 7-ethoxyresorufin-*O*-deethylase (EROD) reaction assay (Rutten *et al.* 1992) using a fluorescence spectrophotometer (see Materials & Methods). Other CYPs such as the closely related 1A2 or 2B are measured by the methoxyresorufin *O*-demethylation (MROD) or pentoxyresorufin *O*-depentylation (PROD) assays, respectively (van Birgelen *et al.* 1996).

Dioxins are formed as waste products of combustion processes, especially from municipal incinerators (Tuppurainen *et al.* 2003). TCDD is highly lipophilic and persists in adipose tissue, allowing for bioaccumulation up the food chain. It is a potent liver carcinogen in rodents and risk assessment of dioxins is based on induction of liver tumors in rats, although there are wide differences in effect between strains (Viluksela *et al.* 2000). Dioxin acts as a promoter, binding the AhR and stimulating cell proliferation.

PCBs are halogenated aromatic hydrocarbons that have been used widely in industry but are now banned from use. PCBs degrade slowly in the environment, are metabolized slowly, and bioaccumulate up the food chain (Kimbrough 1995; Wölfle 1997). Thus, PCBs continue to be important worldwide environmental pollutants (Kimbrough 1995; Wölfle 1997) and they exist as complex mixtures with other polychlorinated chemicals, including dioxins (Vanden Heuvel and Lucier 1993). The biological effects and classification of PCB congeners is based on their molecular structure (Safe 1994). Depending on the position of their outlying chlorine atoms, congeners assume a coplanar or noncoplanar configuration. Coplanar PCBs such as PCB 126 (3,3',4,4',5-pentachlorobiphenyl) are also known as dioxin-like PCBs, binding the

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AhR and having comparable biological effects to TCDD such as induction of the cytochrome P-450 enzymes 1A1 and 1A2. The noncoplanar PCBs such as PCB 153 (2,2',4,4',5,5'-hexachlorobiphenyl) do not bind the AhR. They elicit phenobarbital-like effects such as induction of CYP2B enzymes and hepatic neoplasia in rodents, by binding the constitutive androstane receptor (CAR) (Muangmoonchai *et al.* 2001). These noncoplanar PCBs are among the most common congeners detected in human and wild animal tissues (Jensen 1987; McFarland and Clarke 1989; Safe *et al.* 1985). Past studies have found synergistic neoplastic promoting effects of mixtures of coplanar and noncoplanar PCBs (Bager *et al.* 1995) as well as antagonistic effects (Dean, Jr. *et al.* 2002; Haag-Grönlund *et al.* 1998).

These compounds exhibit sex-related differences in liver carcinogenesis, with females affected more frequently. This marked female-specific hepato-carcinogenicity is likely due to the influence of 17β-estradiol (E2), a hormone found in increased levels in females. Most of the oxidative metabolism of estrogens (mostly hydroxylation) occurs in the liver, catalyzed by the cytochrome P-450 enzymes (Zhu and Conney 1998). Male rats exposed to TCDD and exogenous E2 exhibited increased number and volume fraction of preneoplastic liver foci compared to males given TCDD alone (Wyde *et al.* 2002). Dioxin-like agents are not generally considered to be genotoxic; however, the higher levels of endogenous estrogen in females, leading to increased 4-hydroxy catechol estrogen production and reactive oxygen species (ROS) production, could provide an explanation of gender differences seen in dioxin carcinogenesis (Martucci and Fishman 1993).

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Endogenous estrogens undergo extensive oxidative metabolism by specific CYP enzymes (Martucci and Fishman 1993). CYP1A enzymes are known to catalyze the metabolism of 17β-estradiol to 2-hydroxy and 4-hydroxy catechol forms (Martucci and Fishman 1993). The 4-hydroxy form (4-hydroxyestrone or 4-hydroxyestradiol [4-OHE_2]) may be metabolized via cytochrome P450 to quinones, reactive electrophiles that may undergo redox cycling reactions. These reactions produce ROS, specifically the hydroxyl radical $OH^{\bullet}$, that can cause DNA damage (Cavalieri *et al.* 1997; Han and Liehr 1995).

In contrast, the 2-hydroxy form (2-hydroxyestrone or 2-hydroxyestradiol [2-OHE_2]) has little or no estrogenic activity and increased levels are suggested to be protective against carcinogenesis (Martucci and Fishman 1993; Zhu and Conney 1998). The 2-hydroxy pathway is the dominant form of estrogen oxidative metabolism, although the 4-hydroxy pathway may dominate in some tissues. Catechol-*O*-methyltransferase (COMT), abundant in liver, rapidly methylates 2-catechol and 4-catechol estrogens to their methoxy derivatives (Martucci and Fishman 1993).

To aid in risk assessment for these persistent environmental contaminants, toxic equivalency factors (TEFs) were developed by the World Health Organization and have used the common initial mechanism of AhR binding (Safe 1990). A TEF for a chemical indicates an order of magnitude of the toxicity of a compound relative to TCDD, which is assigned the maximum value of 1.0 (Birnbaum 1994; Finley *et al.* 2003; Toyoshiba *et al.* 2004; van den Berg *et al.* 1998). Other dioxin-like compounds are given equal or lower numbers, with each number proportional to TCDD. For instance, PeCDF is given a value of 0.5 and PCB 126 a value of 0.1 (see Table 2.1 and Table 2.3). TEFs can be used to summarize the dose of a mixture as a simple number, the toxic equivalent dose (TEQ) of

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Chemical	Toxic Equivalency Factor (TEF)
TCDD	**1.0**
PeCDF	**0.5**
PCB 126	**0.1**
1,2,3,7,8-PeCDF	**0.05**
PCB 169	**0.01**
PCB 156	**0.0005**
PCB 118	**0.0001**
PCB 153	**n/a**

PCB 169 = 3,3',4,4',5,5'-hexachlorobiphenyl
PCB 156 = 2,3,3',4,4',5-hexachlorobiphenyl
PCB 118 = 2,3',4,4',5-pentachlorobiphenyl

Table 2.1. Some polyhalogenated hydrocarbons, including all compounds used in these experiments, and their corresponding TEF values.

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a reference compound, by adding together the weighted contributions of different chemicals based on their relative potency (Toyoshiba *et al.* 2004). The TEQ is the sum of the amounts of each compound in the mixture multiplied by the individual TEFs of each compound. A basic assumption of this classification with regard to mixtures is that the effect of the combined chemicals is equivalent to the added effects of the individual chemicals. Most of these chemicals are found not as solitary agents but in complex mixtures with both related and unrelated compounds.

We explored a role for reactive oxygen species in carcinogenesis by measuring the CYP1A1 activity and relative amounts of catechol estrogens in livers from rats exposed to TCDD, PeCDF, the dioxin-like coplanar 3,3',4,4',5-pentachlorobiphenyl (PCB 126), the noncoplanar 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153), a two-way mixture of the PCBs, and a three-way mixture of the AhR-binding TCDD, PeCDF, and PCB 126. The relative CYP1A1 levels were used to match our results to previously published TEFs for these individual chemicals (Vanden Heuvel and Lucier 1993), as well as to added TEFs for the binary & tertiary mixtures.

Materials and Methods

Female Sprague-Dawley rats were orally gavaged five times per week (Monday through Friday) with corn oil solutions to deliver several doses of PCB 126, PCB 153, TCDD, a mixture of PCBs 126 and 153, a mixture of TCDD, PeCDF, and PCB 126, or corn oil as a vehicle (Table 2.2). This study was part of a National Toxicology Program study at Battelle, Columbus OH (Toyoshiba *et al.* 2004). Animals were sacrificed after 13 and 30 weeks of treatment, and two to three grams of fresh liver were snap frozen at

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Dosage Groups

Compound	Doses
TCDD	0 μg/kg
	0.01 μg/kg
	0.10 μg/kg
PeCDF	0 μg/kg
	0.02 μg/kg
	0.20 μg/kg
PCB 126	0 μg/kg
	0.03 μg/kg
	0.30 μg/kg
	1.00 μg/kg
PCB 153	0 μg/kg
	100 μg/kg
	1000 μg/kg
PCB 126 + PCB 153	0/0 μg/kg
	0.10/100 μg/kg
	1.00/1000 μg/kg
TCDD + PeCDF + PCB 126	0/0/0 μg/kg
	0.0033/0.0066/0.0333 μg/kg
	0.033/0.066/0.333 μg/kg

Table 2.2. Doses of chemicals administered to female Sprague Dawley rats five times per week at Battelle Columbus laboratory as part of a National Toxicology Program Research Project (Toyoshiba *et al.* 2004). These doses are designed to represent the known TEFs for the test chemicals, where the doses of the three chemicals are adjusted such that when multiplied by their known TEFs, they would give comparable biological responses.

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CYP1A1 Induction for Ah-receptor Agonists Adjusted for Selected Doses & TEF

COMPOUND	DOSE*	TEF	DOSE X TEF	EROD VALUES	
Low Dose				13 week	30 week
TCDD	0.01	1.0	0.01	240 +/- 30	181 +/- 40
PeCDF	0.02	0.5	0.01	275 +/- 65	343 +/- 62
High Dose					
TCDD	0.1	1.0	0.1	353 +/- 47	279 +/- 59
PeCDF	0.2	0.5	0.1	881 +/- 329	640 +/- 131
PCB126	1.0	0.1	0.1	265 +/- 79	213 +/- 37

* mg/kg body weight

Table 2.3. EROD values of selected doses of CYP1A1-inducing individual compounds using comparable effect doses based on known TEFs (DOSE X TEF). Values within the low dose and high dose groups at 13 or 30 weeks are similar except for those of 0.2 μg/kg PeCDF (high dose). Since TEFs are arbitrarily rounded, a two-fold variation with PeCDF is not out of the realm of what might be expected.

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-70°C and sent to Colorado State University's Department of Environmental and Radiological Health Sciences as part of a grant to Dr. G. Cosma. Liver microsomes were isolated via four differential centrifugations (12000-45000rpm) and stored at -80°C (Nishibayashi and Sato 1968). Microsomes were analyzed for protein concentration using the BCA (bicinchoninic acid) protein assay kit (Pierce Biotechnology, Inc., Rockford, IL USA) and an enzyme immunoassay analyzer (Versamax microplate reader, Molecular Devices, Sunnyvale, CA USA). Briefly, protein samples were pipetted into a 96-well plate, combined with BCA reagent, and loaded into the microplate reader for incubation and analysis, with protein content measured as mg/ml.

Cytochrome P450 1A1 enzyme induction in microsomes was measured by 7-ethoxyresorufin-*O*-deethylase (EROD) assay (Rutten *et al.* 1992) using a Hitachi F-2000 fluorescence spectrophotometer/fluorimeter (Hitachi Instruments, Inc., San Jose, CA USA). Microsomal protein, ethoxyresorufin substrate, and reaction buffer were combined in a 4.5ml clear polystyrene cuvette and warmed to 37°C, in a darkened environment. Baseline fluorescence was measured in the fluorimeter first, then NADPH was added to the cuvette to initiate the color reaction and, after three minutes, dilated resorufin was added to complete the reaction. Specific EROD activity of each sample was measured as pmol resorufin per minute per mg of microsomal protein. All values were compared between dose groups and time points for each chemical and mixture using one-way analysis of variance with Tukey's pairwise comparisons. Interactions between test chemicals at both time points were evaluated using general linear model analysis.

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Hepatic microsomes were incubated with E2 and direct measurement of the estrogen catechol metabolites 2-hydroxyestradiol (2-OHE_2) and 4-hydroxyestradiol (4-OHE_2) was performed using gas chromatography (GC) with electron capture detection (Pinnella *et al.* 2001; Roy *et al.* 1991). This procedure was performed by the Analytical Toxicology Laboratory of the CSU Department of Environmental and Radiological Health Sciences by Dr. J. Tessari and K. Pinnella.

Results

EROD activity (CYP1A1 induction) increased in a dose-responsive fashion in rats treated with Ah-receptor-binding TCDD, PeCDF, and coplanar PCB 126, as well as the three-way mixture of these compounds (Figures 2.1, 2.2, 2.3, 2.4). An exception was with PCB 126 at 13 weeks, when the dose-responsive increase was present in the low and intermediate dose groups, but the mean EROD activities of the 0.3μg/kg and 1.0μg/kg (highest) dose groups were not significantly different from one another. No changes in CYP1A1 activity were observed with the noncoplanar, non-Ah-receptor binding PCB 153 (Figure 2.5). The PCB 153/126 mixture produced a greater-than-additive elevation in EROD activity (Figure 2.6), which was greater than TCDD alone. Similar responses were seen in the 13 and the 30-week treatment groups, with the exception of the groups exposed to 0.3μg/kg PCB 126.

Utilizing known TEFs, equivalency adjusted doses of TCDD, PeCDF, and PCB 126 elicited similar EROD activity, with the exception of the highest PeCDF dose that was elevated above the others (Figure 2.2, Table 2.3). A comparable PCB 126 low dose was not available, so Table 2.3 only includes TCDD and PeCDF at that level. EROD

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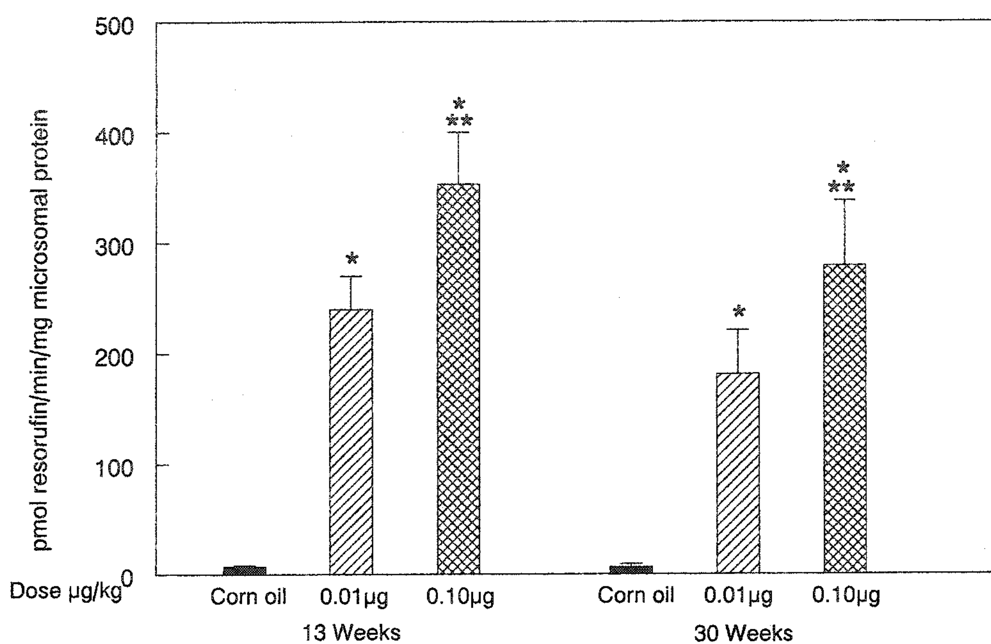

Figure 2.1. Hepatic EROD (CYP1A1) activity in rats exposed to TCDD. Note dose-responsive increases and similar values at both the 13 and 30-week time points.

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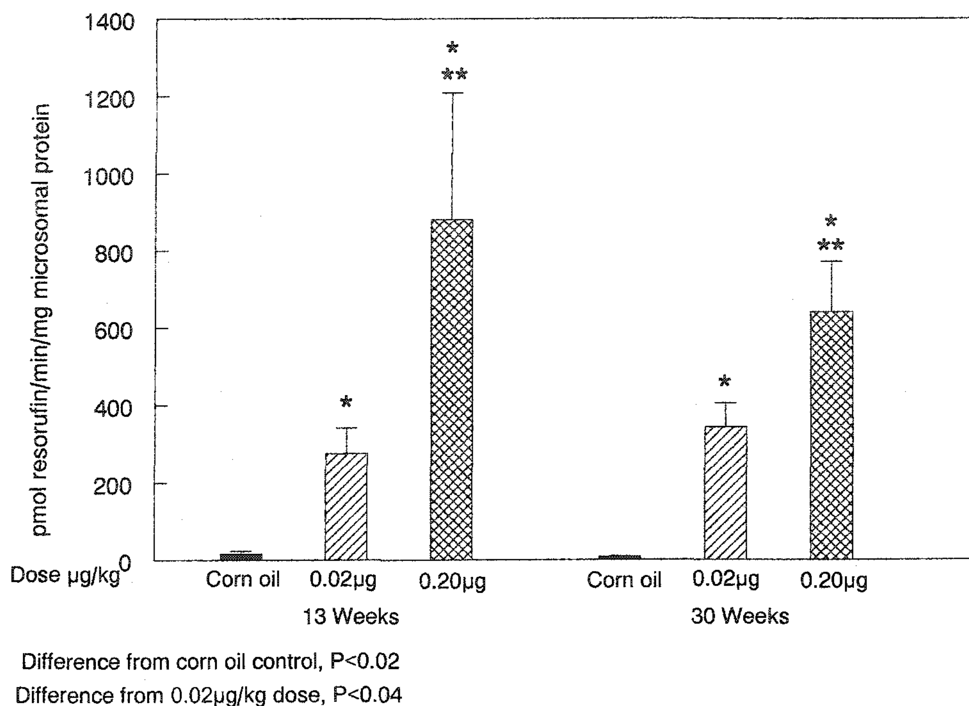

Figure 2.2. Hepatic EROD (CYP1A1) activity in rats exposed to PeCDF. Note dose-responsive increases and similar values at both the 13 and 30-week time points.

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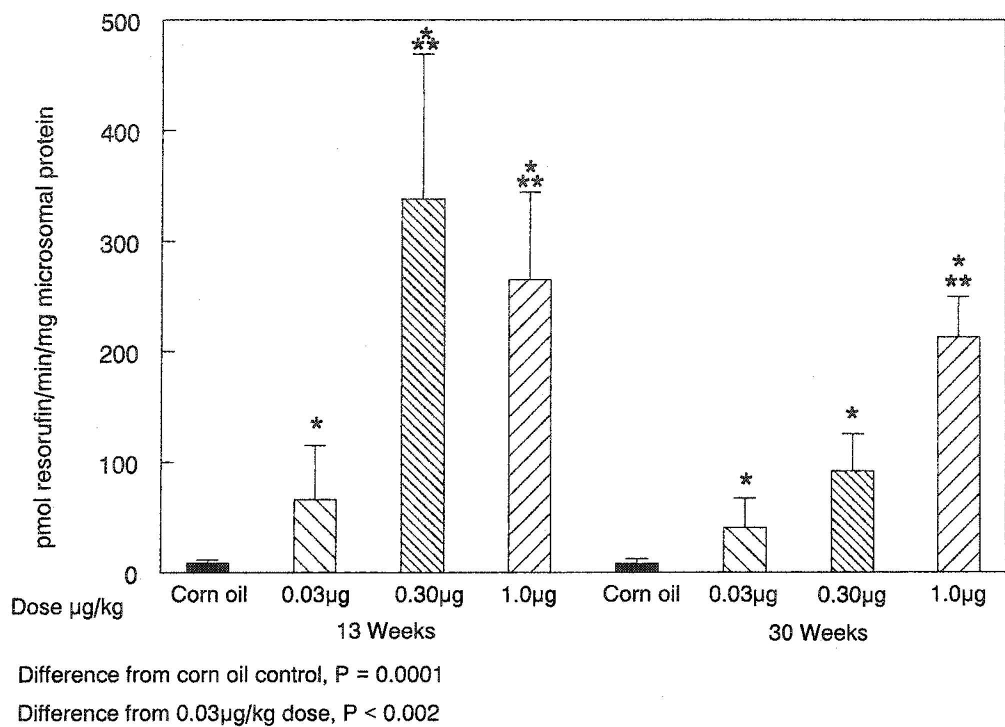

Figure 2.3. Hepatic EROD (CYP1A1) activity in rats exposed to PCB126. Note dose-responsive increase at 30 weeks. The dose-responsive increase at 13 weeks was present in the low and intermediate dose groups, but the 0.3 and 1.0µg groups were not significantly different from one another.

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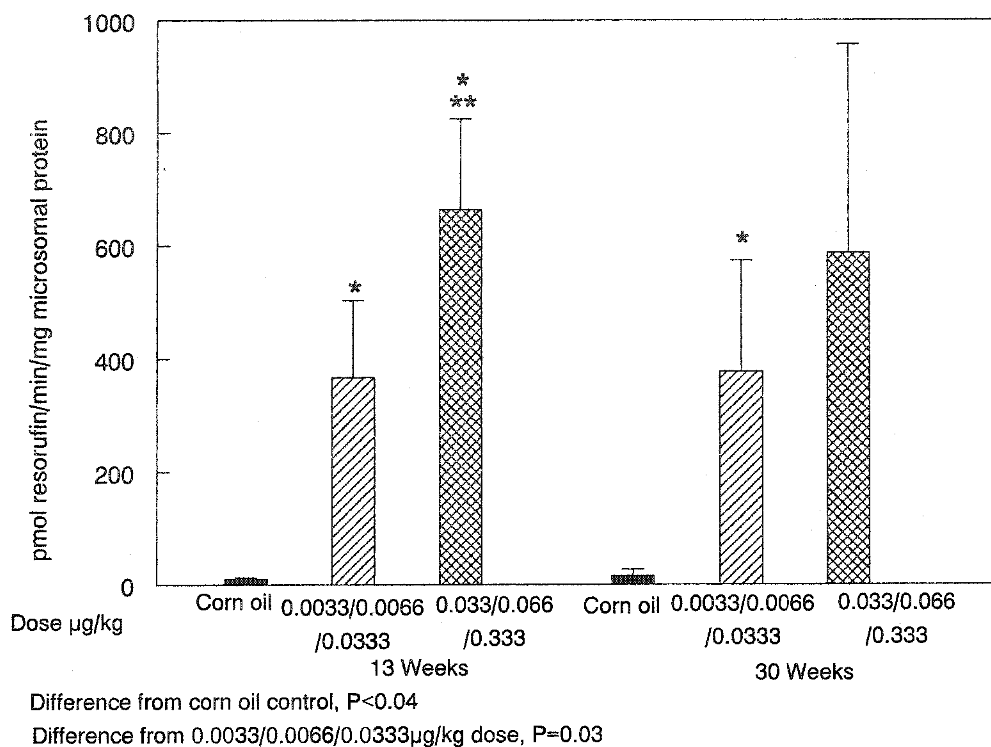

Figure 2.4. Hepatic EROD (CYP1A1) activity in rats exposed to a combination of TCDD, PeCDF, and PCB126. Note dose-responsive increases and similar values at both the 13 and 30-week time points.

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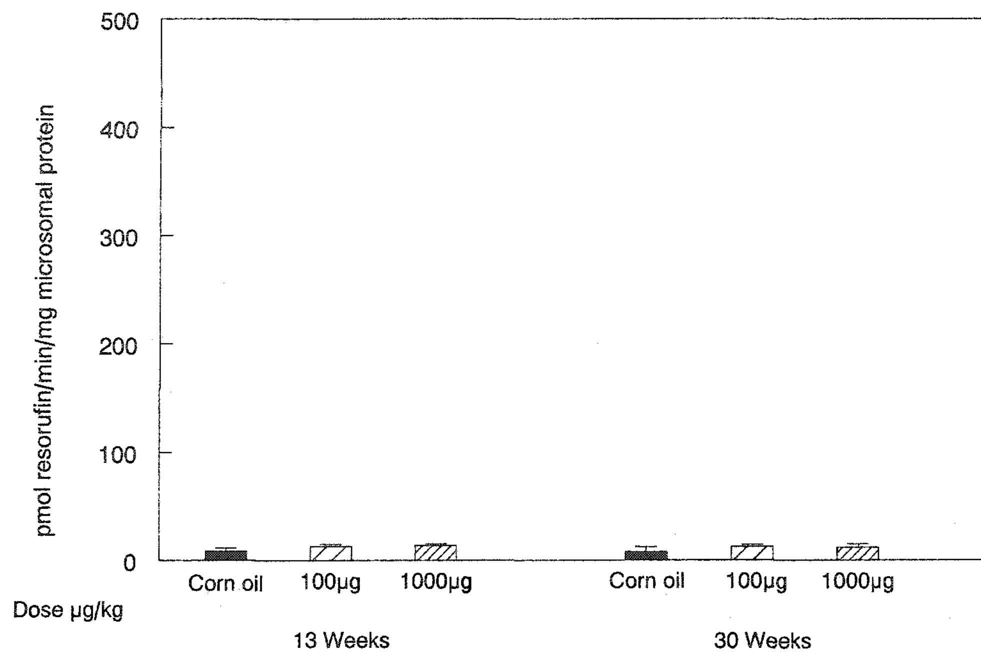

Figure 2.5. Hepatic EROD (CYP1A1) activity in rats exposed to PCB153. No significant differences between controls and PCB153 treatment groups (P>0.05, ANOVA). This lack of response was expected, as PCB153 is noncoplanar and does not bind the Ah receptor.

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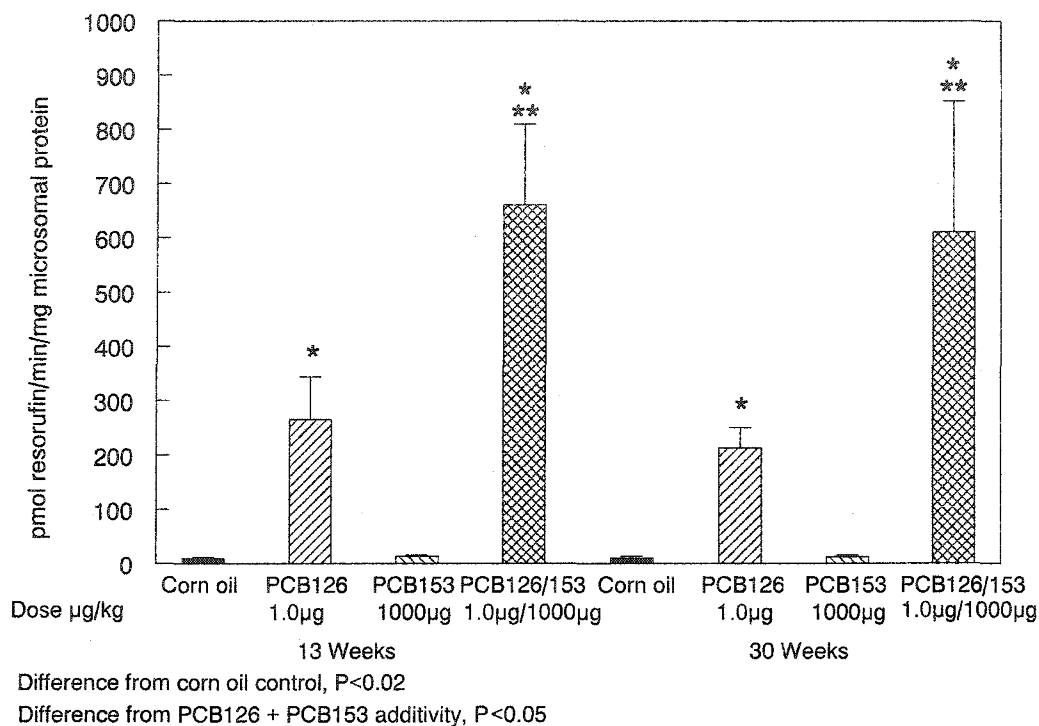

Figure 2.6. Hepatic EROD (CYP1A1) activity in rats exposed to the highest doses of PCB126, PCB153, and a combination of PCBs 126 and 153. Note the significant greater-than-additive interaction in the mixture.

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activities for the three-way mixture of TCDD, PeCDF, and PCB 126 at both doses and both time points were similar to the added EROD activities of the individual chemicals, assuming a linear dose extrapolation (Table 2.4).

Dose-responsive increases in 4-OHE2 were observed with the high dose TCDD, and low and high dose PCB 126, PCB 153, and PCB 126/153 mixture samples. Changes in 2-OHE2 were inconsistent, with some groups increased (PCBs) and some decreased (TCDD). Increases in 4-OHE2 (1.9-fold at 30 weeks) led to decreased 2:4-OHE2 ratio with increasing dose in rats exposed to TCDD, PCB 126, and PCB 126/153 (Pinnella 2000).

Discussion and Conclusions

The similar EROD responses seen in 13 and 30-week treatment groups for all compounds at most of the doses preclude a time-dependent effect over these time periods, suggesting an induction plateau at a steady elevated level. This plateau was achieved by the 13-week first sacrifice point and continued through 30 weeks. Time-course studies with TCDD and/or different PCB congeners in various model systems have demonstrated variable rate of induction of individual CYPs (Lorenzen *et al.* 1997; Parkinson *et al.* 1983; Yeung *et al.* 2003) so it is likely that maximal induction was reached well before our first sampling time.

Enhancement in EROD activity with a mixture of TCDD and PCB 153 has been reported previously (Bannister and Safe 1987; De Jongh *et al.* 1995b). Possible explanations include reports that high doses of PCB 153 can alter the distribution of TCDD, increasing the concentration in liver & decreasing the concentration in adipose

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CYP1A1 Induction in a Mixture of Ah-Receptor Agonists

	13 WEEKS		30 WEEKS	
	LOW DOSE*	HIGH*	LOW DOSE*	HIGH*
EXPECTED ADDITIVE EROD VALUES FOR INDIVIDUAL COMPOUNDS IN 3-WAY MIXTURE (TCDD, PeCDF, PCB126)	340	720	275	550
ACTUAL EROD VALUES FOR 3-WAY MIXTURE	367 +/- 137	664 +/- 161	377 +/- 196	587 +/- 370

* pmol resorufin/min/mg microsomal protein

Table 2.4. Comparison of actual EROD values of 3-way mixture (TCDD/PeCDF/PCB126) with added values of the individual compounds. Linear extrapolation was used to determine individual EROD values for doses equivalent to those used in the mixture, at 13 and 30 weeks.

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tissue (De Jongh *et al.* 1995a; van Birgelen *et al.* 1996). Further, PCB 153 has been shown to increase hepatic AhR levels (Bannister and Safe 1987). Since PCB 126 binds the AhR in a similar fashion to TCDD and exposure induces similar effects, it is not unexpected for a mixture of PCBs 126 and 153 to result in enhanced EROD activity as we observed. This has been reported previously in both male Wistar rats at submaximal, nontoxic doses (Leece *et al.* 1987) and in female Sprague-Dawley rats at higher doses of both chemicals (Bager *et al.* 1995). The AhR may thus be acting not only as a trigger for CYP induction after exposure to these chemicals, but also as a mechanism by which these chemicals are drawn to and bound within hepatocytes, resulting in an overall increase in liver concentration (De Jongh *et al.* 1995b; van Birgelen *et al.* 1996). Alternately, an enzyme induced by AhR binding such as CYP1A2 might act as act to bind and sequester dioxin-like chemicals (Santostefano *et al.* 1996; van Birgelen *et al.* 1996). A previous study examining the formation of altered (preneoplastic) hepatic foci also found evidence of a greater-than-additive effect with PCB 126 and 153 co-exposure (Bager *et al.* 1995). A recent study examining PCB 126 and 153 co-exposure found regional differences in CYP1A1 induction, with centrilobular to panlobular 1A1 staining seen with increasing dose of PCB 126 alone and periportal 1A1 staining with the PCB 126/153 mixture (Chubb *et al.* 2004). Since our study utilized extracted microsomes from all hepatic regions, these types of alterations could not be evaluated, but they illustrate the complex changes occurring in the hepatic tissue with exposure to mixtures of coplanar and noncoplanar PCBs.

Previous experiments in our laboratory have demonstrated increased PCB 153 levels in liver after combined PCB 126/153 exposures but did not find increased PCB 126 levels

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(Dean, Jr. *et al.* 2002). One would expect that PCB 126 would respond similarly to TCDD after PCB 153 co-exposure, resulting in hepatic sequestration, but this was not the case. The mixture of PCBs 126 and 153 did result in a decreased concentration of PCB 126 in the adipose tissue, as reported previously in mixtures of TCDD and PCB 153 (Dean, Jr. *et al.* 2002; van Birgelen *et al.* 1996). Many of the earlier studies examining tissue distribution of these compounds used mice, whereas the current study and the Dean *et al.* studies used the rat, so inherent species differences may have influenced the outcomes. Furthermore, the current study used Sprague-Dawley rats gavaged five times weekly and the Dean *et al.* study used Fischer 344 rats gavaged three times weekly, adding rat strain and dosing schedule variability as elements of uncertainty.

Our data are consistent with the published TEFs for TCDD, PeCDF, and PCB 126 based on our CYP1A1 induction findings (Table 2.3). The higher responses in PeCDF-induced EROD activity, especially in the 0.20μg/kg dose groups, were unexpected. However, the inherent variability in the TEF system may explain some of this elevation. In addition, the fact that only one dose resulted in this elevation and the wide standard deviation in our data suggests that this may not be significant. Several authors have expressed concern regarding the polychlorinated hydrocarbon TEFs, suggesting that they are overly conservative (Birnbaum 1994; DeVito *et al.* 1993; Starr *et al.* 1999). At least with respect to induction of CYP1A1 EROD activity, this experiment suggests that the published TEFs for these particular compounds are accurate.

Our data are very consistent with the assumption of additivity of Ah-binding chemicals in the three-way mixture of TCDD/PeCDF/PCB 126, based on the EROD activities when compared to that of individual components (Figure 2.9). It would be

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interesting to add the non-AhR-binding PCB 153 to the mixture to determine if its EROD-enhancing effects on PCB 126 would be shared by the other chemicals. This would be expected based on PCB 153's assumed effect of increasing AhRs in the liver, increasing the potential binding sites for these chemicals. There may be a limit to how many AhRs can be induced by PCB 153, and this would be evident in a dose-dependent plateau in EROD activity.

Increased 4-OHE2 is observed in tissues where estrogens induce tumors (Cavalieri *et al.* 1997; Liehr and Ricci 1996). Increased 4-OHE2 formation coupled with increased CYP1A1 (EROD) activity, as observed with TCDD, PCB 126, and PCB 126/153, may increase the potential for quinone cycling leading to oxidative DNA damage. This could explain, at least in part, the greater susceptibility of female rats to carcinogenesis by Ah-receptor agonists.

The synergism in CYP1A1 induction by the PCB mixture was not paralleled by a similar response in estrogen metabolism, suggesting that a different pathway may be involved. However, increased CYP1A1 plus increased 4-OHE2 could result in increased quinone cycling, ROS production, and a greater degree of DNA damage. This ultimately might increase the risk of carcinogenic mutations in livers of animals exposed to certain combinations of coplanar and noncoplanar PCBs, and by extension, other AhR binding agents and noncoplanar PCBs. Since the noncoplanar PCBs such as PCB 153 are among the most commonly detected in tissues of humans and wildlife (Jensen 1987; McFarland and Clarke 1989; Safe *et al.* 1985), addition of AhR binding chemicals to these give cause for concern.

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In conclusion, this study supports the known TEFs for the Ah receptor binding chemicals TCDD, PeCDF, and PCB 126, and supports the assumption of additivity for mixtures of these chemicals that act in a similar manner. It also reveals the mechanistic difficulties and risks of extending these assumptions to mixtures containing chemicals that do not bind the AhR, such as PCB 153. The synergistic effect of PCB 153 on the EROD induction of PCB 126, TCDD, and presumably PeCDF suggests limiting the use of the TEF paradigm to dioxin-like chemicals and chemical mixtures.

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CHAPTER 3

Interactions of PCB 126 & Arsenic in Hepatocarcinogenesis

J.T. Painter, L.S. Chubb, C.V. Lane, and S.A. Benjamin

Introduction

Polychlorinated biphenyls (PCBs) are among the most frequently reported chemicals at hazardous waste disposal sites (EPA 2005) and are fifth on the Agency for Toxic Substances and Disease Registry (ATSDR)/Environmental Protection Agency (EPA) Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) Priority List of hazardous substances (ATSDR 2003). PCBs are polycyclic aromatic hydrocarbon (PAH) organochlorines that were widely used in industry and are now worldwide environmental pollutants. PCBs degrade slowly in the environment and are lipophilic and metabolize slowly, resulting in bioaccumulation up the food chain (Kimbrough 1995).

The biological effects and classification of PCB congeners is based on their molecular structure (Safe 1994). Depending on the position of their outlying chlorine atoms, congeners assume a coplanar or noncoplanar configuration. Coplanar PCBs such as PCB 126 (3,3',4,4',5-pentachlorobiphenyl) are also known as dioxin-like PCBs, binding the cytoplasmic aryl hydrocarbon receptor (AhR) and having comparable biological effects to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) such as stimulation of

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cell proliferation in rodents and induction of cytochrome P-450 (CYP) 1A enzymes. PCB 126 is considered the most toxic congener, having the closest molecular structure to TCDD. The noncoplanar PCBs such as PCB 153 (2,2',4,4',5,5'-hexachlorobiphenyl) do not bind the AhR. They elicit phenobarbital-like effects such as cell proliferation and induction of CYP2B enzymes by binding the constitutive androstane receptor (Muangmoonchai *et al.* 2001). PCBs in the environment exist as mixtures of congeners with different physical and biological characteristics that can interact with each other and with other pollutants, causing toxicity.

The carcinogenic effects of PCBs have been widely studied in laboratory animals, where the liver is the target organ. Various studies have shown that, after initiation with a variety of carcinogens, they can act as promoters of hepatocellular neoplasms, including carcinomas (Mayes *et al.* 1998; Moore *et al.* 1994; Silberhorn *et al.* 1990). PCBs are classified as probable (Group 2A) human carcinogens, with suggestive evidence of causation with liver, biliary tract, and breast cancers (Charlier *et al.* 2004; Demers *et al.* 2002; Golden *et al.* 2003; Kimbrough 1995; Siemiatycki *et al.* 2004).

Arsenic (As) is a common groundwater contaminant and is found worldwide. It is common in the USA, especially in southwestern states, and As is among the most frequently reported chemicals at hazardous waste disposal sites (EPA 2005). It is first on the ATSDR/EPA CERCLA Priority List of hazardous substances (ATSDR 2003). Environmental exposures to As usually occur through ingestion of contaminated food and especially water, when natural or manmade sources leach into nearby groundwater.

Arsenic is found both as inorganic and organic forms. Its inorganic forms are the trivalent arsenite, or arsenic trioxide (AsIII), and the pentavalent arsenate (AsV). The

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trivalent arsenite is considered the more toxic of the inorganic forms, in part due to its increased solubility (Aposhian *et al.* 2004; Haschek *et al.* 2002). Organic arsenic is represented by dimethylarsinic acid (DMA) and methylarsonic acid (MMA), the major metabolites after exposure to inorganic As in both humans and rodents (Kenyon and Hughes 2001). Organic arsenic forms from the *in vivo* methylation of As, allowing for eventual elimination from the body.

Arsenic acts by binding to sulfhydryl groups on enzymes and other cellular proteins (Pott *et al.* 2001). Arsenic is classified as a definite (Group 1) human carcinogen, with strong evidence of causation with skin, liver, lung, and urinary bladder cancers (Basu *et al.* 2001; Centeno *et al.* 2002; Chiu *et al.* 2004; Guo *et al.* 2004; Haschek *et al.* 2002; Siemiatycki *et al.* 2004; Tchounwou *et al.* 2004; Yoshida *et al.* 2004).

Although arsenic is a proven human carcinogen, it has been difficult to demonstrate its carcinogenicity in experimental animal studies. The organic metabolite DMA has been found to cause multi-systemic cancers in numerous laboratory animal studies, notably urinary bladder, kidney, liver and thyroid gland (Kenyon and Hughes 2001). DMA is also a potent clastogen, inducing double strand breaks and cross-linking of cellular DNA (Wanibuchi *et al.* 2004). When pregnant mice are exposed to arsenite in drinking water for a brief period during gestation, their progeny develop dose-dependent neoplasms in the liver, lung, adrenal gland, and ovary after reaching adulthood, demonstrating that AsIII can act as a complete carcinogen (Liu *et al.* 2004; Waalkes *et al.* 2004).

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The methods of action of As carcinogenesis are unclear. The strongest experimental evidence suggests production of chromosomal abnormalities, induction of oxidative stress, and activation of selected signal transduction pathways (Huang *et al.* 2004; Kitchin and Ahmad 2003). Other potential carcinogenic pathways are enhancement of cell proliferation and suppression of p53 (Kitchin 2001; Pott *et al.* 2001). Arsenic is thought to act in the promotion and/or progression stages of carcinogenesis (Pott *et al.* 2001). It is a known clastogen, causing large-scale breaks and multilocus deletions in DNA. It is also thought to act as a "co-carcinogen" whereby its interactions with other environmental carcinogens result in a synergistic response.

Acquisition of autocrine growth involving the growth factor transforming growth factor-alpha (TGFα) characterizes altered hepatocytes during the early stages of carcinogenesis in rats and humans (Grisham 1997). TGFα binds to the epidermal growth factor (EGF) receptor and is a potent growth factor for hepatocytes (Burr *et al.* 1996). Although hepatic expression of TGFα is low in normal liver, expression increases from preneoplastic foci to adenomas to carcinomas, suggesting that it is important in neoplastic progression (Burr *et al.* 1996; Dragan *et al.* 1995; Grisham 1997). Expression of TGFα correlates well with areas of progression in chemical carcinogenesis protocols and these areas are most likely to progress to tumors (Burr *et al.* 1996; Dragan *et al.* 1995; Grisham 1997; Steinmetz and Klaunig 1996).

Transforming growth factor-beta (TGFβ) normally inhibits epithelial cell proliferation and promotes differentiation. Experimental data provide *in vivo* evidence of the tumor suppressor activity of TGFβ during chemical hepatocarcinogenesis (Kanzler *et al.* 2001). TGFβ1 is the most abundant and important form in the liver and is synthesized

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by Ito cells and hepatocytes (Roth *et al.* 1997). TGFβ1 is released in a protein-bound, latent form, and the complex cannot bind with its specific receptors until it is bound to the mannose-6-phosphatase/insulin-like growth factor II (M6P/IGFII) receptor on the hepatocyte and is activated by plasmin cleavage (Grisham 1997; Roth *et al.* 1997). Once activated, TGFβ1 binds to three TGFβ cell surface receptor proteins (types I, II, and III) with high affinity. TGFβ1 binds to the type II receptor which then recruits type I receptor into the complex and causes phosphorylation and cytoplasmic kinase activation of the type I receptor. The type III receptor is proposed to be involved with regulating access to I and II. There is experimental evidence that expression of TGFβ1 and TGFβII-r is altered during the promotion stage of hepatocarcinogenesis in the rat, contributing to the development and progression of preneoplastic lesions (Park *et al.* 2001).

Most chemical exposures to humans are not to single chemicals, but rather to mixtures of chemicals by a variety of routes, including drinking water. There has been increasing recognition of the importance of and concerns over the health risks that may be associated with chemical mixture exposure (Calabrese 1995; De Rosa *et al.* 1996). Survey data on groundwater from hazardous waste sites in all regions of the U.S. (De Rosa *et al.* 1996) have revealed a large number of toxic chemicals in a variety of combinations, yet there is relatively little information available on the toxicity and carcinogenicity of mixtures of such agents.

Studies examining the combination of chlorinated hydrocarbons and toxic metals are uncommon and these tend to be of an epidemiological nature (Bordajandi *et al.* 2004; Miao *et al.* 2000; Skaare *et al.* 1990). The purpose of the experiment detailed here was to examine the carcinogenesis of both chlorinated hydrocarbons and metals, individually

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and in mixtures, using PCB 126 and arsenic as the representative model compounds. The general hypothesis is that interactions between a mixture of PCBs and arsenic will result in nonadditive effects on hepatic promotion and progression. Specifically, arsenic will demonstrate negative/antagonist effects in the promotion stage of carcinogenesis and will have positive/pro-carcinogenic effects in the progression stage. PCB 126 will show promotional effects but will have no effect on the progression stage.

Materials and Methods

Female Fischer 344 rats were purchased from Harlan Sprague-Dawley at an age of 30 days and allowed to acclimate to the altitude of Fort Collins, Colorado (5000 feet) for 28 days. Following acclimation they were ear-tagged and randomized by weight into one of nine treatment groups (Figure 3.1). All animals were housed (three per cage) in polycarbonate cages with corncob bedding and stainless steel wire tops and maintained at 25 C with 55% humidity and a 12-hour light/12-hour dark cycle. Control and exposed animals were given food (Harland Tedkad NIH-07 diet, Madison, WI USA) and deionized water *ad libitum*. The clinical state of the animals was assessed twice daily and body weight was assessed three times weekly. This study was conducted in accordance with the National Institutes of Health guidelines for the care of laboratory animals, and animals were housed in a facility fully accredited by the Association for Accreditation of Laboratory Animal Care. Two replicate studies were performed, with the second study started seven months after the cessation of the first, with identical protocols. All data from both studies have been combined for all results, tables, and statistical analyses. Sacrifice time points for each group (A-G) consisted of six animals, resulting in a

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cumulative total of twelve animals per time point per group for analysis (Figure 3.1). Groups H and J contained three animals per sacrifice time point, for a total of six per time point per group for analysis.

Experimental study design was based upon the Ito medium-term bioassay (Ito *et al.* 1989a) and modified to accommodate our extended exposure time (Figure 3.1 and Table 3.1). Rats were injected intraperitoneally with saline (Group A) or 200mg/kg diethylnitrosamine (DEN, Sigma Chemical Company, St. Louis, MO USA) (Groups B-J) at Day 0. All animals began oral gavage with corn oil solution three times weekly (Monday, Wednesday, Friday) (Bestfoods 100% Pure Mazola corn oil, Englewood Cliffs, NJ USA) at Day 14, two weeks after DEN injection. Three groups (C, E, and G) received 10μg/kg PCB 126 in corn oil (Lot#081699MT-AC, AccuStandard, Inc., New Haven, CT USA), and the six remaining groups received corn oil alone for the duration of the study. Also at Day 14, two groups (D and E) were started on sodium metaarsenite (trivalent As, Lot #CR 05726 PQ, Aldrich Chemical Company, Inc., Milwaukee, WI USA) in the *ad libitum* drinking water at 75 parts per million (ppm). This was diluted to 7.5ppm after seven weeks of exposure to minimize subacute toxicity. Two groups (F and G) began sodium metaarsenite treatment at Day 56 (Week 8) of the study. The dose was started at 75ppm and, after eight weeks of exposure reduced to 7.5ppm. Two groups received PCB126 in combination with arsenic, with As begun concurrently w/PCB126 gavage in one group (E), or six weeks later in the second group (G). The final two groups received neither PCB 126 nor As, and instead were injected intraperitoneally at Day 56 with either saline (Group H) or with 150mg/kg of the hepatic carcinogen and

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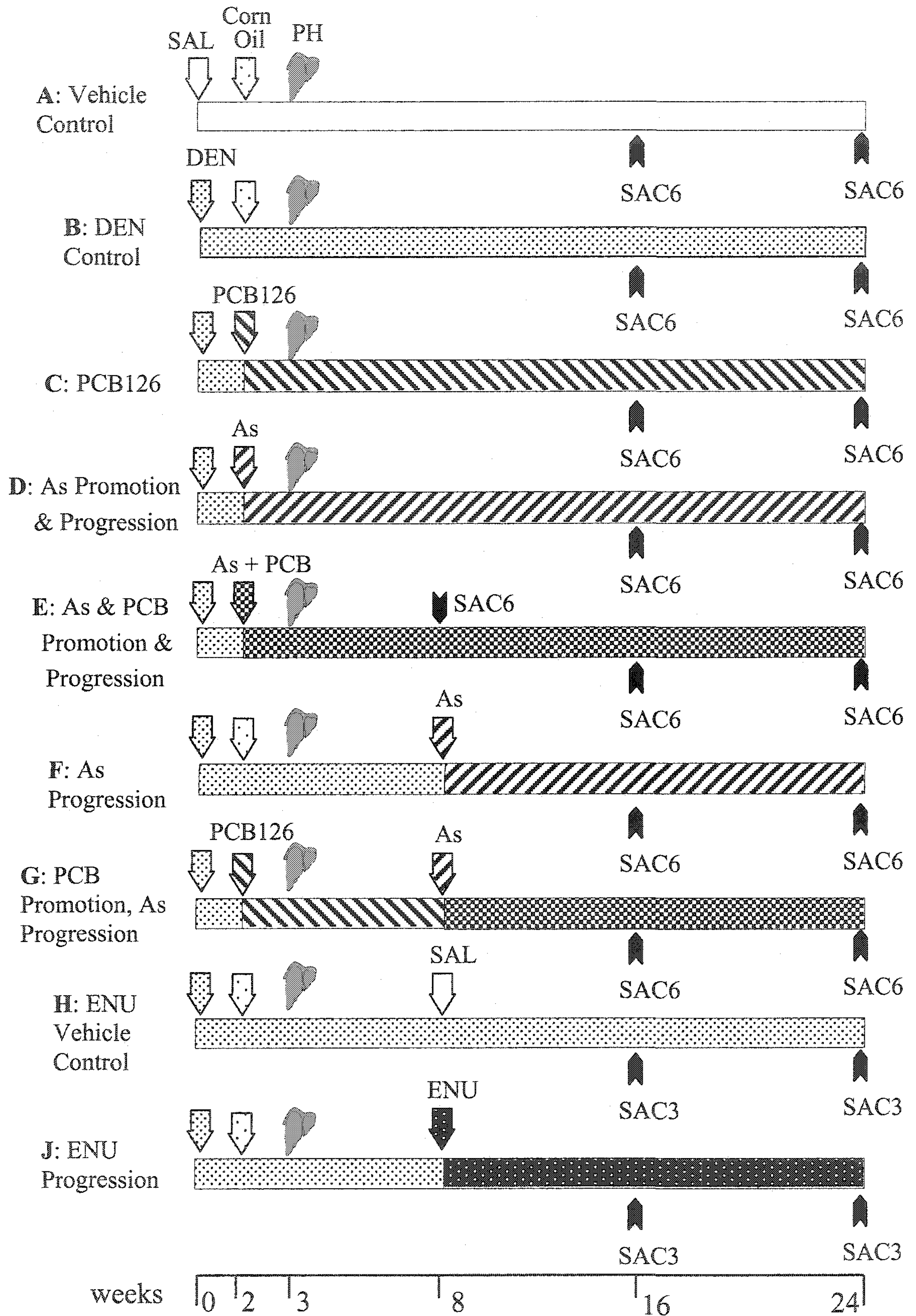

Figure 3.1. Study design. Note both letter designation and brief description of groups. SAC6 = sacrifice 6 rats at this time point. SAL = saline IP. DEN = diethylnitrosamine IP. PH = partial hepatectomy. ENU = ethylnitrosourea IP.

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Chemical	Dose
DEN	200mg/kg IP, single dose
PCB 126	10μg/kg PO 3 times weekly in corn oil gavage
Arsenic (sodium metaarsenite, 3^{+})	75ppm in drinking water for first 7-8 weeks, followed by 7.5ppm in drinking water until sacrifice (group-dependent)

Table 3.1. Doses and treatment schedule for test chemicals used in this study. PO = *per os*.

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tumor progressor ethylnitrosourea (ENU, N-nitroso-N-ethylurea, Sigma Chemical Company, St. Louis, MO USA) (Dragan *et al.* 1993; Pereira *et al.* 1985).

All animals received a two-thirds partial hepatectomy at Day 21 of the study, with removal of the median and left lateral lobes of the liver. Post-operative pain management consisted of Tylenol® (200mg/kg) and codeine (60mg/kg) administered in the drinking water for 72 hours. Rats from all groups were sacrificed after 112 and 168 days (16 and 24 weeks) of treatment (post-DEN), and a single group of PCB/As early (Group E) animals was sacrificed at 56 days (8 weeks) to compare to previous studies in our laboratory (Dean, Jr. *et al.* 2002; Pott *et al.* 1998). Euthanasia was performed via aortic exsanguination following isofluorane anesthesia.

At necropsy, four representative liver sections were harvested from each animal, one each from the right posterior lobe and caudate lobe and two from the right anterior lobe. These were placed in 10% neutral buffered formalin (NBF) for fixation. Additional tissues placed in NBF included jejunum, kidneys, spleen, heart/lungs, stomach, pancreas, and thymus (if present), in addition to any lesions. Jejunal samples were used as internal positive controls for later cell proliferation and apoptosis staining. Samples of liver were pooled in groups of three and frozen for analysis of PCB126 levels. Body, liver, and thymus weights were recorded. All liver sections were embedded in paraffin and serially sectioned at 5μm thickness and mounted on positively charged microscope slides (two sections per slide). The first slide from each section of each animal was stained with hematoxylin and eosin (H&E).

Unstained sections were used for immunohistochemical staining for glutathione-S-transferase placental form (GST-P), TGFα, TGFβ, TGFβII-receptor, proliferating cell

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nuclear antigen (PCNA), and apoptosis. These sections were deparaffinized in xylene and rehydrated by passage through an alcohol series. With the exception of apoptosis slides (see below), endogenous peroxidase was quenched in 3% H_2O_2 for five minutes. The slides were rinsed with deionized (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 Na_2HPO_4). For antigen retrieval, liver sections were microwaved at full power until boiling in a citrate buffer (Biogenex Labs, San Ramon, CA USA). After boiling, sections were microwaved at a reduced power for ten minutes. A standard avidin biotin (ABC) protocol was followed using an immunoperoxidase kit (Vector Laboratories, Burlingame, CA USA). Individual procedures for antibody use, incubation times and tissue handling are briefly described below.

GST-P primary antibody (The Binding Site Inc., San Diego, CA USA) incubations were performed at 37 C for one hour. TGFα primary antibody (EMD Biosciences, La Jolla, CA USA) incubation was performed at room temperature for five hours, followed by secondary antibody (biotinylated anti-mouse IgG, Vector Laboratories, Burlingame, CA USA) incubation at room temperature for 30 minutes and a final 30 minute room temperature incubation with streptavidin horseradish peroxidase (Zymed Lab. Inc., San Francisco, CA USA). To detect TGFβ1 and TGFβII-r, rabbit polyclonal antibodies against TGFβ1 and TGFβII-r (Santa Cruz Biotechnology, Inc., Santa Cruz CA USA) were incubated at 4 C overnight at a dilution of 1:200 and 1:50, respectively. PCNA primary antibody (DAKO Corp., Carpenteria, CA USA) is incubated for 60 minutes at 37 C, followed by secondary antibody incubation at 37 C for 30 minutes.

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DNA apoptotic fragmentation was evaluated by optimizing the recommended method of a commercial apoptosis detection kit (ApopTag® Peroxidase In Situ Apoptosis Detection Kit, Serologicals Corporation, Norcross, GA USA). After deparaffination, liver sections were digested in 25μg/ml Proteinase K solution (Roche Diagnostics Corp., Indianapolis, IN USA) for 25 minutes at room temperature. Approximately 100μl of equilibration buffer containing digoxigenin-conjugated nucleotides (supplied by kit) was applied to each section for at least five minutes. Sections were then incubated with ApopTag® terminal transferase enzyme in a humidified slide chamber for 140 minutes at room temperature. Slides were then placed in a dish containing stop-wash buffer (supplied by kit) and incubated in a 37 C water bath for 30 minutes, followed by a PBS wash.

In all cases, a final incubation with the chromagen 3-amino-9-ethyl carbazole (AEC; Biomeda Corp., Foster City, CA USA) was used. All slides were counterstained with Gill's Hematoxylin (Lerner Laboratories, Pittsburgh, PA USA) and mounted with coverslips with water-based mounting media (Supermount, Biogenex, San Ramon, CA USA) for histologic evaluation.

Area and number of GST-P and TGFα positive foci were measured using an Olympus BX51 light microscope (Olympus Optical Co., LTD., Tokyo, Japan) coupled with an Optronics DEI-750CE microscope mounted digital camera (Optronics, Goleta, CA USA) and a stage-mounted Microcode II Digital Readout (Boeckeler Instruments, Inc., Tuscon, AZ USA). Image analysis software was the BIOQUANT NOVA for Windows 98 (Version 5.00.8) computerized histomorphometry program (B&M Biometrics Inc., Nashville, TN USA), installed in an AOpen PIII-700 computer (AOpen,

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Inc., Taipei, Taiwan). Measurements consisted of manually outlining each liver section and its stained foci and utilizing the computer software to compute the total liver area and total focus area. Since all cells in a focus did not stain positively for TGFα, a digital overview from the GST-P results was superimposed in order to best approximate the overall area of the focus, not just the TGFα positive portion. Foci were considered positive for TGFα if the focus could accurately be matched to a previously traced GST-P positive focus of greater than 200μm diameter and at least three hepatocytes stained positive for TGFα. Results for the control and treatment groups were expressed as areas of large foci (greater than 200μm in diameter) in mm^2 per area of liver sections examined in cm^2 or number of large foci per cm^2. Formation of foci larger than 200μm in diameter have correlated very well with the development of hepatocellular carcinomas in medium-term liver bioassays (Ito *et al.* 1988; Ito *et al.* 1989b). Final quantitative analysis was performed using an Excel spreadsheet (Microsoft Corporation, Redmond, WA USA).

Representative samples of liver tissues were pooled (three rats per sample, two samples per sacrifice point for each group) and snap frozen for analysis of PCB 126 levels. A HP-5890 Series II Plus gas chromatograph and HP-6890 series auto-injector (Hewlett-Packard Company, Palo Alto, CA USA) with electron capture detection and was used to determine PCB levels in liver samples. Tissues were extracted using a modification of published procedures (Mills *et al.* 1963). Analysis was performed with a DB-5 capillary column (crosslinked 5% phenylmethylsilicone, 30m X 0.32mm, Supelco, Bellefonte, PA USA). Each set of samples included one reagent blank and a series of spiked controls for calibration. This was performed by M. Lohitnavy of the Quantitative

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and Computational Toxicology Group of the CSU Department of Environmental and Radiological Health Sciences.

Numbers and area of GST-P and TGFα foci greater than 200μm in diameter were analyzed using nonparametric analysis of variance (ANOVA, Kruskal-Wallis test). This was followed by comparisons of the PCB and As treatment groups to DEN and saline control groups via unpaired t-test with Welch correction and a two-tailed p value. Changes in organ and body weights were analyzed by one-way ANOVA using Tukey-Kramer multiple comparisons procedure. All statistical tests were performed using the GraphPad Instat software package, version 3.00 (GraphPad Software, Inc., San Diego, CA USA). All results are expressed as means +/- standard deviation.

Results

Clinical signs

Over the course of the study, several adverse clinical effects were seen in animals in some of the treatment groups. Infrequent deaths of animals (three total), unrelated to group assignment, occurred after partial hepatectomy but prior to the specified sacrifice times. Necropsy findings for these early deaths consisted of severe cirrhosis of the liver and corresponding icterus due to accidental ligation of the bile ducts during surgery. These animals were not incorporated in final analyses. Rats in the arsenic-exposed treatment groups (D, E, and G) exhibited progressive clinical signs of wasting and dehydration, beginning at approximately five weeks after the start of As treatment. Two animals died, one after 5.5 weeks of exposure, the other after seven weeks, both exhibiting marked dermal and subcutaneous hemorrhage on the tail in addition to their

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severely wasted condition. After reduction of the As concentration in the drinking water to 7.5ppm the surviving rats recovered without further acute signs or mortality. At final sacrifices at all time points (56, 112, and 168 days), rats with exposure to both concentrations of As exhibited more visible dermal and subcutaneous hemorrhages and bruising of the tail compared to non-As-exposed animals (but less severe than that seen with the Week 8 As deaths). No other significant clinical signs were observed in rats over the course of the experiment.

Since all data on weights or liver foci from the ENU-treated and ENU control animals did not differ from the DEN controls, the ENU data are not shown in any of the figures.

Body and Tissue Weights

A separate group of PCB/As early animals was sacrificed at 56 days (8 weeks) for comparison to earlier studies. All data on the 8-week sacrifice group are shown in Table 3.2. The remaining data on all of the treatment groups sacrificed at 112 days (16 weeks) or 168 days (24 weeks) are shown in Figures 3.2 - 3.7.

Body weights for animals in Groups A (saline control), B (DEN control), H (saline IP control) and J (ENU control) were not significantly different from one another at both the 16-week and 24-week sacrifice points (Figures 3.2 and 3.3). Animals in all groups exposed to PCB 126 and/or As exhibited decreased body weights at sacrifice when compared to the saline or DEN control animals. At the 16-week sacrifice time, all treatment groups showed significant decreases in body weight, with the most significantly lowered weights in animals receiving both PCB and As late. By 24 weeks,

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Test	Result
Body Weight	**139.400 +/- 12.294g**
Liver Weight	**5.441 +/- 0.547g**
Thymus Weight	**0.097 +/- 0.020g**
Kidney Weight	**1.154 +/- 0.080g**
Spleen Weight	**0.348 +/- 0.106g**
GST-P Large Foci Area	**0.853 +/- 0.172mm^2/cm^2 liver**
GST-P Large Foci Number	**10.880 +/- 3.061 per cm^2 liver**
TGFα Large Foci Area	**0.277 +/- 0.107mm^2/cm^2 liver**
TGFα Large Foci Number	**2.540 +/- 0.445 per cm^2 liver**
PCB 126 Level	**602.25 +/- 350.57ng/g liver**

Table 3.2. All data on the 8-week sacrifice Group E, PCB/As early.

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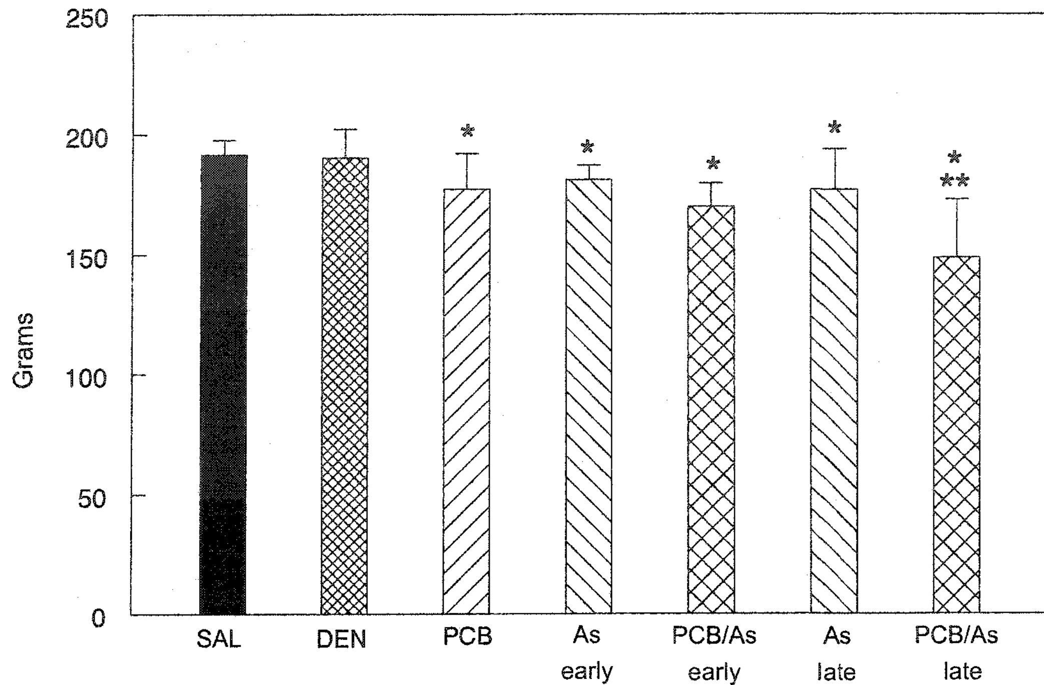

Figure 3.2. Body weights of exposed rats, 16 weeks. Decreased body weights were seen in all the treatment groups when compared to controls, and the PCB-As late group showed significantly reduced body weights compared to PCB alone.

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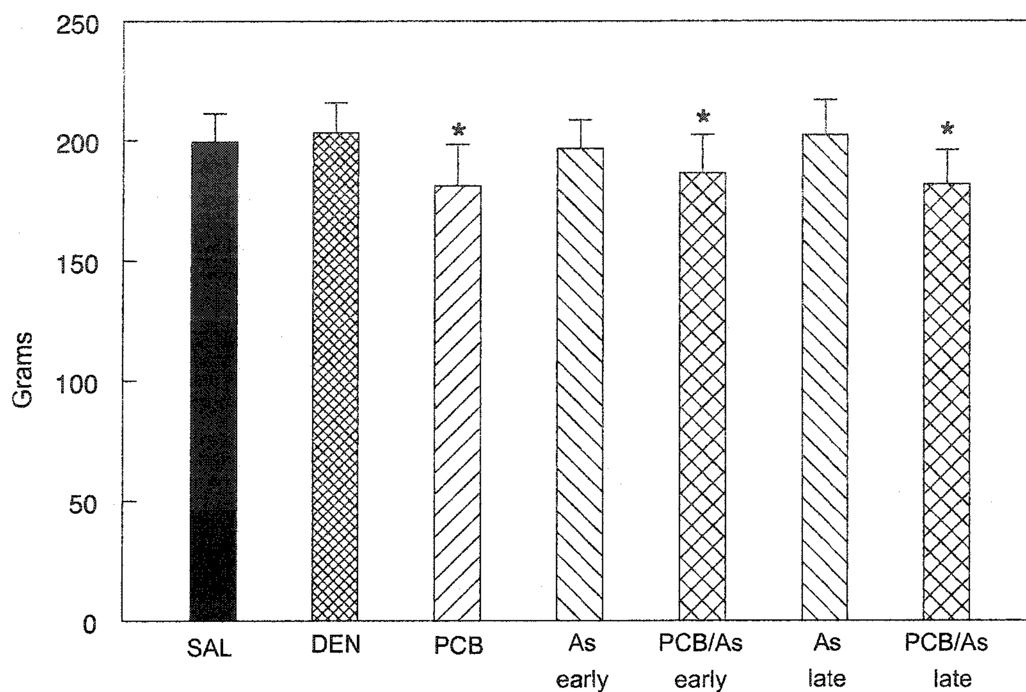

* Difference from DEN & SAL control, P<0.05

Figure 3.3. Body weights of exposed rats, 24 weeks. Decreased body weights were seen only in groups exposed to PCB 126, alone or in combination with As.

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only rats receiving PCB had statistically significant lower body weights. Animals in the PCB/As-early Group E sacrificed at 8 weeks had lower body weights than Group E animals sacrificed at 16 and 24 weeks, with increasing values over time (8 weeks vs. 16, $P < 0.0001$; 16 weeks vs. 24, $P = 0.0102$).

Liver weights for animals at 16 weeks in Groups A (saline control), B (DEN control), and D (As early, starting at 14 days) were not significantly different from one another (Figure 3.4). Both Groups C (PCB only) and E (PCB + As early) exhibited significantly increased liver weights. Groups F (As late, starting at 56 days) and G (PCB + As late) showed decreased liver weights when compared to the DEN control group, but not the saline control group. At 24 weeks, liver weights of rats in Groups A (saline control), B (DEN control), D (As early), and F (As late) were not statistically different from one another (Figure 3.5). Also at 24 weeks, all three groups receiving PCB, alone and in combination with As, exhibited increased liver weights. Animals in the PCB/As-early Group E sacrificed at 8 weeks had lower liver weights than Group E animals sacrificed at 16 and 24 weeks, with increasing values over time (8 weeks vs. 16, $P < 0.0001$; 16 weeks vs. 24, $P < 0.0001$).

Thymus weights were decreased in animals exposed to PCB 126 with or without As (Groups C, E, G) when compared to DEN (B) and saline (A) controls at both sacrifice time points (Figures 3.6 and 3.7). At 16 weeks, there was a statistically significant decrease in thymus weights for rats in Group G (PCB + As late) when compared to those in Group C (PCB only) (Figure 3.6). Animals in the PCB/As-early Group E sacrificed at 8 weeks had the highest thymus weights among the three Group E time points, followed by the 16-week animals and finally the 24-week animals with the lowest weights. For all

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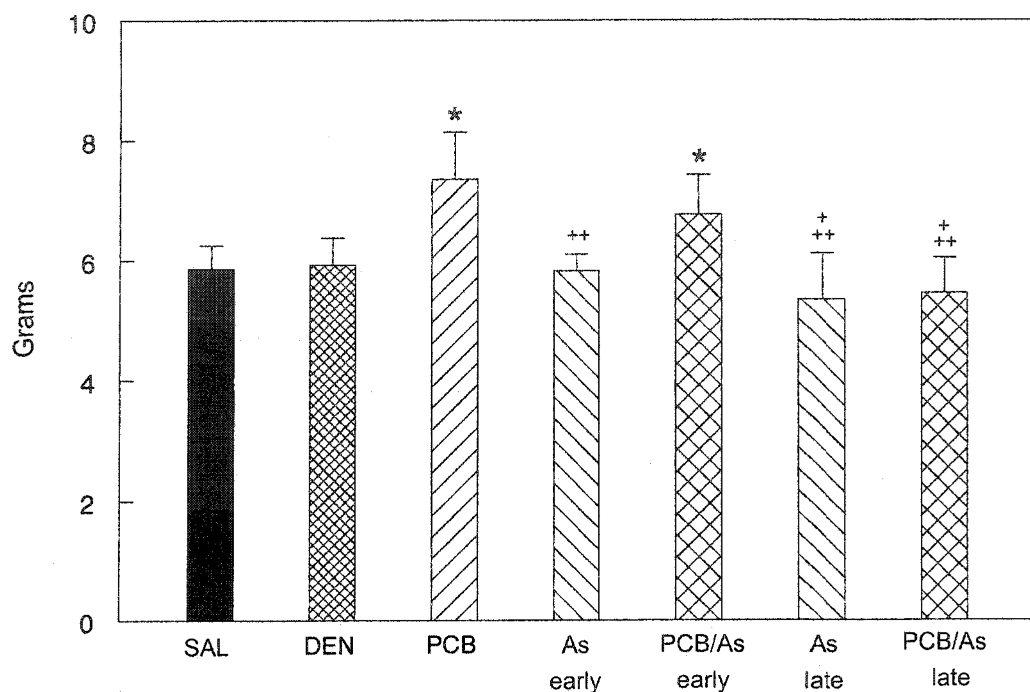

* Difference from DEN & SAL control, P<0.005

+ Difference from DEN control only, P<0.05

++ Difference from PCB & PCB/As early, P<0.001

Figure 3.4. Liver weights, 16 weeks. Increased liver weights were seen with animals in two of the groups exposed to PCB 126 compared to saline and DEN control groups. There was a significantly decreased liver weight of animals in the two As-only groups and the PCB-As late group when compared to PCB alone.

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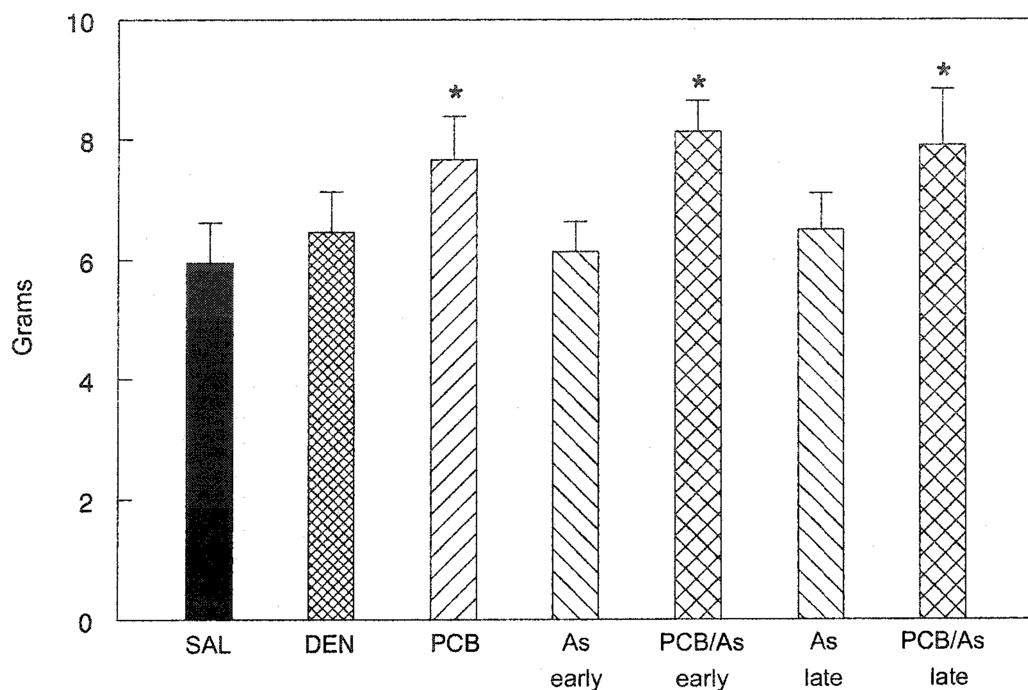

Figure 3.5. Liver weights, 24 weeks. Increased liver weights were seen with animals in all groups exposed to PCB 126 compared to saline and DEN control groups. The decreased liver weights relative to PCB alone seen in the As-only and PCB/As late groups at 16 weeks are absent at 24 weeks.

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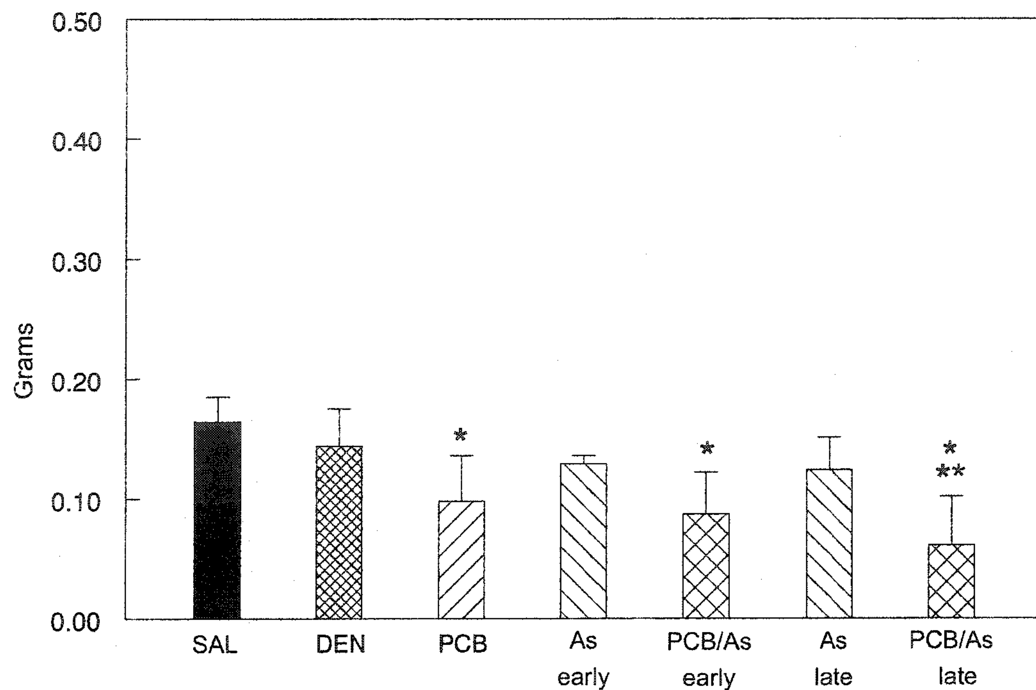

* Difference from DEN & SAL control, P<0.005

** Difference from PCB alone, P<0.05

Figure 3.6. Thymus weights, 16 weeks. Decreased thymus weights are seen in all groups exposed to PCB 126, alone or in combination with As. In addition, the rats of the PCB/As late Group G exhibited thymus weights significantly below those of animals exposed to PCB 126 as a single agent.

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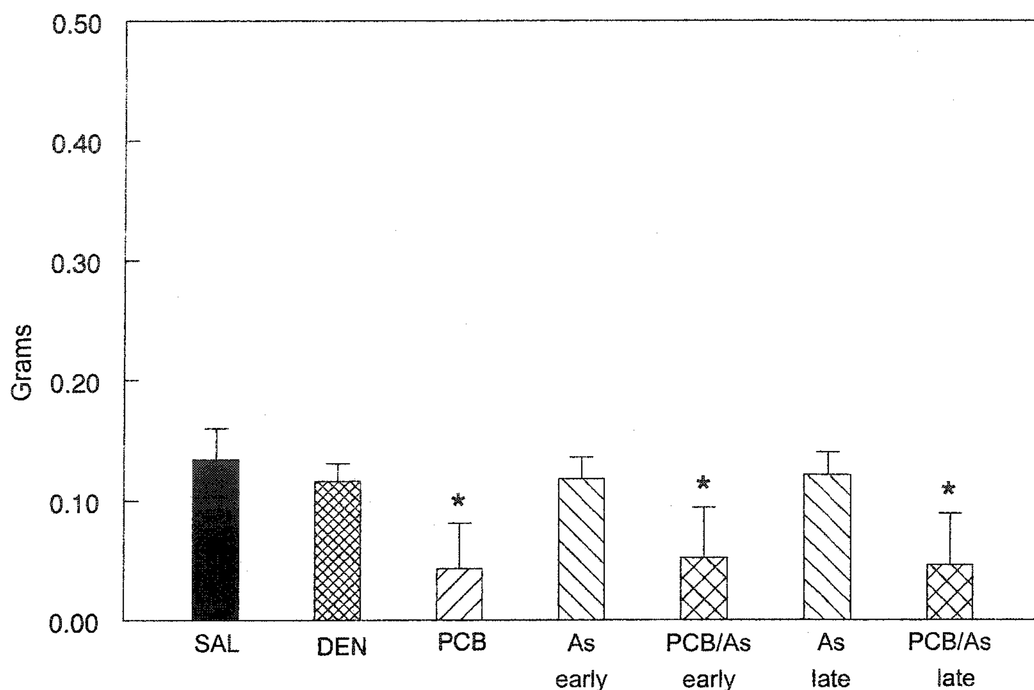

Figure 3.7. Thymus weights, 24 weeks. Decreased thymus weights are seen in all groups exposed to PCB 126, alone or in combination with As. Note the general decreases in thymus weight for all groups compared to the 16-week samples, likely a result of normal physiologic thymic involution with advancing age and maturity.

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experimental groups, thymus weights decreased a small amount between 16 and 24 weeks, a result of normal physiologic thymic involution with advancing age and maturity. Kidney weights were essentially unaffected by treatment, except for a significant decrease at 16 weeks with Group G (PCB + As late) when compared to DEN (B) and saline (A) controls (G = 1.184g +/- 0.102, B = 1.272g +/- 0.091, A = 1.284g +/- .083, $P < 0.05$). With regards to splenic weight, the only significant difference at 16 weeks was an increased weight in Group C (PCB only) compared to Group A (saline control) (C = 0.546g +/- 0.052, A = 0.493g +/- 0.029, $P < 0.01$). At 24 weeks, Group A (saline control) exhibited a statistically significant decrease in splenic weight compared to all other groups (A = 0.494g +/- 0.024, next lowest value D = 0.554g +/-.047, $P < 0.05$).

Histologic evaluation

Histologic examination of tails performed in a sample of the arsenic-exposed animals revealed dermal and subcutaneous congestion and hemorrhage. This was more pronounced in the two animals that died from As toxicity prior to the dilution of As in the drinking water at 8 weeks (see Materials and Methods).

Routine histologic screening (H&E stain) of the livers of rats sacrificed at the defined time points revealed no significant findings other than preneoplastic foci. Animals that died prior to their assigned sacrifice times due to biliary ligation exhibited marked hepatocyte loss with fibrosis, biliary hyperplasia and multifocal nodular hepatocyte regeneration. Preneoplastic foci (foci of altered hepatocytes) were primarily of the eosinophilic, clear-cell, or mixed-cell type (Figures 3.8, 3.9, and 3.10).

All rats that died prior to designated sacrifice times were excluded from all further analyses.

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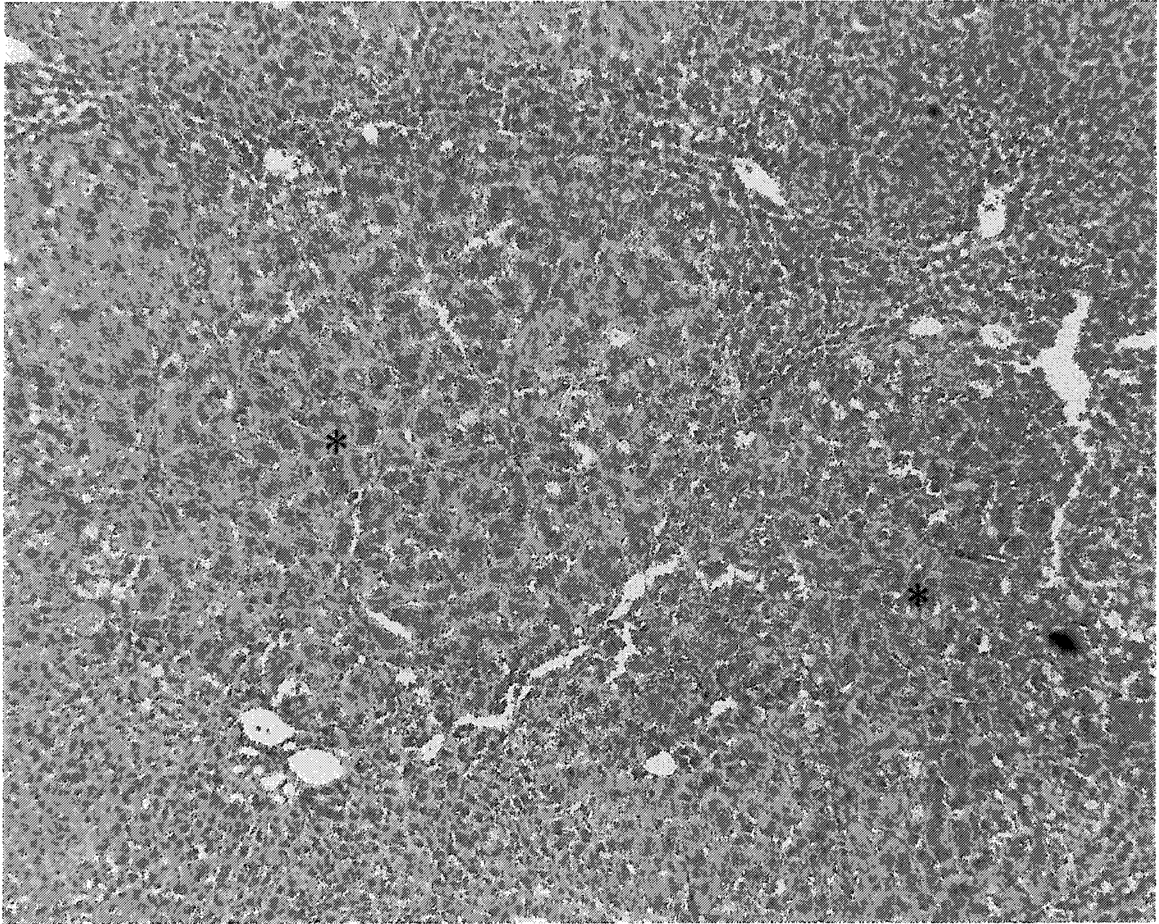

Figure 3.8. Photomicrograph of two eosinophilic foci of altered hepatocytes (*) from a rat exposed to 22 weeks of PCB 126. Hematoxylin & eosin stain, 40X magnification.

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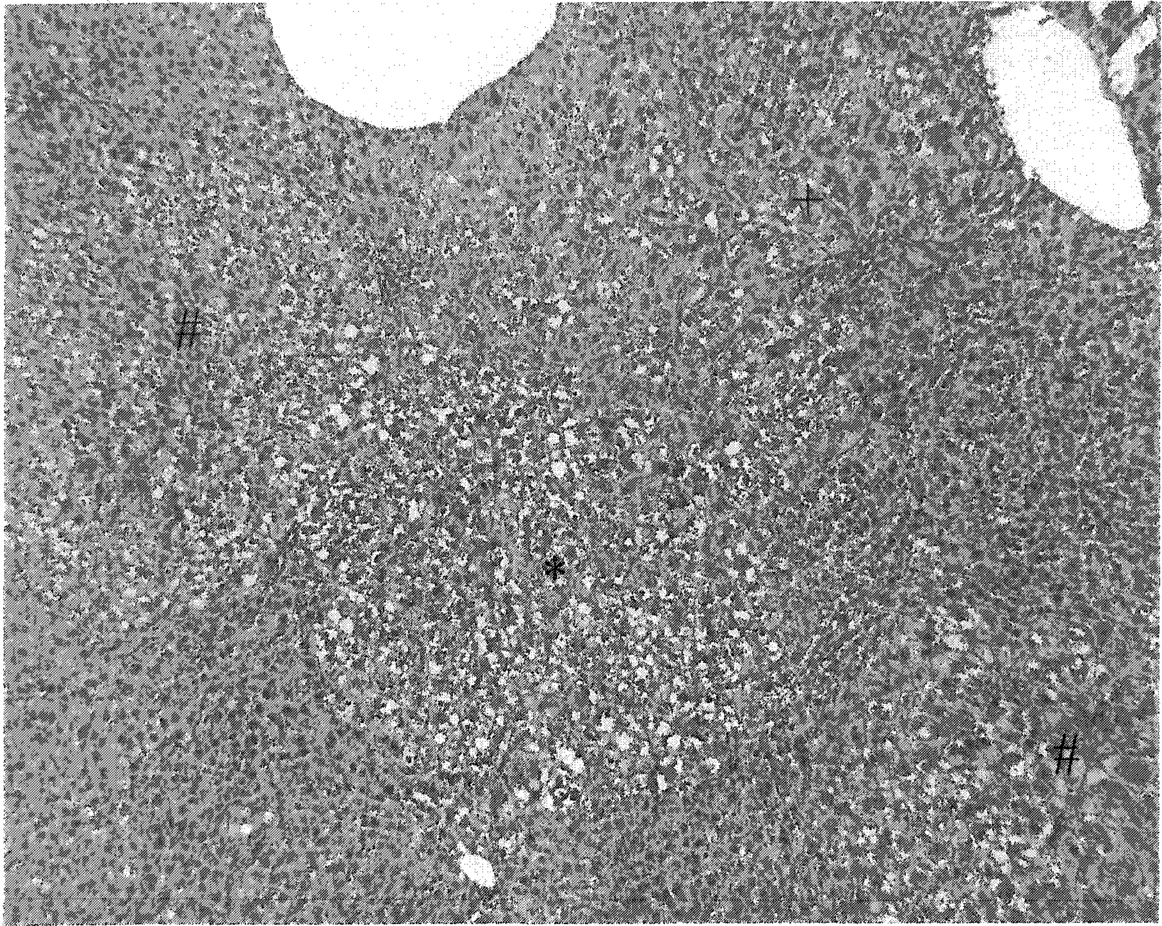

Figure 3.9. Photomicrograph of a clear cell preneoplastic focus (*), an eosinophilic cell focus (+), and two mixed cell foci (#) in the liver of a rat in the PCB/As early Group G, sacrificed at 24 weeks. Note the compression of the surrounding hepatic parenchyma.

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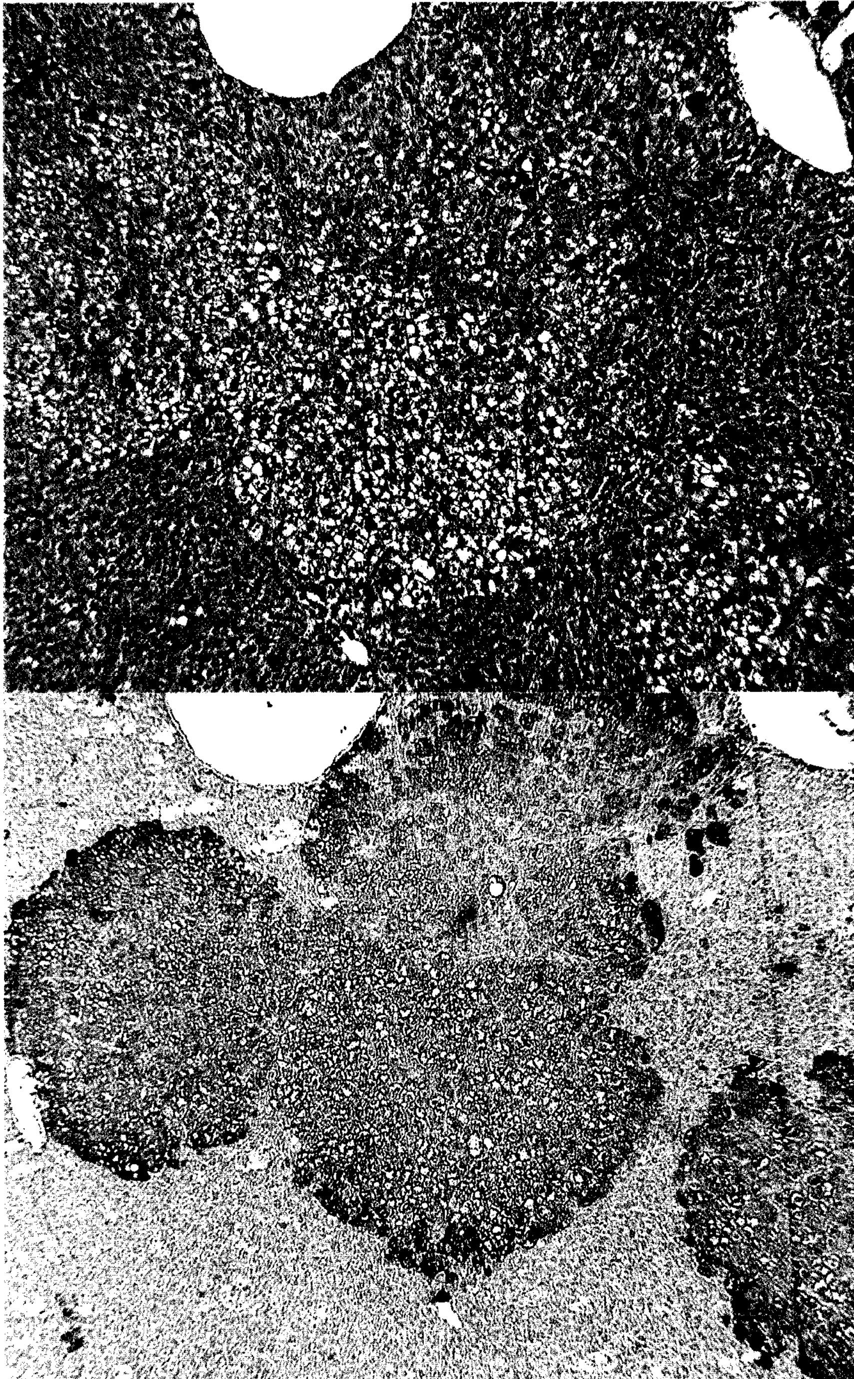

Figure 3.11. Photomicrograph of multiple preneoplastic foci in the liver of a rat in the PCB/As early Group E, sacrificed at 24 weeks (see Fig. 3.9). H&E (top) vs. GST-P (bottom). Note the easily discernible demarcation of the foci from the surrounding tissue using GST-P immunostain that is less clear with H&E.

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GST-P

Animals exposed to DEN as a single agent (DEN control, Group B) showed increased number and relative area of large (>200μm diameter) GST-P positive preneoplastic liver foci (Figure 3.11) when compared to saline alone (Group A) at all sacrifice points (Figures 3.12 - 3.15). Foci smaller than 200μm diameter in general followed the trends of the larger, but these were not analyzed morphometrically since they showed no real differences and measurement was less accurate than for the larger foci. Experimental groups exposed to PCB 126, alone (Group C) or in combination with As (Groups E and G), exhibited both increased number and relative area of GST-P positive foci, compared to DEN and saline control groups at both 16 and 24 weeks. Arsenic as a single agent started early (Group D) or late (Group F) did not increase or decrease number or area of GST-P foci compared to the DEN control group at either the 16 or 24-week time points. In general, the number and relative area of preneoplastic foci increased with time between the 16- and 24-week sacrifice time points. Within the PCB/As early Group E, there were significant increases in both relative area and number of GST-P+ foci from the 8-week sacrifice point to the 16- and then 24-week points (Area: 8 weeks vs. 16, $P = 0.0007$; 16 weeks vs. 24, $P = .0033$; Number: 8 weeks vs. 16, $P = 0.0081$; 16 weeks vs. 24, $P = 0.0237$).

Groups exposed to PCB/As early and late (Groups E and G) exhibited statistically significantly decreased relative area of GST-P preneoplastic foci at 16 weeks compared with the PCB only treatment (Group C) (Figure 3.12). The PCB/As late group showed a similar change at 24 weeks (Figure 3.13). Numbers of foci were similarly reduced at 16 weeks for both the PCB/As groups (Figure 3.14) and in the 24 week PCB/As late group

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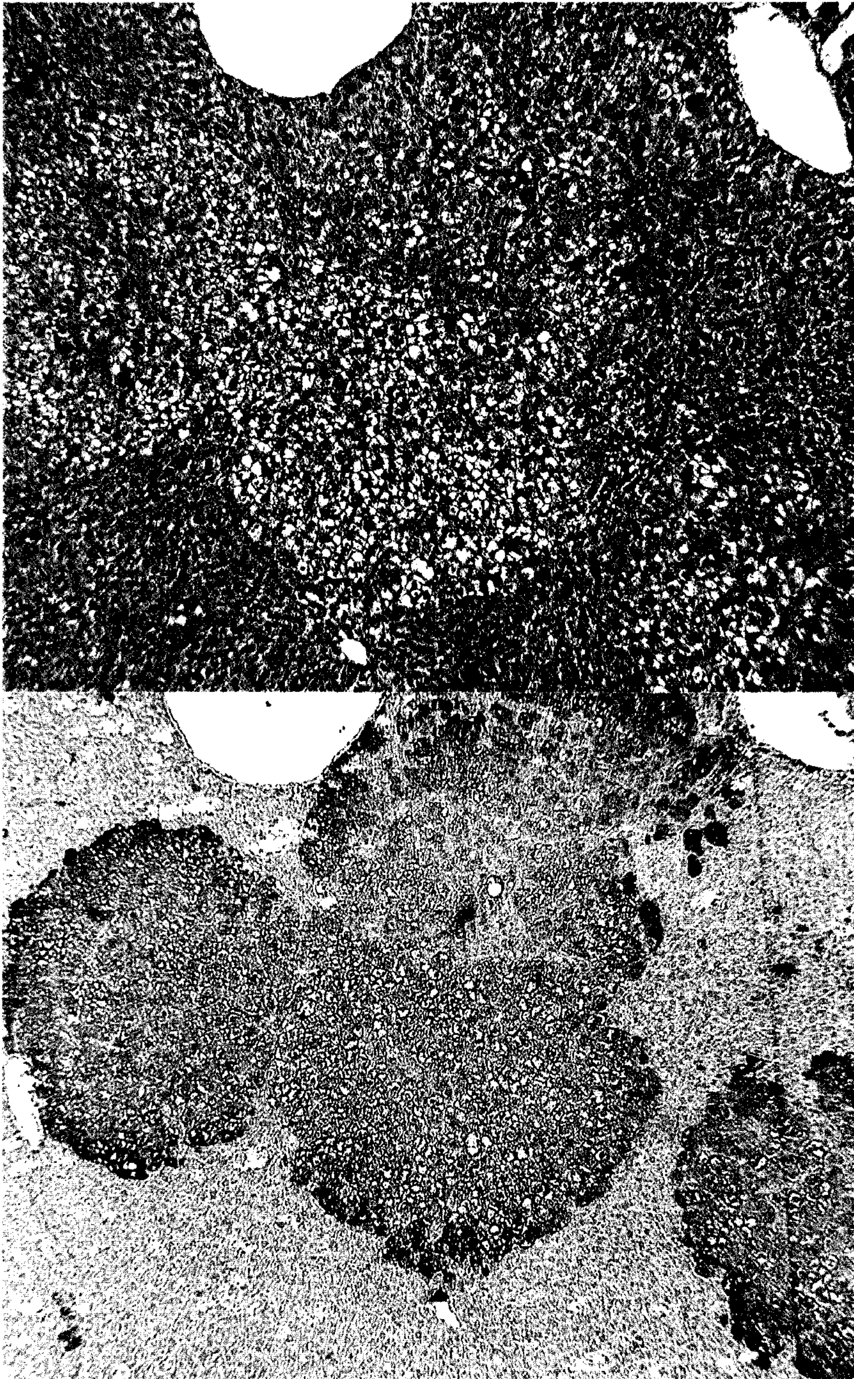

Figure 3.11. Photomicrograph of multiple preneoplastic foci in the liver of a rat in the PCB/As early Group E, sacrificed at 24 weeks (see Fig. 3.9). H&E (top) vs. GST-P (bottom). Note the easily discernible demarcation of the foci from the surrounding tissue using GST-P immunostain that is less clear with H&E.

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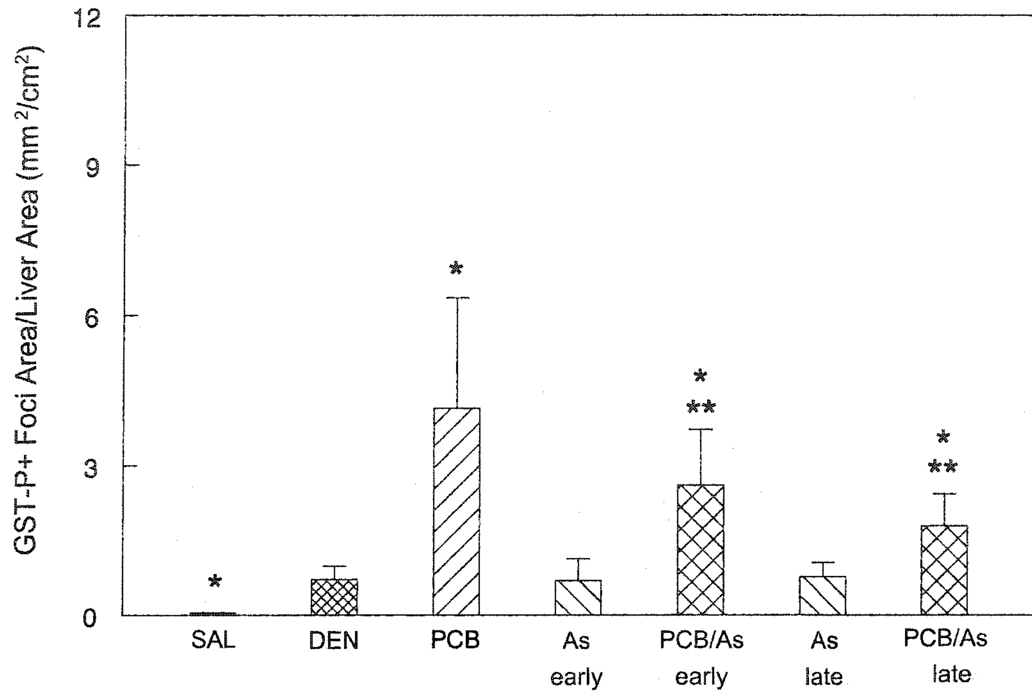

* Difference from DEN control, P<0.0005

** Difference from PCB126 alone, P<0.05

Figure 3.12. Relative area of GST-P+ foci > 200μm, 16 weeks. The three groups exposed to PCB 126 all exhibit increased relative area of GST-P+ foci compared to DEN and saline control groups. There is an inhibitory effect of concurrent As exposure with PCB.

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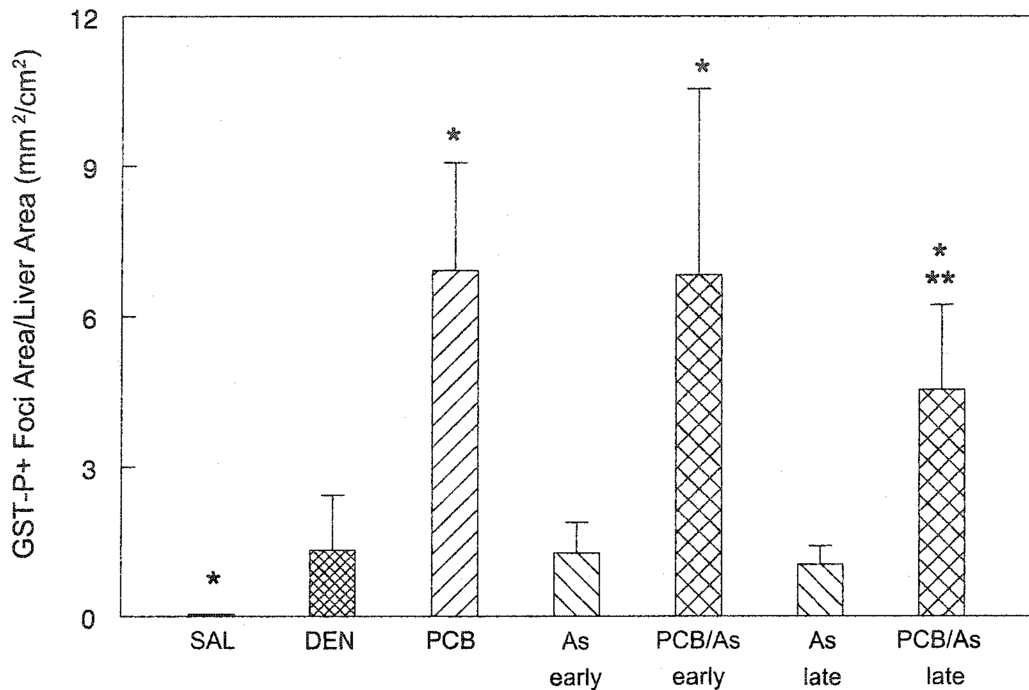

Figure 3.13. Relative area of GST-P+ foci > 200μm, 24 weeks. The three groups exposed to PCB 126 all exhibit increased relative area of GST-P+ foci compared to DEN and saline control groups. The inhibitory effect of concurrent As exposure with PCB seen at 16 weeks is now limited to the PCB/As late group.

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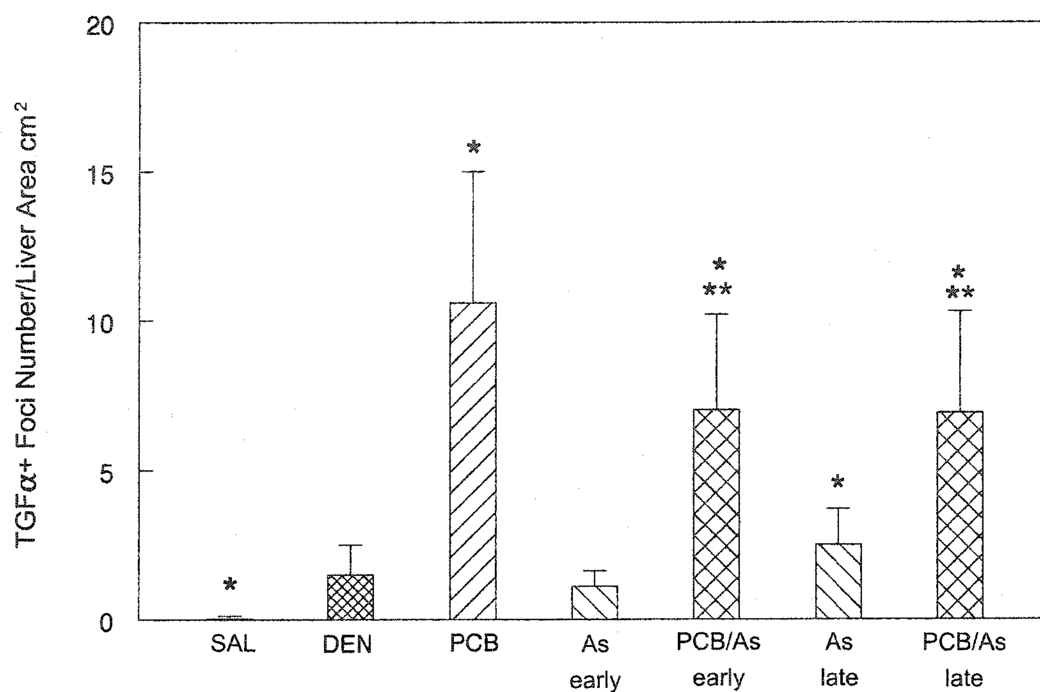

* Difference from DEN control, P<0.05

** Difference from PCB126 alone, P<0.04

Figure 3.14. Relative GST-P number, 16 weeks. The number of GST-P+ preneoplastic foci is increased in all three PCB-exposed groups compared to DEN and saline control groups.

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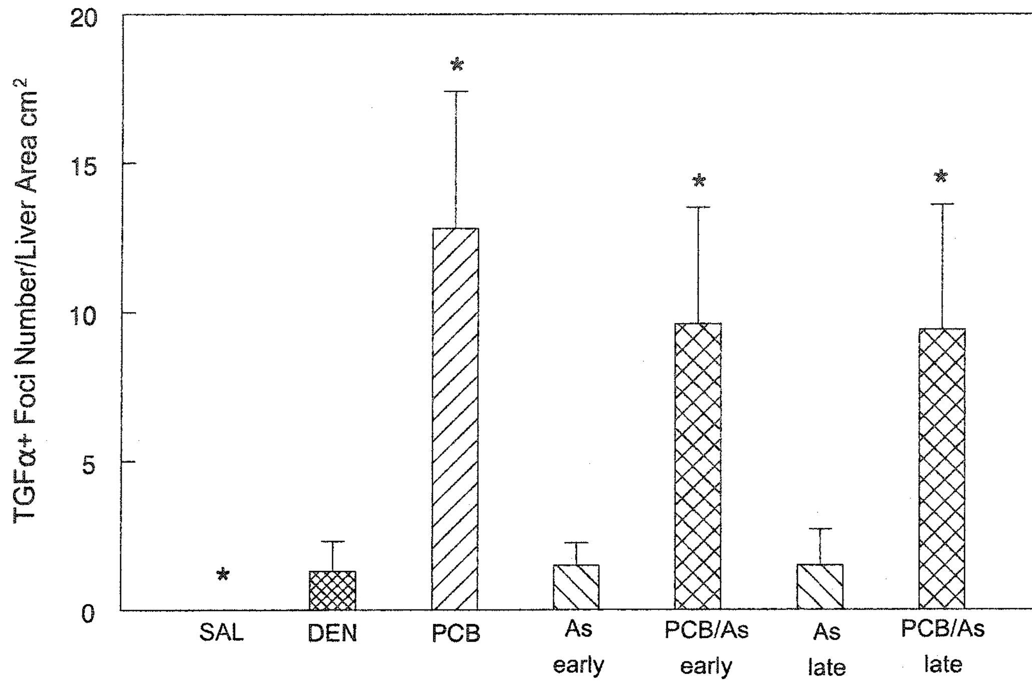

* Difference from DEN control, P<0.0001

Figure 3.15. Relative GST-P number, 24 weeks. The number of GST-P+ preneoplastic foci is increased in all three PCB-exposed groups compared to DEN and saline control groups.

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compared with the PCB only Group C (Figure 3.15), but this was not statistically significant. There was no significant difference in area or number of foci between the DEN controls and the two As-only treated groups, either the As-early (Group D) or the As-late (Group F) at either time point.

TGFα

Larger GST-P+ preneoplastic foci were much more likely to stain positively for TGFα, and only those greater than 200μm in diameter were included for morphometric analysis. Within the subset of larger foci, the larger the focus, the more likely it was to stain for TGFα. Of the GST-P+ large foci, approximately 40% also stained positively for TGFα and these comprised approximately 60% of the total focus area (Figure 3.16). Staining characteristics among the foci varied, with many of the largest ones having a more peripheral pattern to their TGFα staining, while the smaller (still greater than 200μm) tended to stain more diffusely (Figure 3.17). Nonspecific staining for TGFα was present on the periphery of sections and around blood vessels. These areas were not incorporated unless part of a previously-found GST-P+ focus.

Immunohistochemical staining for the positive growth factor TGFα tended to mirror the GST-P morphometrical results. At both 16 and 24 weeks, animals exposed to DEN as a single agent (DEN control, Group B) showed increased number and relative area of large (>200μm diameter) TGFα positive preneoplastic liver foci when compared to saline alone (Group A) (Figures 3.18 - 3.21). The two As-only groups early and late (D and F) had similar numbers and relative areas of TGFα+ foci when compared to DEN controls (Group B) at both time points. One exception was a small but statistically

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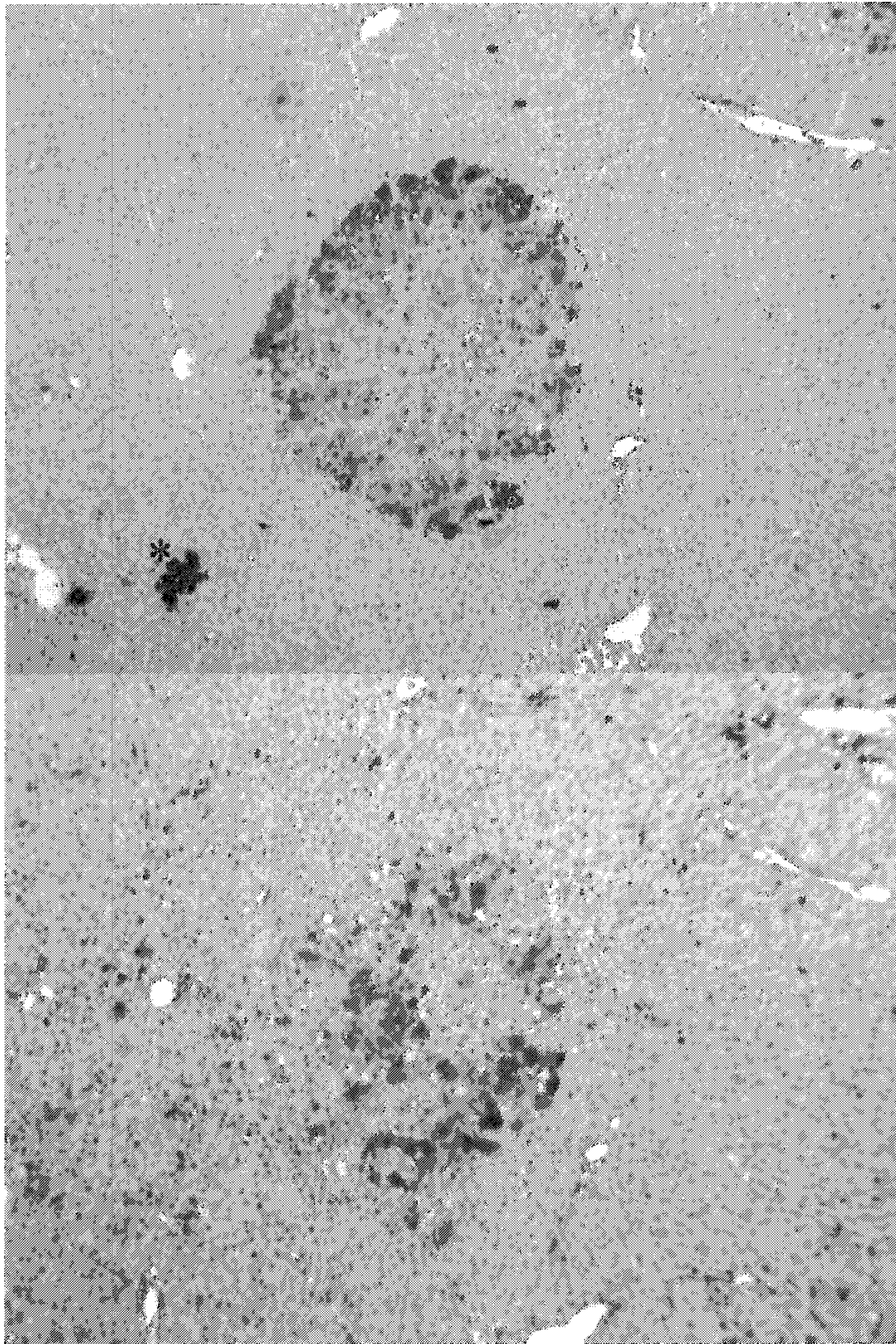

Figure 3.16. Photomicrograph of preneoplastic focus in the liver of a rat in the PCB/As early Group E, sacrificed at 24 weeks. GST-P (top) vs. TGFα (bottom). Note relative uniformity of GST-P stain vs. TGFα, and small GST-P+ focus (*) that is TGFα negative. 40X magnification.

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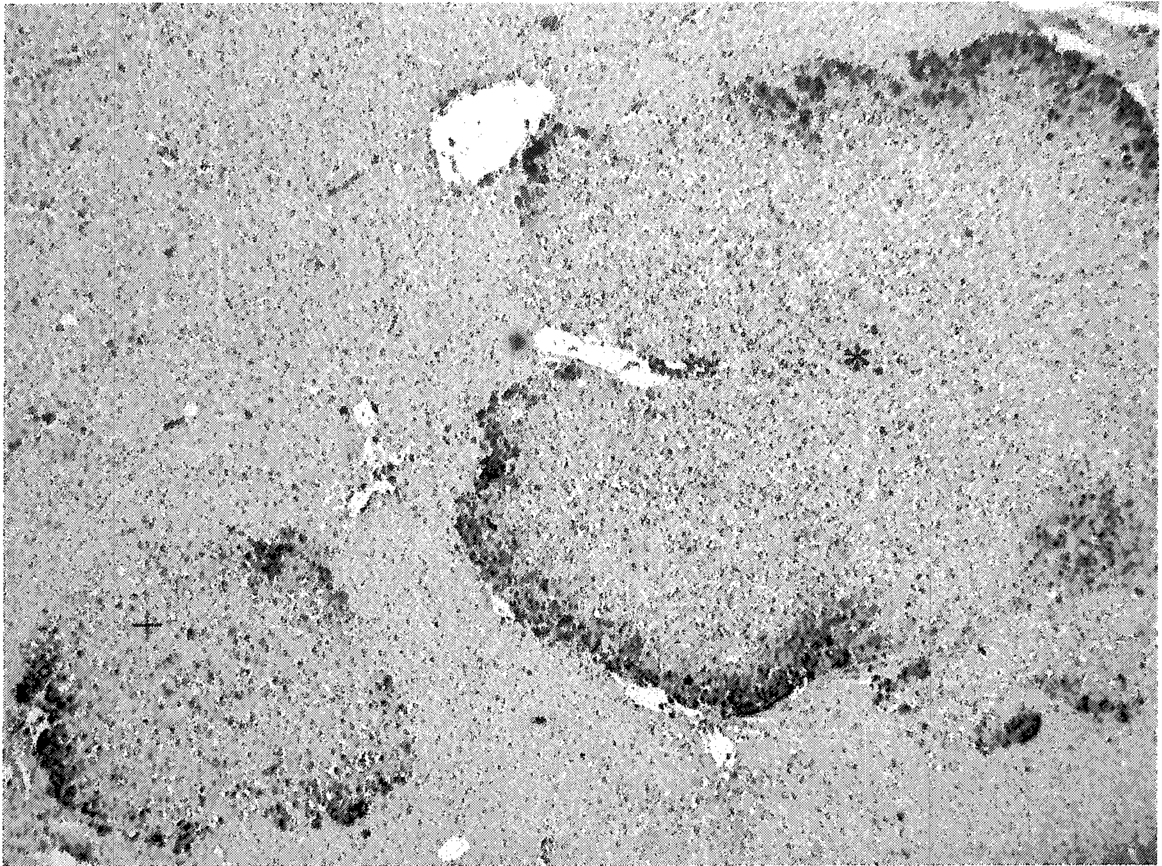

Figure 3.17. Photomicrograph of two preneoplastic foci in the liver of a rat in the PCB/As late Group G, TGFα stain. Note the marked peripheral staining pattern in the larger (*) of the two foci. Although the smaller focus (+) has peripheral staining as well, it also has more visible staining of the interior.

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significant increase in foci number at 16 weeks for the As-late Group F but this was absent at 24 weeks.

Both number and relative area of TGFα+ foci were significantly decreased in both groups with concurrent PCB-As exposure (E, G), compared to PCB 126 alone (C) at 16 weeks (Figures 3.18 and 3.20). While the area of both the PCB/As groups was also decreased at 24 weeks, this was not a statistically significant difference. These findings paralleled the reduction of GST-P positive foci in the PCB/As groups. Comparing the two PCB/As early or late groups (E, G) revealed no significant differences between them. At 24 weeks, foci area was significantly less in the PCB/As late group compared with the PCB/As early group (figure 3.19) but foci number was equivalent (Figure 3.21). Animals in Group E (PCB/As early) at the 8-week sacrifice point had significantly less relative area and number of TGFα+ foci when compared to either the 16- or 24-week sacrifice points (Area: 8 weeks vs. 16, $P = 0.0008$, 8 weeks vs. 24, $P = 0.0006$; Number: 8 weeks vs. 16, $P = 0.0011$, 8 weeks vs. 24, $P = 0.0003$).

TGFβ1 & TGFβII-r

Immunohistochemical stain for TGFβ1 revealed little difference in staining intensity between the preneoplastic foci (GST-P+ and TGFα+) and the surrounding hepatic tissue. Nonspecific positive staining was widespread, especially on the periphery of the individual liver sections, surrounding blood vessels, within randomly scattered hepatocytes, and within sinusoids. Although all of the tissue section had a pale red stain throughout, the truly positive cells were colored bright red and stood out from the background. These randomly positive cells were scattered in equal numbers both within and outside foci.

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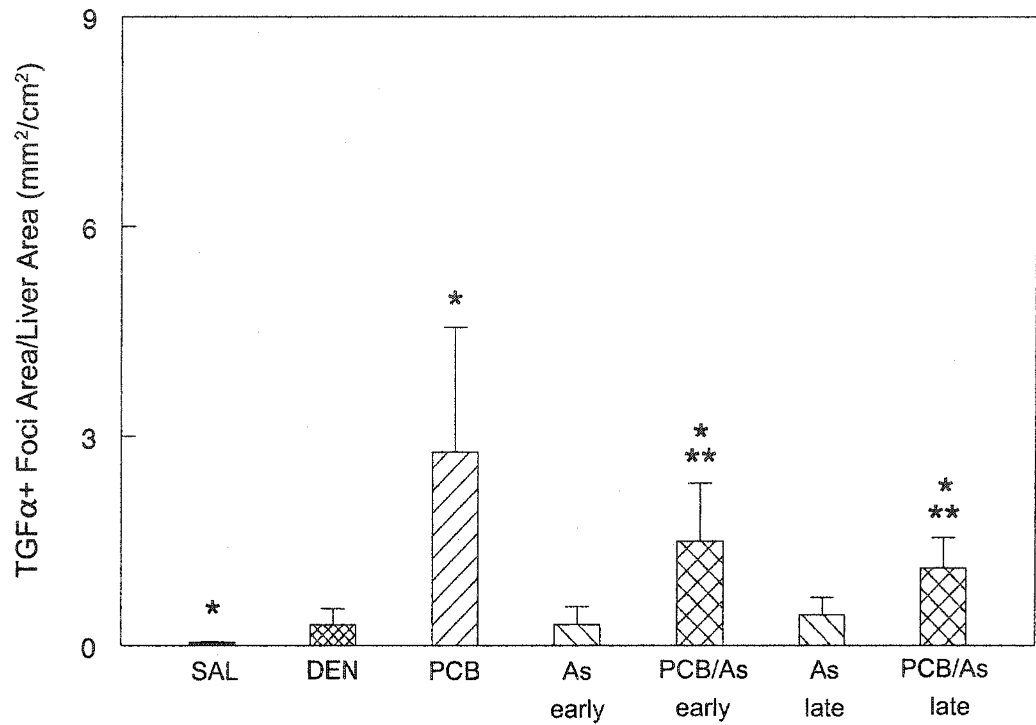

Figure 3.18. Relative area, TGFα positive foci, 16 weeks. Note significant increases in all three groups exposed to PCB 126, compared to DEN and saline control groups, and significant inhibitory effect of concurrent As exposure.

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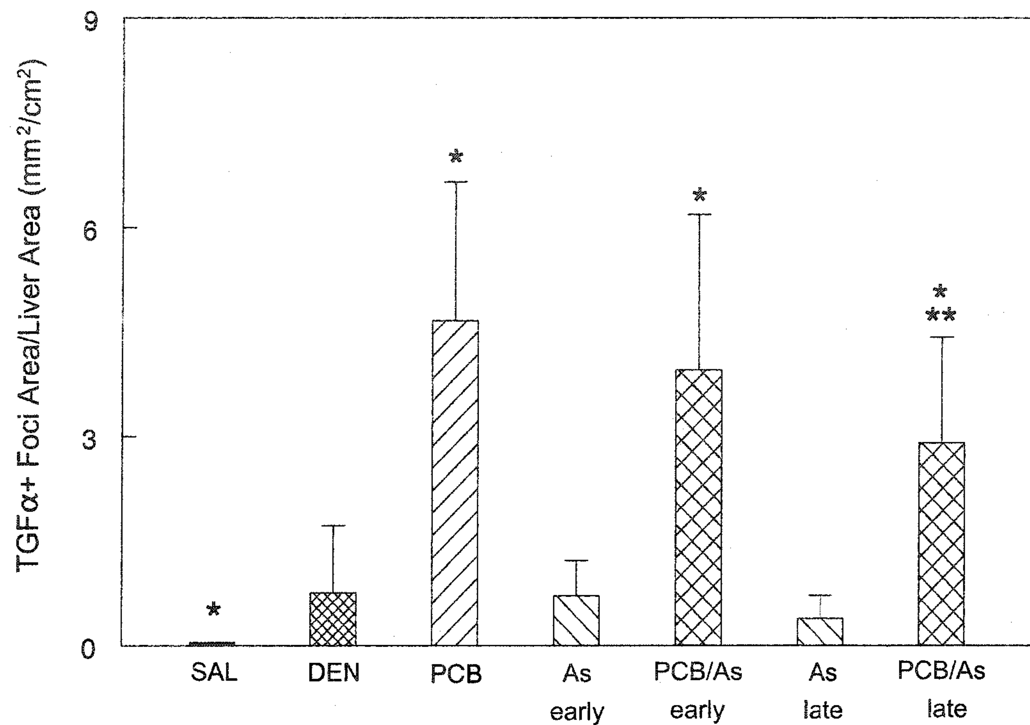

Figure 3.19. Relative area, TGFα positive foci, 24 weeks. Note significant increases in all three groups exposed to PCB 126, compared to DEN and saline control groups, with significant inhibitory effect of concurrent As exposure only present in the PCB-As late group.

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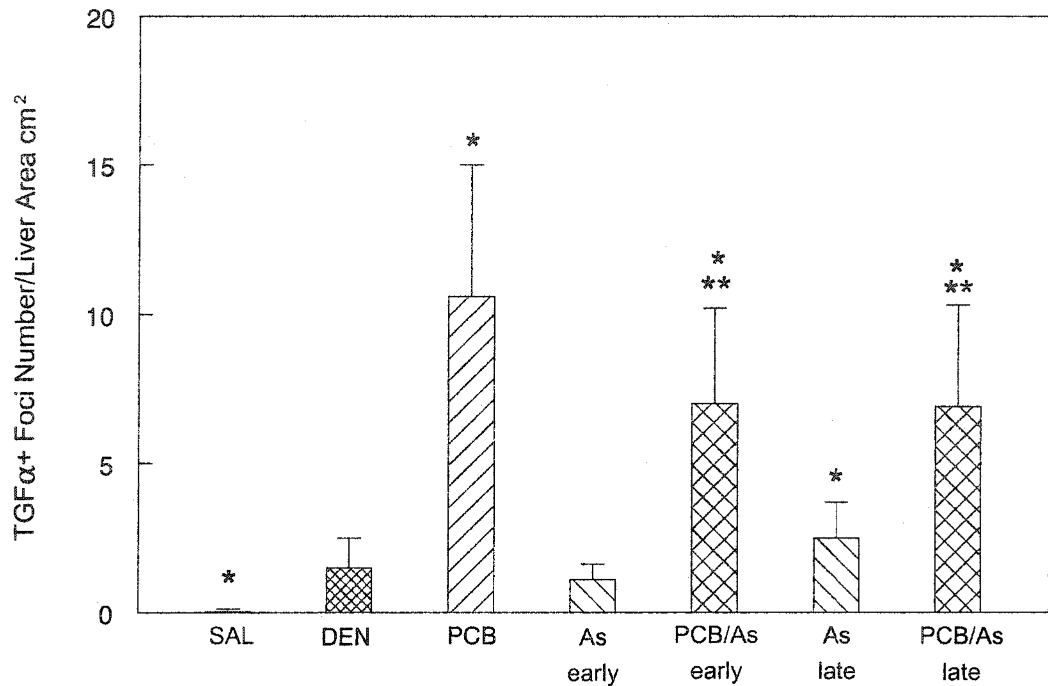

Figure 3.20. Relative number, TGFα positive foci, 16 weeks. All PCB-exposed groups, as well as the As-only late group, show increased numbers of TGFα+ foci compared to DEN and saline control groups. Note the inhibitory effect in both of the PCB/As groups compared with PCB treatment alone.

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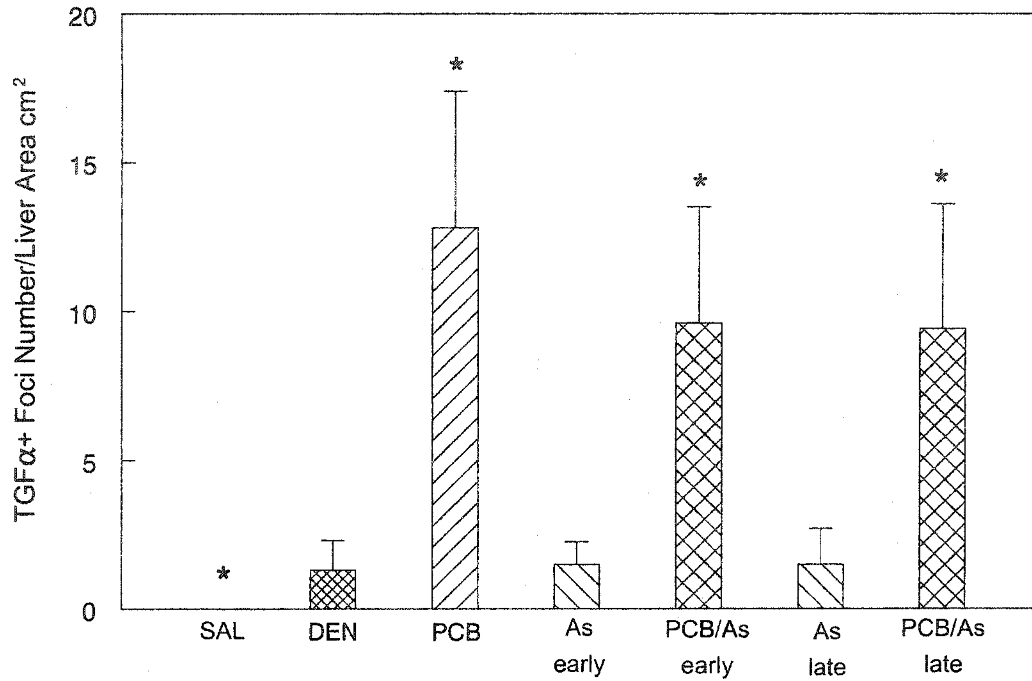

Figure 3.21. Relative number, TGFα positive foci, 24 weeks. All PCB-exposed groups exhibit increased numbers of TGFα+ foci compared to DEN and saline control groups. The inhibitory effect of As with PCB seen at Week 16 is no longer statistically significant.

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Most of the hepatic tissue stained positive for TGFβII-r at a low intensity, with increased staining on the periphery of the liver sections. A limited subset, less than 10%, of large (greater than 200μm diameter) preneoplastic foci did not stain for TGFβII-r as intensely as the surrounding hepatic tissue (Figure 3.22). This finding was independent of chemical exposure, and there was no apparent difference between GST-P+ and GST-P+/TGFα+ foci in lack of TGFβII-r staining.

PCNA

Staining for this marker of cell proliferation was increased within preneoplastic foci when compared to normal hepatic parenchyma as reported previously (Dean, Jr. 2003). This increased PCNA labeling was equally increased in foci previously stained positive for GST-P only and GST-P/TGFα. Specifically within TGFα+ foci, areas of intense TGFα staining at the periphery of larger foci (Figure 3.23) often showed increased PCNA staining compared to central areas. These central areas still had increased staining compared to normal hepatic tissue. Scattered GST-P+/TGFα negative foci also exhibited increased amounts of PCNA staining.

Apoptosis

Although internal control sections of small intestine invariably demonstrated marked positive staining for apoptotic cells, very few hepatocytes stained positively. These positive hepatocytes were scattered randomly throughout the liver sections with no localization within altered foci or surrounding normal hepatocytes. When rare small clusters of positively stained cells were found, they tended to be in centrilobular areas

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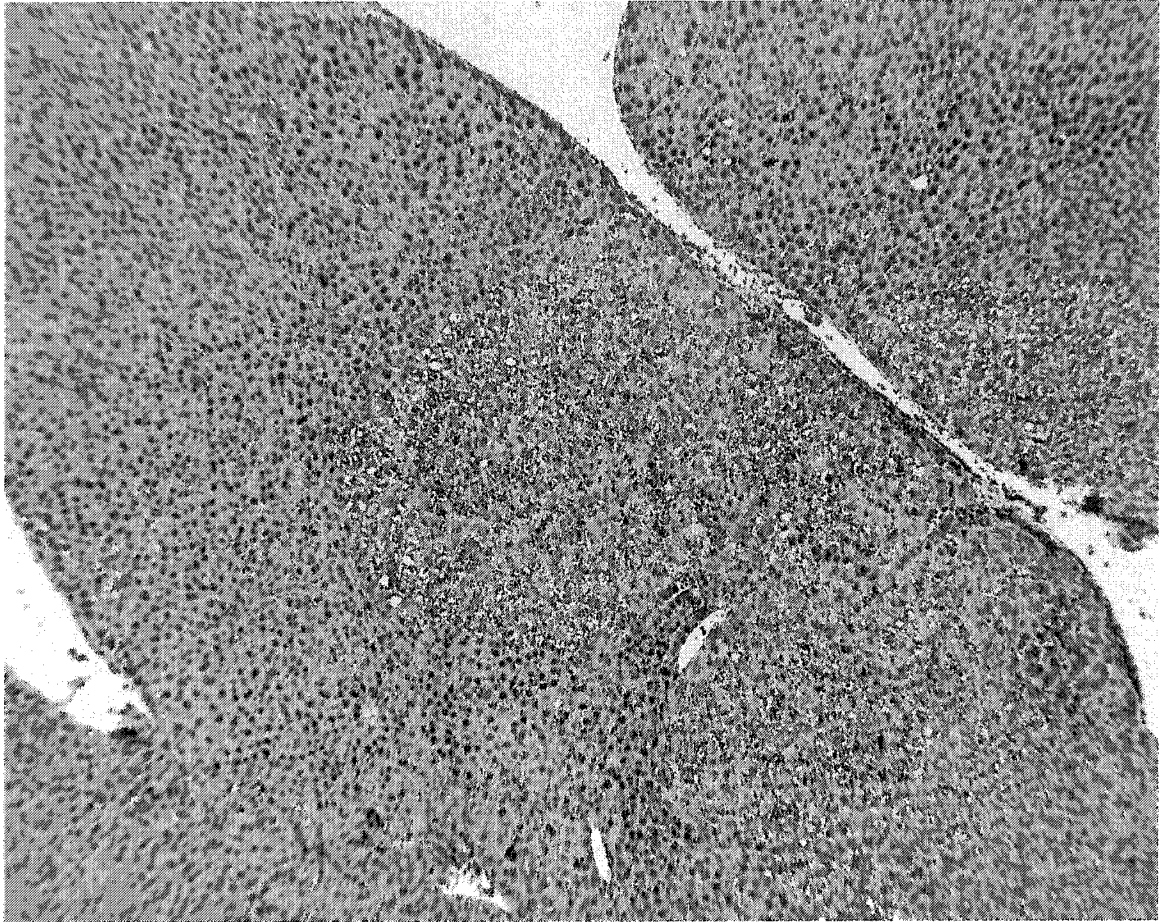

Figure 3.22. Photomicrograph of a large preneoplastic focus in the liver of a rat exposed to PCB 126 only (Group C), TGFβII-r stain, 40X magnification. Note decreased intensity of stain within the focus compared to the surrounding normal hepatic tissue.

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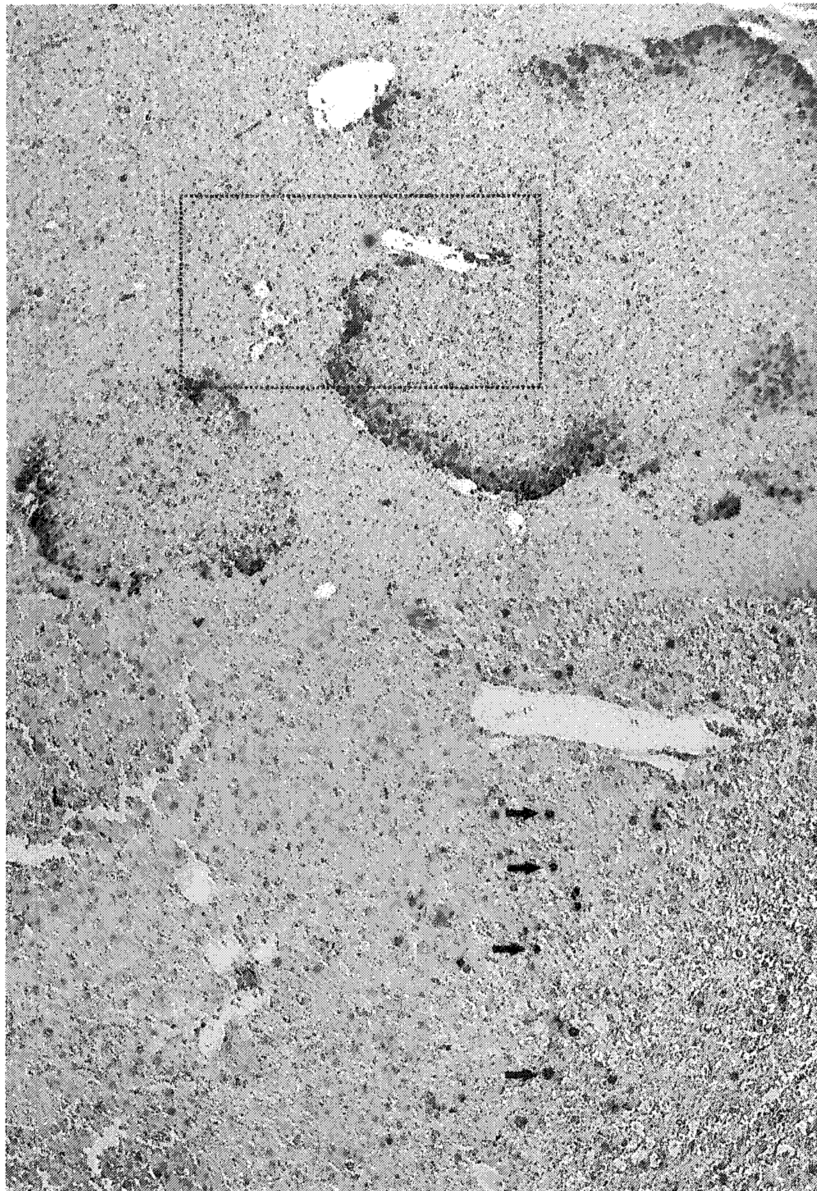

Figure 3.23. Photomicrograph of two preneoplastic foci in the liver of a rat in the PCB/As late Group G, TGFα stain, 40X (top). Note the boxed area, which is magnified to 100X and stained with PCNA, with positive nuclei marked with arrows (bottom). This demonstrates increased cell proliferation along the periphery of the large focus matched with increased TGFα stain.

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with no predilection for preneoplastic foci. Nonspecific staining was occasionally present on the periphery of liver sections.

PCB 126 levels

High levels of PCB 126 in liver tissue were demonstrable in all the groups receiving PCB, both as a single chemical (Group C) and in combination with As (Groups E and G) at 8, 16, and 24 weeks (Figure 3.24). At 16 and 24 weeks the PCB-only Group C exhibited the least amount of PCB 126 in liver. The two groups treated with PCB plus As had higher level of PCBs at all time points with the greatest amount in the PCB/As late Group G, however, none of these differences were statistically significant. The two arsenic only groups had very small levels of PCB on analyses that were above the DEN baseline of zero. The only saline control group available for analysis was at 24 weeks and that was negative for PCBs.

Discussion and Conclusions

Postoperative rat deaths at various time points prior to scheduled sacrifice points were uncommon (three per 204 rats, 1.5%) but not unexpected. The partial hepatectomy procedure is delicate, and accidental ligation of the bile ducts can occur when removing portions of the liver. This resulted in cirrhosis (hepatocyte loss, fibrosis, biliary hyperplasia, and attempted nodular hepatocyte regeneration), seen histologically in the affected rats. The degree of compromise, or what proportion of bile ducts is ligated, and the ability of the individual animal to develop ancillary bile channels, determines the possibility of cirrhosis and early death. The variation in time between partial hepatectomy and mortality (7-11 weeks) reflects the degree of biliary compromise for

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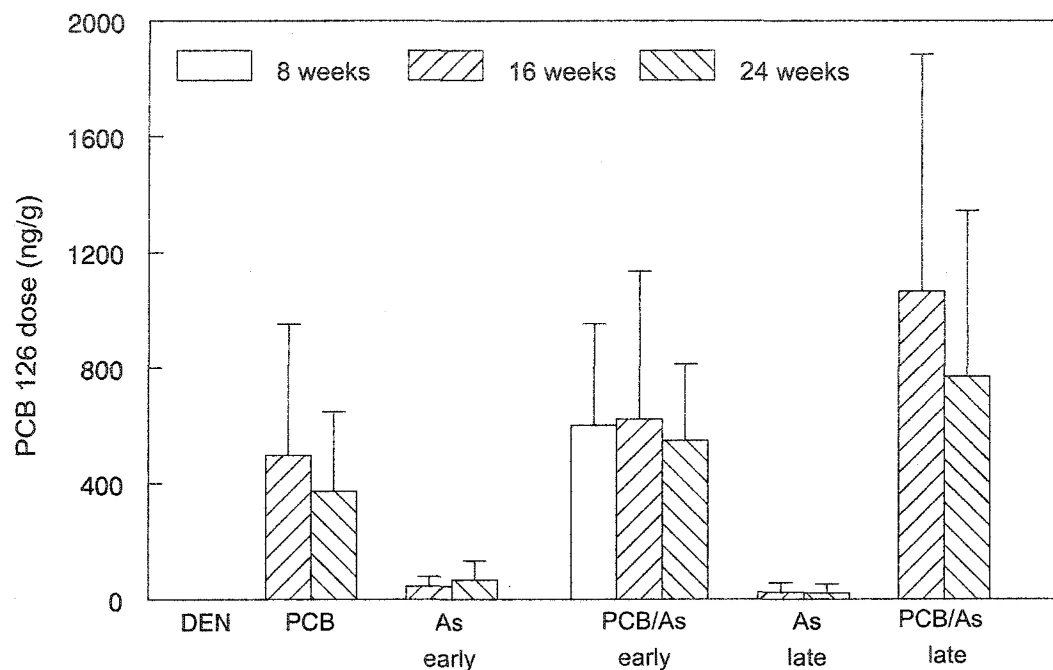

Figure 3.25. PCB 126 levels in livers of experimental animals. Note increasing levels with arsenic co-exposure, especially in the PCB/As late Group G (not statistically significant). No saline control results were available.

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each animal. Since the livers of these animals would contain findings more typical of cirrhosis than any chemical effect on preneoplastic foci, and because the rats failed to survive to their designated sacrifice points, they were excluded from all further analyses.

The subacute toxicity and deaths of rats exposed to 75ppm As in the drinking water was unexpected and had not been seen in previous studies in our laboratory, including those studies with similar exposure concentrations and duration, but different chemical (non-PCB) mixtures (Pott 2003; Pott *et al.* 1998). The two rats that died were both in Group E (PCB/As early) and exhibited marked dehydration and wasting as well as subcutaneous hemorrhages on the tail. Although most animals in the As-only groups showed wasting and tail hemorrhages of varying severity, there were no other deaths and all rats improved once the As concentration was reduced to 7.5ppm. Since PCB-exposed animals did have decreased body weights at both 16 and 24 weeks, perhaps this particular combination of PCB 126 and As resulted in more severe toxicity in the PCB/As early group. Similar to the early rat deaths from cirrhosis, the animals that died were excluded from further analyses.

The decrease in body weight and increase in liver weight seen in animals in all the PCB-exposed groups reflects the common clinical signs of wasting and hepatomegaly seen in individuals exposed to dioxin-like agents (Birnbaum 1994; Safe 1994). Additionally, the reduced thymus weights exhibited by PCB-exposed animals is part of the immune impairment seen with chronic PCB exposure (Safe 1994). The decrease in thymus weights seen in all experimental groups between 16 and 24 weeks was likely a result of normal physiologic thymic involution with advancing age and maturity. Increased splenic weight seen in all groups (B through J) exposed to DEN instead of

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saline is presumably a result of the DEN treatment, although no other gross abnormalities were seen with spleens at sacrifice.

Our current and previous studies (Dean, Jr. *et al.* 2002) have shown PCB 126 to be an effective agent of promotion of hepatic preneoplasia. The marker of promotion used in this study was the expression of GST-P by preneoplastic foci. Foci 200μm and larger expressing this marker have been shown to be more likely to progress to areas of overt neoplasia (Ito *et al.* 1989a; Ogiso *et al.* 1985). Exposure of rats to PCB 126, alone and in combination with arsenic, produced the largest relative size and number of GST-P+ preneoplastic foci. This supports our hypothesis that PCB 126 is acting as a promoting agent by increasing the relative size and number of these GST-P+ foci.

Trivalent inorganic arsenic as a single agent did not increase the relative area or number of GST-P+ hepatic foci when compared to DEN controls. It also did not decrease the size or number of DEN-induced preneoplastic foci. This finding was of interest, since our laboratory previously reported that As demonstrated antagonistic effects on hepatic promotion as a single agent or in chemical mixtures (Pott 2003; Pott *et al.* 1998).

Arsenic did, however, act in an antagonistic fashion when combined with PCB 126 at certain time points. The mixture of the two chemicals did result in decreased relative area of large (>200μm) GST-P+ foci at 16 weeks when As was administered concurrently with PCB from the start of the study (PCB/As early) or added in to the mix eight weeks after PCB dosing began (PCB/As late). This effect was only present in the PCB/As late group by the 24-week time point. This suggests that the role of As in antagonism of GST-P+ focus formation is time-dependent since the anti-promotional

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effect seen after 8 weeks in previous studies (Pott 2003) and 16 weeks in the current study was reduced or absent after 24 weeks. An alternate explanation may be that the intense wasting and dehydration seen at the 75ppm concentration in drinking water also suppresses the growth of preneoplastic foci and once the animals stabilize after reducing the dose to 7.5ppm, the foci can grow in size. The combination of As and PCB did not suppress the number of GST-P+ foci, only the area, which also supports the hypothesis that As inhibits growth of these foci beyond a certain size rather than the number of foci appearing. Addition of As to non-PCB chemical mixtures has been shown to result in antagonistic effects on promotion (Pott *et al.* 1998), but this is the first demonstration of its ability to antagonize the promotional effects of a mixture containing PCBs. This finding is in agreement with our hypothesis that As would have a negative or antagonistic effect at the promotion stage. However, this effect in this hepatocarcinogenicity model appears to be limited to arsenic as part of a chemical mixture, not as a single agent, at least in our current study. Arsenic as part of a mixture may be acting in this stage of carcinogenesis to decrease cell proliferation within foci and/or increase apoptosis, countering the effects of the promoter PCB 126. Arsenic has also been speculated to act by influencing phenotypic maturation, whereby preneoplastic cells revert back to a normal phenotype, otherwise known as cellular remodeling (Pott *et al.* 1998; Tatematsu *et al.* 1983), which is a potential mechanism by which preneoplastic foci may revert back to normal liver. Since we saw more As toxicity in our study than had been reported in previous experiments with the same dose and time of As exposure, it is possible that the difference in As toxicity might have influenced the proliferative response of foci as well, particularly in the As-only groups. It is not clear why there was more toxicity in this

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study since the same procedures for As dosing and design were used. It is possible that the particular non-PCB chemicals used in the Pott et al study decreased the As toxicity, or that the particular combination of PCB 126 and As used here (no rats in the As-only groups died, although they exhibited signs of toxicity) contributed to a more lethal response.

Expression of TGFα was being used as a marker of progression, the third stage of carcinogenesis, in this model. Numerous studies have shown that expression of TGFα correlates well with areas of progression and these TGFα+ areas are most likely to progress to tumors (Burr *et al.* 1996; Dragan *et al.* 1995; Grisham 1997; Steinmetz and Klaunig 1996). Results show that TGFα positivity (excluding nonspecific and perivascular staining) is restricted to a subset of GST-P positive preneoplastic foci. Of GST-P+ foci greater than 200μm diameter, approximately 40% also stained positive for TGFα, and these tended to be the larger foci. This 40% value for the number of foci positive for both markers accounts for 60% of the total relative area. This suggests that these TGFα+ foci are growing more rapidly that those that stain for GST-P only and that these foci had taken an additional step in the carcinogenic process by developing the ability to self-produce the known hepatocyte growth factor TGFα and enter the progression stage. In gaining this phenotypic characteristic, the cells of these GST-P+/TGFα+ foci would no longer be dependent on exogenous growth factors to proliferate. This might suggest that TGFα is a more important phenotypic marker for progression at this stage, although as discussed below, decreased TGFβII-r expression may also be a factor. The two alterations could be occurring as parallel processes with additive but equally important roles.

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We found a difference in TGFα staining characteristics, with the largest of the foci (500μm – 3mm diameter) often showing a peripheral staining pattern while the more moderately-sized (200μm – 1mm diameter) ones exhibited a more diffuse pattern. Staining for PCNA, a marker for cell proliferation, also tended to be more pronounced in these peripheral zones of TGFα positivity in the largest foci. This suggests a more actively dividing subpopulation of altered hepatocytes, positioned along the "leading edge" of an expanding TGFα+ focus. This would parallel one of the characteristics of overt neoplasms, where the tumor outgrows its blood supply on the interior, leading to necrosis, while the peripheral neoplastic cells continue to receive oxygen and nutrients and thus continue to divide. To apply this concept to these preneoplastic foci, the interior altered cells are the most distant from pre-existing hepatic vasculature while the expanding peripheral cells receive the most nutrients. The moderately-sized foci would still allow for diffusion of nutrients to the interior, providing the stimulus for proliferation. This might partially explain the potential rapid expansion of the larger altered foci. Infrequent large foci possessed both intense peripheral TGFα staining and a robust TGFα positivity throughout the focus, and these foci may be the most aggressively growing of those found and those most likely to progress to overt neoplasms.

Our hypothesis was that arsenic's role as an anti-promotion agent would not carry into the progression stage and that its clastogenicity would facilitate a more rapid accumulation of genetic mutations and transit through the late stages of carcinogenesis. Rather than support this, however, the results of the TGFα staining in the PCB-only (C) compared with the PCB/As groups (E, G) mirror the GST-P results. Specifically, the presence of As in the mixture decreased the relative area of large (>200μm) TGFα+ foci

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in both the PCB/As early (E) and PCB/As late (G) groups compared to PCB alone (Group C), at both 16 and 24 weeks. If As were acting as a progressing agent, one would expect an increase in the staining of a putative marker of progression such as TGFα. Furthermore, the presence of As also resulted a decreased number of TGFα+ foci in both PCB/As groups at 16 weeks, relative to PCB as a single agent. This contrasts with the GST-P results, where As had no effect on the number of foci. This effect was absent by 24 weeks however, which again suggests that arsenic's role in suppression of preneoplastic foci is time-dependent and may reverse after a long enough period.

The role of PCB in the carcinogenic process is thought to be limited to the promotion stage, as it exhibits no evidence of direct mutagenicity typically seen with initiating or progressing agents (Safe 1994; Silberhorn *et al.* 1990). Our hypothesis that PCB would only exert promotional effects may be questioned in light of the TGFα results, where the PCB-only Group C consistently showed higher relative area and number of TGFα+ foci when compared to the PCB/As Groups E and G. If PCB were truly only acting in promotion, one would expect a relative decrease in area and number of TGFα+ foci in comparison with the PCB/As groups. In fact, the ratio of TGFα+ to GST-P+ foci was similar for all treatment groups at 40%. This suggests that the most consistent characteristic of a focus to enter the progression stage is not the particular chemical mixture causing its existence, but its size, as the largest of the foci regardless of group designation tended to be TGFα+. An alternate explanation would be that TGFα might not be a good marker for progression, and may instead be a marker for late promotion, where PCB exposure would still have an effect and As would not.

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The TGFβ1 and TGFβII-r stains proved to be difficult to interpret, due to the large amount of background and nonspecific staining throughout the hepatic tissues. Despite this, there were interesting trends to the TGFβII-r staining patterns. Infrequent large preneoplastic foci, both solely GST-P+ and more so GST-P+/TGFα+, exhibited clearly decreased level of TGFβII-r staining compared to surrounding normal tissue. This suggests an additional mutation that may be a marker of progression in addition to (or in lieu of) TGFα. Preneoplastic cells that are resistant to the growth-inhibitory effects of TGFβ would have a significant growth advantage over normal hepatocytes. Furthermore, the TGFβ produced by either normal or altered hepatocytes would only inhibit the surrounding normal cells, stimulating their apoptosis and allowing for more rapid expansion of the altered cells. A preneoplastic cell population possessing both autocrine growth stimulation by TGFα and resistance to growth inhibition by TGFβ would have a significant growth advantage and this cellular phenotype would be more likely to continue through the progression stage of carcinogenesis and potentially develop into an overt neoplasm. In the earlier study by Dean (Dean, Jr. 2003), foci induced at 8 weeks by PCB 126 showed increased staining for TGFβ1 and decreased staining for TGFβII-r. It was suggested that these were early reflections of the negative selection hypothesis allowing the early foci to expand at the expense of normal hepatic tissue (Andersen *et al.* 1995). In the current study, we did not demonstrate clear differences in TGFβ1 staining inside and outside foci but did still demonstrate decreased receptor staining. Since our data were at 16 and 24 weeks, this might suggest that the increased TGFβ production within the foci is a more important factor at early times and becomes less of a factor as TGFα takes over with its growth stimulatory role. It is well reported that lack of the

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TGFβII-r is an important characteristic even in fully developed cancer cells (Im *et al.* 2001; Park *et al.* 2001; Rossmanith and Schulte-Hermann 2001) no matter what the source of the TGFβ might be. Under normal conditions TGFβ is produced from nonparenchymal supporting cells of the liver rather than from the hepatocytes, so lack of hepatocyte production in foci would not decrease the importance of the lack of TGFβII receptors. It is also possible that as TGFα expression is increased, this leads to or is accompanied by a concomitant reduction of TGFβ1 expression in the same cells. This hypothesis would need to be tested.

The increased labeling of PCNA within preneoplastic foci is not unexpected, as part of the growth advantage of a focus of altered hepatocytes is increased cell proliferation above that of unaltered normal cells, and this has been reported previously for PCBs (Dean, Jr. 2003). In our study, increased PCNA was found in both GST-P+ and GST-P+/TGFα+ foci, demonstrating that cell proliferation is increased with or without increased expression of the specific growth factor TGFα. However, the more intense PCNA labeling around the periphery of many of the largest TGFα+ foci supports the above theory that these cells on the leading edge of an advancing lesion have even greater production of growth-stimulatory TGFα. The increased PCNA labeling reflects a downstream mitogenic response of these peripheral cells to that excess TGFα. For these more advanced foci, the additional stimulation by autocrine production of TGFα provides a more significant advantage than the GST-P+ foci, shown by these bands of PCNA labeling.

The lack of apoptosis staining throughout the liver sections might suggest that the role of TGFβ as an inducer of apoptosis may not be a part of the process. One would

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expect that the positive staining of TGFβ1 throughout the liver sections would translate into increased apoptosis. We did not confirm such an increase in either TGFβ1 or apoptosis is this study, however. Further, apoptosis is an infrequent event and was only evaluated at one point in time in this study. The TUNEL method employed here is widely used to detect apoptosis, but it is not a full measure of that process (Levin *et al.* 1999). The exact mechanism by which TGFβ induces apoptosis is not completely understood, and direct measurement of the apoptosis-effector protein caspases might provide a more thorough assessment to evaluate the role of TGFβ1 in this model.

High levels of PCB 126 were found in tissues of all animals exposed to PCB 126, alone (Group C) or in combination with As (Groups E and G), at all three time points. Interestingly, the levels of PCB 126 were higher in those animals exposed to both PCB 126 and As, and the highest levels were reached in the PCB/As late Group G. These differences were trends and were not statistically significant, primarily because the samples from the different animals in a group were pooled for analysis and standard deviations were very large. The individual chemicals or mixtures did not cause any microscopically visible alterations that may have affected liver retention of PCB, and hepatomegaly was seen in the livers of all animals exposed to PCB. The 16-week PCB/As late group, which quantitatively had the highest PCB concentration, had significantly decreased liver weights compared to PCB alone or PCB/As early groups. This lower liver weight may have contributed to a relative increase in PCB concentration in that group. In addition, the rats in this group (G) exposed to PCB 126 and As late showed decreased body weights compared with Groups C and E. If this decrease in body

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weight was due to a decrease in adipose tissue content, one of the main storage areas of PCBs (Jensen 1987), the body burden of PCB may have shifted to the liver.

Within each group, the PCB concentration did not increase over time, as one might expect with a steady accumulation of chemical with chronic exposure. This might reflect achievement of a steady-state level of hepatic PCB. Since adipose tissue is also known to accumulate PCBs (Dean, Jr. *et al.* 2002; Jensen 1987), adipose levels would have been desirable but were not available for this study, to possibly account for the maintenance of the hepatic steady-state.

The concentration of our 8-week samples from Group E (PCB/As early) was less than that reported for PCB 126 only in a previous study (Dean, Jr. *et al.* 2002) but within one standard deviation. The difference could be due to the combination of chemicals in our study, although the co-administration of PCB and As resulted in an overall increase in hepatic PCB levels.

Very low levels of PCB 126 were found in the two As-only groups (D and F). Rats in these groups were only gavaged with corn oil alone, never with PCB. The DEN control groups and the single saline control group for which results were available showed no accumulation of PCBs. During analysis, samples were run through the gas chromatography column in group order, beginning with Group A and followed by Group B, then C and so forth. Between groups, ethyl acetate solvent was rinsed through the sampling needle and dosing syringe five times (two pre-sample, three post-sample) to minimize contamination of subsequent samples. Given that the initial control groups of A and B showed no PCB levels, and the As-only groups following the PCB-containing Groups C and E showed low levels, it is likely that the five-time rinsing of the needle and

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syringe was inadequate for complete decontamination and therefore these low levels in the As-only groups were a result of contamination error.

The experimental design for this study included an additional sacrifice point at 8 weeks within the PCB/As early Group G. This time point, referred to as "E8," was added in order to facilitate comparisons between this study of PCB 126 and As co-exposure with two earlier studies in our laboratory that contained data on PCBs and arsenic as single agents from 8-week studies (Dean, Jr. *et al.* 2002; Pott 2003). We erred in not including a set of our own DEN and saline controls at the 8-week time point, thus prohibiting comparison of our E8 data with many of the other data in this study. Therefore, comparisons within this study incorporating E8 data will only be made with other Group E findings from the 16 and 24-week sacrifice points. When compared with the rats exposed to PCB 126 alone in the Dean *et al* 2002 study, our E8 animals exhibited decreased body and liver weights, decreased GST-P+ relative area and number, and decreased concentrations of PCB 126 in liver. The differences in these results may be relative, as all values are decreased, or they may reflect the influence of As in the chemical mixture (see discussion below). Compared to the Pott *et al* study, our E8 group exhibited markedly increased area and numbers of large GST-P+ foci compared to 8-week As only animals, as would be expected.

Experimental Group J was designed to act as a positive control for progression, using an intraperitoneal injection of ethylnitrosourea, a known hepatic carcinogen and tumor progressor (Dragan *et al.* 1993; Pereira *et al.* 1985). The failure of this group to have any significant differences from its control group H (saline injected IP) or from the DEN control Group B is difficult to explain. At the time of the injection, the compound

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was difficult to suspend in solution, which may have affected its absorption or allowed for more effective clearance from the body. Since our ENU dose was higher (150mg/kg vs. 100mg/kg) to that in previous studies (Dragan *et al.* 1993), insufficient dose is not a likely cause.

In conclusion, we have identified several of the phenotypic characteristics of a subpopulation of altered hepatocytes that are more advanced than others in the progression stage of carcinogenesis. Those characteristics are increased expression transforming growth factor-alpha and decreased expression of transforming growth factor-beta II receptor. Both might provide these cells with a competitive growth advantage and make them more likely to continue through the carcinogenic process and form an overt neoplasm. Exposing rats *in vivo* to different combinations of PCB 126 and arsenic influences the development of these altered hepatic foci and may alter the expression of the growth-stimulatory TGFα.

Acknowledgements

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Dean and Wendy Pott for performing the initial PCB and As experiments, respectively.

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CHAPTER 4

CONCLUSION AND FUTURE DIRECTIONS

Toxic chemicals such as polycyclic aromatic hydrocarbon (PAH) organochlorines and arsenic are common pollutants around hazardous waste sites and may be found throughout the environment. PAHs such as polychlorinated biphenyls (PCBs), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin), and 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) are lipophilic and metabolize slowly and thus persist in organic tissues in the environment and bioaccumulate up the food chain (Kimbrough 1995). The biological effects and classification of PCB congeners is based on their molecular structure (Safe 1994). Coplanar PCBs such as PCB 126 (3,3',4,4',5-pentachlorobiphenyl) are also known as dioxin-like PCBs, binding the cytoplasmic aryl hydrocarbon receptor (AhR) and having comparable biological effects to TCDD such as stimulation of hepatocyte proliferation in rodents and induction of cytochrome P-450 (CYP) 1A enzymes (Burke *et al.* 1994; Safe 1994). PCB 126 is considered the most toxic congener, having the closest molecular structure to TCDD. The noncoplanar PCBs such as PCB 153 (2,2',4,4',5,5'-hexachlorobiphenyl) do not bind the AhR. They elicit phenobarbital-like effects such as hepatocyte proliferation and induction of CYP2B enzymes (Muangmoonchai *et al.* 2001). PCBs in the environment exist as mixtures of congeners with different physical and

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biological characteristics that can interact with each other and with other pollutants, causing toxicity (Vanden Heuvel and Lucier 1993).

TCDD and PCBs exhibit sex-related differences in liver tumor carcinogenesis, with females affected more frequently. This marked female-specific hepato-carcinogenicity is likely due to the influence of 17β-estradiol (E2), a hormone found in increased levels in females. Most of the oxidative metabolism of estrogens (mostly hydroxylation) occurs in the liver, catalyzed by the cytochrome P-450 enzymes (Zhu and Conney 1998). CYP1A enzymes are known to catalyze the metabolism of 17β-estradiol to 2-hydroxy and 4-hydroxy catechol forms (Martucci and Fishman 1993). The 4-hydroxy form (4-hydroxyestrone or 4-hydroxyestradiol [4-OHE_2]) may be metabolized via cytochrome P450 to quinones, reactive electrophiles that may undergo redox cycling reactions. These reactions produce reactive oxygen species (ROS), specifically the hydroxyl radical $OH^{\bullet}$, that can cause DNA damage (Cavalieri *et al.* 1997; Han and Liehr 1995). The 2-hydroxy form (2-hydroxyestrone or 2-hydroxyestradiol [2-OHE_2]) has little or no oxidative activity and increased levels are suggested to be protective against carcinogenesis (Martucci and Fishman 1993; Zhu and Conney 1998). Dioxin-like agents are not generally considered to be genotoxic; however, the higher levels of endogenous estrogen in females, leading to increased 4-OHE_2 and ROS production, could provide an explanation of gender differences seen in dioxin carcinogenesis (Martucci and Fishman 1993).

The carcinogenic effects of PCBs have been widely studied in laboratory animals, where the liver is the target organ. Various studies have shown that, after initiation with a variety of carcinogens, they can act as promoters of hepatocellular neoplasms, including

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carcinomas (Mayes *et al.* 1998; Moore *et al.* 1994; Silberhorn *et al.* 1990). PCBs are classified as probable (Group 2A) human carcinogens, with suggestive evidence of causation with liver, biliary tract, and breast cancers (Siemiatycki *et al.* 2004).

To aid in risk assessment for these persistent environmental contaminants, toxic equivalency factors (TEFs) were developed by the World Health Organization and have used the common initial mechanism of AhR binding (Safe 1990). A TEF for a chemical indicates an order of magnitude of the toxicity of a compound relative to TCDD, which is assigned the maximum value of 1.0 (Birnbaum 1994; Toyoshiba *et al.* 2004; van den Berg *et al.* 1998). Other dioxin-like compounds are given equal or lower numbers, with each number proportional to TCDD. A basic assumption of this classification with regard to mixtures is that the effect of the combined chemicals is equivalent to the added effects of the individual chemicals.

In Chapter 2, we explored a role for reactive oxygen species in carcinogenesis by measuring the CYP1A1 activity and relative amounts of catechol estrogens in livers from rats exposed to TCDD, PeCDF, the dioxin-like coplanar PCB 126, the noncoplanar PCB 153, a two-way mixture of the PCBs, and a three-way mixture of the AhR-binding TCDD, PeCDF, and PCB 126. We measured the activity of CYP1A1 by determining the ability of isolated liver microsomes to catalyze the 7-ethoxyresorufin-*O*-deethylase (EROD) reaction assay (Rutten *et al.* 1992). The relative CYP1A1 levels were used to match our results to previously published TEFs for these individual chemicals (Vanden Heuvel and Lucier 1993), as well as to added TEFs for the binary & tertiary mixtures. In addition, microsomes were incubated with E2 and direct measurement of the estrogen

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catechol metabolites 2-hydroxyestradiol (2-OHE_2) and 4-hydroxyestradiol (4-OHE_2) was performed (Roy *et al.* 1991).

As expected, we found that EROD activity increased in a dose-responsive fashion in rats treated with Ah-receptor-binding TCDD, PeCDF, and coplanar PCB 126, as well as the three-way mixture of these compounds. Not surprisingly, no changes in CYP1A1 activity were observed with the noncoplanar, non-Ah-receptor binding PCB 153.

The PCB 126/153 mixture produced a greater-than-additive elevation in EROD activity, which was greater than TCDD alone. This was an interesting finding, since PCB 153 showed little activity as a single agent. Enhancement in EROD activity with a mixture of TCDD and PCB 153 has been reported previously (Bannister and Safe 1987; De Jongh *et al.* 1995b). Possible explanations include reports that high doses of PCB 153 can alter the distribution of TCDD, increasing the concentration in liver & decreasing the concentration in adipose tissue (De Jongh *et al.* 1995a; van Birgelen *et al.* 1996). Further, PCB 153 has been shown to increase hepatic AhR levels (Bannister and Safe 1987). Since PCB 126 binds the AhR in a similar fashion to TCDD and exposure induces similar effects, it is not unexpected for a mixture of PCBs 126 and 153 to result in enhanced EROD activity. This has been reported previously in both male Wistar rats (Leece *et al.* 1987) and in female Sprague-Dawley rats (Bager *et al.* 1995). The AhR may thus be acting not only as a trigger for CYP induction after exposure to these dioxin-like chemicals, but also as a mechanism by which these chemicals are drawn to and bound within hepatocytes, resulting in an overall increase in liver concentration (De Jongh *et al.* 1995b; van Birgelen *et al.* 1996). Alternately, an enzyme induced by AhR binding such as CYP1A2 might act as act to bind and sequester dioxin-like chemicals

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(Santostefano *et al.* 1996; van Birgelen *et al.* 1996). A previous study examining the formation of preneoplastic foci in the liver also found evidence of a greater-than-additive effect with PCB 126 and 153 co-exposure (Bager *et al.* 1995), although other experiments have found antagonistic interactions (Dean, Jr. *et al.* 2002; Haag-Grönlund *et al.* 1998) as well as differential localization of CYP1A1 induction (Chubb *et al.* 2004). These conflicting findings are confounded by varying experimental protocols as well as use of different rat strains and underscore the complexities involved in examining the effects of chemical mixtures, even with closely related chemicals such as PCB congeners.

Utilizing known TEFs, equivalency adjusted doses of TCDD, PeCDF, and PCB 126 elicited similar EROD activity. Activity for the three-way mixture of TCDD, PeCDF, and PCB 126 was similar to the added EROD activities of the individual chemicals. Our data are consistent with the published TEFs for TCDD, PeCDF, and PCB 126 based on our CYP1A1 induction findings. This provides support for the assumption of additivity inherent in the TEF classification, at least for this mixture of AhR-binding chemicals. The non-AhR-binding PCB 153 was not evaluated in combination with TCDD or PeCDF, but given the response its mixture with PCB 126, the addition of PCB 153 to either (or a combination of the two) would likely have produced a synergistic EROD response. Given the conflicting results of previous studies reported above, however, this EROD enhancement would not be a certainty and could be dependent on the potential number of additional AhR or other receptors that PCB 153 is able to induce. This suggests that applying the TEF concepts to complex mixtures involving chemicals other than AhR binders may not be appropriate, even to those as closely related chemically as PCB 153. Additionally, risk assessment based on the TEF concept, while

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appropriate for mixtures containing only AhR-binding chemicals, may be underestimating hazards of mixtures of unrelated compounds and should not be applied under these circumstances. Given that environmental mixtures are most often complex and contain a number of unrelated chemicals, the utilization of TEFs may often not be practical.

Dose-responsive increases in 4-OHE2 were observed with TCDD, PCB 126, and PCB 126/153 samples. Changes in 2-OHE2 were inconsistent, with some groups increased and some decreased. The use of catechol estrogen ratios is one method used to estimate risk, as 4-OHE2 is known to increase cancer risk, while 2-OHE2 is thought to decrease cancer risk (Liehr and Ricci 1996). Overall increases of 4-OHE2 led to decreased 2:4-OHE2 ratio with increasing dose in rats exposed to TCDD, PCB 126, and PCB 126/153. Increased 4-OHE2 formation (and decreased 2:4-OHE2 ratio) coupled with increased CYP1A1 (EROD) activity, as observed with TCDD, PCB 126, and PCB 126/153, may increase the potential for quinone cycling leading to oxidative DNA damage. This ultimately might increase the risk of carcinogenic mutations in livers of animals exposed to certain combinations of coplanar and noncoplanar PCBs, and by extension, other AhR binding agents and noncoplanar PCBs. This could explain, at least in part, the greater susceptibility of female rats to carcinogenesis by Ah-receptor agonists.

Arsenic (As) is a common groundwater contaminant and is found worldwide. Environmental exposures to As usually occur through ingestion of contaminated food and especially water, when natural or manmade sources leach into nearby groundwater. Arsenic is found both as inorganic and organic forms. Its inorganic forms are the

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trivalent arsenite, or arsenic trioxide (AsIII), and the pentavalent arsenate (AsV). The trivalent arsenite is considered the more toxic of the inorganic forms (Aposhian *et al.* 2004; Haschek *et al.* 2002). Arsenic is classified as a definite (Group 1) human carcinogen, with strong evidence of causation with skin, liver, lung, and urinary bladder cancers (Siemiatycki *et al.* 2004).

Exposure of pregnant mice to arsenite has resulted in production of various dose-dependent neoplasms in their adult progeny, demonstrating that AsIII can act as a complete carcinogen (Liu *et al.* 2004; Waalkes *et al.* 2004). The methods of action of As carcinogenesis are unclear. The strongest experimental evidence suggests production of chromosomal abnormalities, induction of oxidative stress, and activation of selected signal transduction pathways (Huang *et al.* 2004; Kitchin and Ahmad 2003). Other potential carcinogenic pathways are enhancement of cell proliferation and suppression of p53 (Kitchin 2001; Pott *et al.* 2001). Arsenic is thought to act in the promotion and/or progression stages of carcinogenesis (Pott *et al.* 2001). It is also thought to act as a "co-carcinogen" whereby its interactions with other environmental carcinogens result in a synergistic response.

Acquisition of autocrine growth involving the growth factor transforming growth factor-alpha (TGFα) characterizes altered hepatocytes during the early stages of carcinogenesis in rats and humans (Grisham 1997). Expression of TGFα correlates well with areas of progression in chemical carcinogenesis protocols and these areas are most likely to progress to tumors (Dragan *et al.* 1995). Transforming growth factor-beta (TGFβ) normally inhibits epithelial cell proliferation and promotes differentiation. TGFβ1 is the most abundant and important form in the liver (Roth *et al.* 1997) and binds

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to three TGFβ cell surface receptor proteins (types I, II, and III) with high affinity. There is experimental evidence that expression of TGFβ1 and TGFβII-r is altered during the promotion stage of hepatocarcinogenesis in the rat, contributing to the development and progression of preneoplastic lesions (Dean, Jr. 2003; Park *et al.* 2001).

The purpose of the experiment detailed in Chapter 3 was to examine the carcinogenesis of PCB 126 and arsenic as single agents and in combination. The general hypothesis was that interactions between a mixture of PCBs and arsenic would result in nonadditive effects on hepatic promotion and progression. Specifically, arsenic would demonstrate negative/antagonist effects in the promotion stage of carcinogenesis and would have positive/pro-carcinogenic effects in the progression stage. PCB 126 would show promotional effects but would have no effect on the progression stage.

The marker of promotion used in this study was the expression of glutathione-S-transferase (placental form, GST-P) by preneoplastic foci. Foci 200μm and larger expressing this marker have been shown to be more likely to progress to areas of overt neoplasia (Ito *et al.* 1989). Exposure of rats to PCB 126, alone and in combination with arsenic, produced the largest relative size and number of GST-P+ preneoplastic foci. This supports our hypothesis that PCB 126 is acting as a promoting agent by increasing the relative size and number of these GST-P+ foci.

Arsenic as a single agent did not increase or decrease the relative area or number of GST-P+ hepatic foci when compared to DEN controls. Antagonistic effects of As were seen when As was combined with PCB 126. The mixture of the two chemicals resulted in decreased relative area of large (>200μm) GST-P+ foci at 16 weeks in both PCB/As groups. This area effect was only present in the PCB/As late group by the 24-

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week time point, suggesting a time-dependent effect since the anti-promotional effects seen after 8 weeks in previous studies (Pott 2003) and 16 weeks in the current study were reduced or absent after 24 weeks. It is possible that the toxicity seen in rats at the 75ppm As concentration in drinking water may have suppressed the growth of preneoplastic foci, resulting in significant decreases of foci area at 16 and 24 weeks when compared with the PCB-only group. The combination of As and PCB did not significantly suppress the number of GST-P+ foci, which also supports the hypothesis that As inhibits growth of these foci beyond a certain size rather than the number of foci generated. Addition of As to non-PCB chemical mixtures containing 1,2-dichloroethane, vinyl chloride, and trichloroethylene has been shown to result in antagonistic effects on promotion (Pott *et al.* 1998; Pott 2003), but this is the first demonstration of its ability to antagonize the promotional effects of a mixture containing PCBs. This finding is in agreement with our hypothesis that As would have a negative or antagonistic effect at the promotion stage, at least as part of a mixture with PCB 126. To achieve an antagonistic effect on promotion, As could be inhibiting cell proliferation and/or enhancing apoptosis to counter the promotional effects of PCB 126.

We used the enhanced expression of TGFα as a marker of progression in this study. Of GST-P+ foci greater than 200μm diameter, 40% also stained positively for TGFα with these accounted for 60% of the total relative area, demonstrating that these tended to be the largest foci. This suggests that these TGFα+ foci are growing more rapidly that those that stain for GST-P only and that these foci had taken an additional step in the carcinogenic process by developing the ability to self-produce the known hepatocyte growth factor TGFα and enter the progression stage. In gaining this

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phenotypic characteristic, the cells of these GST-P+/TGFα+ foci would no longer be dependent on exogenous growth factors to proliferate and would have achieved a competitive growth advantage over the neighboring normal and hepatocytes positive for GST-P only.

The more intense peripheral TGFα+ staining found in some of the largest of the foci (500μm - 3mm diameter) combined with enhanced peripheral PCNA labeling suggests the development of a population of most actively dividing cells along the "leading edge" of these preneoplastic foci. Since some of the foci ranging from 200μm - 1000μm diameter had intense staining distributed more diffusely throughout the focus, this pattern may reflect better nutritional availability for these more rapidly dividing cells. Neoplasms do not grow beyond a certain size (approximately 2mm diameter) without the formation of additional circulatory channels to allow the transfer of oxygen and nutrients via the blood supply, and it is logical to presume a similar paradigm occurring with the largest of the preneoplastic foci. Smaller TGFα+ foci contain a more uniform distribution of rapidly dividing cells, and the proximity of normal hepatic circulatory channels could allow for more efficient diffusion of oxygen and nutrients to all of these cells. In the largest foci, cellular demands are just as great, but those cells in the deep interior of the focus may not have the same access to the pre-existing blood supply, and therefore these interior cells may be less able to produce growth factor and divide. The infrequent largest foci with diffuse TGFα and enhanced PCNA staining throughout might be in a naturally better-perfused area of the hepatic lobule or might have formation of some new blood channels. These largest foci with diffuse TGFα staining may well be those most likely to go on to form frank neoplasms. This theory would need to be tested

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and confirmed, possibly by examining distinct characteristics of central compared to peripheral altered cells within the foci and morphometric evaluation of blood channel/sinusoid formation.

Some of the >200μm preneoplastic foci exhibited clearly decreased levels of TGFβII-r staining compared to surrounding normal tissue. Many of these foci were also TGFα+, but some were only GST-P+. This suggests an additional mutation that may be a marker of progression in addition to (or in lieu of) TGFα. Preneoplastic cells that are resistant to the growth-inhibitory effects of TGFβ would have a significant growth advantage over normal hepatocytes. Furthermore, the TGFβ produced by either normal or altered hepatocytes would only affect the surrounding normal cells, stimulating their apoptosis and allowing for more rapid expansion of the altered cells, an application of the "negative selection hypothesis" (Andersen *et al.* 1995). TGFβ1 staining in these tissues was uniform within foci and normal hepatic tissue. Although we did not find increased production of TGFβ1 within the foci, this has been found previously (Dean, Jr. 2003) for preneoplastic foci after six weeks of PCB 126 treatment. Even if the altered hepatocytes were not producing excess TGFβ1 at the later times in the current study, it would be present due to normal nonparenchymal cell production and would influence the normal hepatocytes with fully functioning receptors more so than altered hepatocytes with decreased TGFβII-r. It also is possible that the increased TGFβ1 production seen at eight weeks by Dean *et al* was diminished by the time of our assessment at 16 and 24 weeks. A preneoplastic cell population possessing both autocrine growth stimulation by TGFα and resistance to growth inhibition by TGFβ would have a significant growth advantage

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and this cellular phenotype would be more likely to continue through the progression stage of carcinogenesis and potentially develop into an overt neoplasm.

High levels of PCB 126 were found in tissues of all animals exposed to PCB 126, alone or in combination with As. The levels of PCB 126 were higher in those animals exposed to both PCB 126 and As, and the highest levels were reached in the PCB/As late group. This may be explained by the lower liver weights of animals exposed to As, leading to a relative increase in PCB concentration. PCB levels in adipose tissue, another depot for body PCB burden (Dean, Jr. *et al.* 2002; Jensen 1987) were not available, so shifts in PCB levels between body tissues could not be determined. Arsenic-exposed animals exhibited decreased body weights, and if this decreased adipose tissue content, PCB bound to fat may have been liberated and taken up by the liver. The PCB content in liver did not change between time points despite ongoing exposure, suggesting a maximal hepatic storage capacity. Whether excess PCB 126 was stored in the adipose tissue, stored in some other tissue, or excreted could not be determined in this study.

We have determined that the phenotypic characteristics of increased expression of TGFα and decreased expression of TGFβII-r in a subpopulation of altered hepatocytes suggest a more advanced stage of carcinogenic progression. Both phenotypes might provide these cells with a competitive growth advantage and make them more likely to continue through the carcinogenic process and form an overt neoplasm. Exposing rats *in vivo* to different combinations of PCB 126 and arsenic influences the development of these altered hepatic foci and may alter the expression of the growth-stimulatory TGFα.

The overall hypothesis for this research is that the effects of mixtures of toxic chemicals commonly found at hazardous waste sites cannot necessarily be predicted from

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the actions of the individual chemicals. We have demonstrated this hypothesis to be true in two studies of interactions between PCBs, dioxins and arsenic evaluating several different endpoints.

The primary hypotheses presented in this dissertation were as follows:

Hypothesis 1. A mixture of Ah-receptor agonists such as TCDD, PeCDF and PCB 126 will show an additive interaction with respect to oxidative metabolism and induction of cytochrome P450 1A1 enzyme. In Chapter 2, we demonstrated that this was the case, as the three-way mixture of TCDD, PeCDF, and PCB 126, all AhR-binding agents, did exhibit additivity in mixtures, supporting the TEF concept.

Hypothesis 1a. The addition of non-AhR-binding compounds such as noncoplanar PCBs to mixtures containing dioxin-like chemicals will result in a non-additive interaction. Adding noncoplanar PCB 153 to the dioxin-like PCB 126 did result in a synergistic response with regard to CYP 1A1 induction, in that EROD values for the mixture were greater than what would be expected by additivity. This does question the use of the TEF concept of additivity for these two chemicals using EROD, and it can be theoretically extrapolated to the addition of noncoplanar PCBs to other AhR binding agents. Testing other combinations of similar chemicals would likely confirm this.

Hypothesis 2. Induction of catechol estrogen metabolites in female rats may help explain gender differences in TCDD and PCB hepatocarcinogenesis. The increases in 4-OHE2 catechol estrogen seen with TCDD, PCB 126, and PCB 126/153 in female rats supports this hypothesis. A good method by which to further examine this would be to perform the experiment in male rats, including a group with estrogen supplementation, to compare results.

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Hypothesis 3. The general hypothesis is that interactions between a mixture of PCB 126 and arsenic will result in nonadditive effects on hepatic promotion and progression.

Hypothesis 3a. Arsenic will demonstrate negative/antagonistic effects in the promotion stage of carcinogenesis and will have positive/pro-carcinogenic effects in the progression stage. The first of these sub-hypotheses is partially supported by our results. Contrary to prior experiments with shorter exposures (Pott 2003), the As-only groups in this study were no different from DEN controls with regards to area and number of GST-P+ foci. Thus arsenic as a single agent was not decreasing GST-P+ focus formation. In the PCB/As mixture, arsenic did demonstrate a time-dependent decrease in GST-P+ foci relative area, especially at 16 weeks. This effect was not present in one of the groups at 24 weeks, and As had no effect on the number of foci at either time point. The second of these sub-hypotheses is not supported by our results. TGFα staining, our primary marker of progression, correlated with GST-P positivity and size of the focus rather than with As exposure. Similar to the GST-P results, As co-exposure decreased the relative area of the TGFα+ foci compared to PCB alone at both time points. If As were acting as a progressor, one would expect an increase in TGFα+ foci with co-exposure, not a decrease. For both GST-P and TGFα studies, there appeared to be a lessening of arsenic's antagonistic effects with increased time, perhaps partially explaining the differences from the Pott *et al* studies. Extending the time course of such studies even later (perhaps to one year) would be a way to determine if the suppressing role of As reverses at a later time or if other progression events might be triggered by the clastogenic activity of As.

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Hypothesis 3b. PCB 126 will show promotional effects but will have no effect on the progression stage. Similar to previous studies in our laboratory and elsewhere (Dean, Jr. *et al.* 2002; Silberhorn *et al.* 1990), PCB 126 exposure elicited formation of GST-P+ foci, the marker of promotion. However, the PCB-only group consistently showed the highest relative area and number of TGFα+ foci, suggesting that PCB 126 might have effects on progression as well. If PCB 126 were only acting in promotion, we would expect a relative decrease in area and number of TGFα+ foci in comparison with the PCB/As groups. The ratio of TGFα+ to GST-P+ foci remained at 40% for all treatment groups. This suggests that the most consistent characteristic of a focus to enter the progression stage is not the particular chemical mixture causing its existence, but its size, as the largest of the foci regardless of group designation tended to be TGFα+.

Our investigation into the carcinogenic mechanisms of these chemicals was thorough but incomplete. Future experiments need to further characterize the potential oxidative injury component of dioxin-like chemical exposure and might start with comparison studies of male rats to confirm that catechol estrogen production reflects higher endogenous estrogen in females. Although EROD is a proven and adequate method for measuring CYP1A1 induction, incorporating direct measurements such as Western immunoblot and/or gas chromatography would provide direct evidence of CYP 1A1 protein. Since our component of the overall NTP experiment was limited to EROD and catechol estrogen detection, physiologic responses to the chemical exposures were unavailable. Designing a study to include all parameters would allow for a more complete assessment of the effects.

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The two most critical changes that should be added to any subsequent studies investigating the findings described in Chapter 3 would be adding several different lower doses of As exposure and extending the duration of the study. Additional As doses would allow for a dose response assessment and would likely avoid the toxicity issues we found with extended 75ppm exposure, allowing for consistent dosing throughout the experiment. This could help determine if some of our PCB/As effects were due to the subacute toxicity of As or due to a more fundamental antagonistic property of the chemical. Furthermore, the use of a lower dose would be more applicable to human exposures where the environmentally relevant concentrations are well below 75ppm. Extending the duration of the study could allow for the confirmation and assessment of a time-dependent As effect on promotion and progression, and would ideally result in the formation of overt neoplasms that could be assessed for type and characteristics. With regard to tissue processing, large numbers of serial liver sections should be cut and stored, to preclude any disruption in focus assessment as additional immunohistochemical evaluations are added.

Since our TGFβ1 and TGFβII-r stains were more difficult to evaluate than expected, immunohistochemical staining for downstream proteins induced by receptor binding such as CDK inhibitors p21 and p27 could be employed. We could infer binding of the TGFβII-r or lack thereof by detection of these known downstream cell cycle inhibitors. Since a functional TGFβI-r is also required for successful TGFβ1 binding, this receptor should also be examined by immunohistochemistry to obtain a more complete picture of TGFβ's effect on the foci or surrounding tissues.

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Much of the discussion presented concerned the microenvironment of the preneoplastic focus compared to surrounding normal tissue, and even the cellular characteristics of subpopulations of altered cells within the foci. The utilization of laser capture microdissection could allow for the physical manipulation and separation of these cell populations by physically excising foci. Once separated, DNA from microdissected cells could be amplified for functional exons by polymerase chain reaction (PCR) and specific cell characteristics could be determined via gene expression and or protein expression microarray analysis to more thoroughly explore the differences between these cells. The microarray technique has been used previously to determine genetic variation in As-exposed tissues, demonstrating overexpression of cyclin D1, alpha-fetoprotein, *c-myc*, and estrogen receptor-alpha in tumors of mice exposed to As (Chen *et al.* 2004; Liu *et al.* 2004), all of which could contribute to As carcinogenesis. Furthermore, the Liu *et al* study found significant changes in some CYPs and other genes in normal tissues of these exposed mice. We could therefore use this technique to compare control to exposed rats as well as normal to altered cells, e.g. inside vs. outside foci and GST-P+ vs. TGFα+. This could allow for a rapid assessment of the specific genetic markers and protein products between the different cell populations and provide a targeted list of phenotypic markers on which to focus further investigation of progression. It might also provide genomic reinforcement for our current findings with regard to differences in expression of TGFα and TGFβII-r.

Comparative genomic hybridization (CGH) is a technique by which one can analyze large scale alterations in the genetic material, as might be expected as a result of As exposure due to arsenic's clastogenicity (Nacheva *et al.* 1998; Weiss *et al.* 1999).

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DNA would be isolated from both focus cells and normal adjacent cells at any stage of mitosis and each cell type labeled. The altered and normal DNA are hybridized to metaphase cells from rat lymphocytes. Gains of genetic material such as acquisition of an oncogene, and losses such as deletion of a tumor suppressor gene, are indicated by changes in the ratio of color labels on the hybridized cells. This is a method that could be used to highlight any genetic changes that As (or even dioxin-like chemicals) might induce, especially in the later stages of carcinogenesis. While this might not have been fruitful with our relative short study duration, it certainly would be indicated on a longer study, especially with overt neoplasms as an endpoint. Furthermore, it would allow the determination of relatively specific genomic alterations that could be compared between treatment groups. For instance, this could help answer the questions of arsenic's clastogenic effects as a single agent compared to it in a mixture with PCB 126, and allow us to examine how those effects might change at the chromosome level over time. Since the work presented here suggested a time-dependent effect with arsenic's antagonism on promotion appearing to decrease as the progression stage is reached, CGH would allow the generation of a more complete picture of what genetic alterations might be involved.

The studies described in this dissertation provided a glimpse into the workings of chemical carcinogenesis and allowed an assessment of some of the chemicals involved and potential mechanisms by which they exert their effects. With both halogenated aromatic hydrocarbons (PCBs and TCDD) and arsenic continuing to be present in the environment and exposure to humans an ongoing concern, experiments to explore the toxic and carcinogenic properties of these agents will continue to be necessary. We investigated only a few of the processes involved, including oxidative injury and

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expression of transforming growth factors alpha and beta. The carcinogenic process is extremely complex, and numerous other factors could exert their influence on the transition through the stages of carcinogenesis. It is likely that the growth factors TGFα and TGFβ are merely two small parts of a vast web of parallel and interconnecting pathways, some pro-carcinogenic and some anti-carcinogenic, and it is the overall effect of all of these myriad factors that ultimately determines carcinogenic outcome.

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