BENZO(A)PYRENE-MEDIATED EFFECTS ON CELLULAR ADHESION AND SIGNAL TRANSDUCTION PATHWAYS IN ENDOMETRIAL CANCER

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2001
This dissertation is dedicated to my parents Joan and Donald McGarry for all of their love, support, and encouragement in helping me to achieve my dreams.
ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the many people who have contributed to my dissertation work over the past four years. First, and foremost, I would like to thank my mentor, Dr. Kathleen Shiverick, for her continuous efforts to challenge, support and guide me throughout my graduate school years. Her enthusiasm and love for science has been an inspiration to me, and has made my graduate learning so enjoyable. Her high expectations and encouragement in presenting my research at national and local conferences has helped me in developing my critical thinking, problem solving, and communication skills. She has also generously shared her time in discussing my experiments and has provided extensive support in helping me to accomplish my professional and personal goals. Her genuine compassion, warmth, and concern for others have made her a role model to me in both my research and personal life, and I feel truly fortunate to be her student.

I would also like to thank my committee members, Dr. Michael Bubb, Dr. Naser Chegini, Dr. Stephen Roberts, and Dr. Dietmar Siemann, for their dedication and support of my research over the past four years. Individually, and as a group, they have shared excellent suggestions and advice that has stimulated my thoughts and have deepened my understanding. Their enthusiasm and encouragement have contributed to my success and enjoyment of research.

I would further like to thank Theresa Medrano from our laboratory for her outstanding technical assistance and advice, as well as her friendship. Her patience,
kindness, and concern for others make her a pleasure to work with. I also thank all of the past and present members of Dr. Shiverick’s laboratory for their friendship and support, including fellow graduate students Von Samed and Renita Handayani.

In addition, I would like to thank Dr. Maria Grant for her generous assistance with the Boyden chamber attachment assay technique, as well as Dr. Grantley Charles for his prior experience, advice, and humor. In addition, I would like to thank Dr. Martha Campbell-Thompson and Dr. Michael Bubb for generously sharing their time, expertise and resources for my immunocytochemistry and actin immunostaining experiments. In addition, I thank Melissa Chen from the flow cytometry core laboratory for her kindness and help with the cell cycle phase distribution analysis, and Juan and Dr. Henry Baker for assistance and suggestions on cDNA microarray data analysis. Finally, I would like to thank Judy Adams and the entire staff in the Department of Pharmacology for their exceptional administrative and personal support. I am additionally grateful for the financial support of the Superfund Basic Research Program graduate fellowship award.

Finally, I would like to extend my deepest gratitude and love to my parents, who have continuously believed in me and encouraged me to do my best. They have given me the encouragement and support I needed to follow my dreams.
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Cigarette smoking in women has been linked with a decreased incidence of endometrial cancer and endometriosis, whereas exposure to the dioxin 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has been associated with an increased incidence of endometriosis in animal models. This study characterized the cellular and molecular effects of BaP, a cigarette smoke toxicant, and dioxin on three human uterine endometrial cancer cell lines (RL95-2, HEC-1A, and HEC-1B).

Experiments show that BaP completely inhibited RL95-2 and HEC-1A cellular attachment and decreased membrane β-catenin and EGF receptor protein levels; in contrast, HEC-1B cells were unaffected. HEC-1A cells also showed decreased α-catenin levels with BaP treatment, whereas HEC-1B cells remained unaffected. TCDD did not affect RL95-2 cell attachment, β-catenin, or EGF-R levels. BaP, but not TCDD, further produced subcortical actin aggregates and decreased cadherin levels in RL95-2 cells,
whereas neither chemical altered overall actin and vinculin levels. Furthermore, BaP induced an enhanced cell cycle response in HEC-1A and RL95-2, but not HEC-1B cells, whereas TCDD had no effect.

HEC-1A (BaP-responsive) and HEC-1B (BaP-unresponsive) cells were compared for key morphological differences. HEC-1A cells expressed higher levels of α-catenin and had thick, cortical, filamentous actin, whereas HEC-1B cells exhibited nuclear-localized actin and had non-continuous intercellular boundaries. CYP1A1 was induced in all three cell lines upon BaP and TCDD treatment, and therefore did not account for the cell-specific and BaP-specific effects. Microarray experiments to define profiles of gene expression following treatment of HEC-1A and HEC-1B cells, as well as for BaP, TCDD, and t-butylhydroperoxide treatment of RL95-2 cells, indicated that BaP does not significantly induce stress-regulated genes by 6 hours.

Overall, results show that the Ah-Receptor ligand BaP alters a complex array of signal transduction pathways in human endometrial adenocarcinoma cell lines, resulting in profound alterations of cellular adhesion, cytoskeleton, and cell cycle. RL95-2, HEC-1A, and HEC-1B cells are presented as useful models for determining alternate cellular responsiveness to xenobiotic exposure.
Evidence indicates that cigarette smoking in women is associated with a decreased incidence of endometrial cancer and endometriosis. The environmental contaminant benzo(a)pyrene (BaP), a major toxicant in cigarette smoke, has been shown to inhibit the growth of human uterine endometrial RL95-2 cells in culture (Charles, 1997). Exposure to the dioxin 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has been associated with an increased incidence of endometriosis in animal models, and possibly women. The present study explored cellular and molecular mechanisms which underlie the growth inhibitory effects of BaP, a known carcinogen and AhR ligand, on uterine endometrial cancer cell lines. One hypothesis herein tested is that BaP exerts growth inhibitory effects by altering the delicate balance of cellular adhesion and cytoskeletal proteins, thereby altering cellular attachment and invasion. An additional hypothesis investigated was that BaP acts by disrupting the integrity of the adherens complex proteins by altering the phosphorylation status of β-catenin. Finally, the role of cell cycle alterations and the induction of AhR-dependent and AhR-independent enzymatic pathways for BaP mediated effects, including the generation of an oxidative stress response, were investigated using BaP-responsive and BaP-unresponsive endometrial cancer cell lines. Many experiments involved a comparison of the effects of BaP with those of TCDD, a non-metabolized AhR ligand.
The studies presented here evaluated the usefulness of three human, uterine endometrial cancer cell lines as *in vitro* models to elucidate the cellular and molecular effects of BaP and TCDD. Experiments were conducted to characterize the effects of BaP and TCDD treatment on key cellular adhesion molecule protein levels, localization, and interactions in relation to cellular attachment and invasion. Levels of the major proteins involved in cell-cell adhesion (cadherin, β-catenin, α-catenin, vinculin, and actin), as well as epidermal growth factor receptor (EGF-R) were quantitated in RL95-2, HEC-1A, and HEC-1B cell lines. The localization of key cellular proteins was visualized using light and confocal microscopy. The effects of BaP on the phosphorylation levels of cellular adhesion proteins and the integrity of the adhesion complex were evaluated in HEC-1A and HEC-1B cells.

Further studies were performed to characterize the effects of BaP and TCDD on gene expression in three endometrial cancer cell lines through two separate signal transduction pathways, the AhR and oxidative stress. Because BaP and TCDD activate AhR-mediated genes, studies were performed to determine whether the observed differences in gene expression between BaP and TCDD were due to the differential activation of enzymes involved in xenobiotic metabolism and the induction of an oxidative stress response in cells. The effects of BaP and TCDD on cellular mRNA and protein levels of the enzymes CYP1A1, CYP1B1, PGHS-1, and PGHS-2 were characterized in each cell line. Further comparison of major gene induction pathways of BaP with TCDD, a non-metabolized AhR ligand, and t-butylhydroperoxide, a classic oxidative stress inducer, were evaluated using cDNA microarray technology. A biochemical measure for oxidative stress, the oxidation of dichlorodihydrofluorescein
diacetate, was employed to characterize the xenobiotic effects on intracellular oxidative stress.

The temporal effects of BaP and TCDD on cell cycle phase distribution in RL95-2, HEC-1A, and HEC-1B cells were determined using flow cytometry analysis with propidium iodide fluorescence at 6, 12, 24, and 48 hours. Analysis of the alternate signal transduction pathways for BaP-mediated effects on the HEC-1A and HEC-1B cell lines was carried out using cDNA microarray technology. Gene expression data were analyzed for fold-change induction or repression of individual genes, as well as for common patterns of gene expression across multiple replicates and chemical treatments. The differential response of the respective cell lines to BaP treatment serves as a predictive model for biomarkers of effect for xenobiotic exposure on the human uterine endometrium, as well as for differential tumor cell response to exposure to these prototype environmental toxicants.

Carcinogenesis and Human Health

There are an estimated 200 different types of cancer known to exist in humans, all characterized by an uncontrolled cellular growth and the capacity to metastasize to distant sites, thereby adversely affecting the health of the patient (Thomas, 1993). Animal studies indicate that the formation of cancer involves multiple stages, characterized as initiation, promotion, and progression (Guyton and Kensler, 1993). The initiation stage of carcinogenesis involves irreversible alteration of a cell, typically by mutation. Initiation can occur through oxidative DNA modification that results in potentially deleterious mutations. The resulting mutations may alter the function of key genes
relating to carcinogenesis, such as oncogenes or tumor suppressor genes. Promotion, the second stage of cancer development, involves the clonal expansion of a mutated cell. Typically, promotion will occur through the perturbation of signal transduction pathways, including changes in methylation, accumulation of intracellular calcium, inhibition of intercellular communication, or alterations in gene expression or apoptosis (Yoshida and Ogawa, 2000). The ultimate stage of carcinogenesis, progression, involves the process whereby malignant transformation occurs and cellular growth proceeds uncontrolled. Progression typically occurs through multiple stages, as it has been estimated that 10 or more mutational changes occur in most cancers (Barrett, 1993). Oxidative stress has been implicated in carcinogenesis by producing alterations in signaling pathways which lead to mutagenicity through oxidative modification of genetic material, stimulation of the initiated cell during tumor promotion, and progression to uncontrolled growth and malignancy (Guyton and Kensler, 1993; Yoshida and Ogawa, 2000).

Pathophysiology of Uterine Cancer

Endometrial cancer, the most common gynecologic malignancy in the United States, remains the fourth leading cancer incidence in females and the eighth leading cause of new cancer deaths in women (American Cancer Society, 1998). Whereas an estimated 36,100 cases of cancer of the uterine corpus, usually of the endometrium, were diagnosed in 1998, incidence rates have been relatively constant since the middle 1980s at about 21 per 100,000 (American Cancer Society, 1998). The estimated deaths from endometrial cancer of 6,300 in 1998 coincide with the relatively constant mortality rates since 1989 of about 3 per 100,000 (American Cancer Society, 1998). Furthermore,
endometriosis, a painful and chronic uterine disease often associated with infertility, has been detected in 10 to 15 percent of all premenopausal women undergoing gynecological surgery (Haney, 1990), as well as 25 percent of all women in their thirties and forties (Chalmers, 1980).

Endometrial adenocarcinoma occurs during the reproductive and menopausal years, with 20 to 25 percent of cases being diagnosed before the onset of menopause (Creasman, 1997). Initially associated with abnormal uterine bleeding or spotting, endometrial cancer later produces pain or systemic symptoms. There are two primary types of endometrial cancers, estrogen-dependent and estrogen-independent. The estrogen-dependent form of endometrial cancer is associated with estrogen-related exposures such as estrogen replacement therapy, tamoxifen, early menarche, late menopause, nulliparity, and failure to ovulate. The estrogen-independent form of endometrial cancer appears to lack direct hormonal influence, and could potentially arise due to a compromised immune system with age. Additional risk factors associated with endometrial cancer include infertility, diabetes, gallbladder disease, dietary factors, hypertension, and obesity (American Cancer Society, 1998). Endometrial cancers are routinely treated with surgery, radiation, hormones and/or chemotherapy, and result in a relatively high rate of survival if diagnosed early.

It has been shown that smoking is consistently associated with an independent reduction in risk for endometrial cancer. Epidemiological studies indicate that cigarette smoking reduces the risk of endometrial cancer by as much as 50 percent (Meek and Finch, 1999). It is believed that the seemingly protective effect of cigarette smoking on endometrial cancer involves an anti-estrogenic mechanism, wherein women who smoke
appear as if estrogen deficient (Baron, 1996; Baron et al., 1990). In addition, current smokers and postmenopausal women experience the greatest reduction in cancer risk (Baron et al., 1990; Lesko et al., 1985). The reduction may be attributed to three primary mechanisms: a reduction in luteal phase estrogens, early menopause, and differences in metabolism of estrogens among smokers (Burke et al., 1996). In addition, smoking has been shown to alter prostaglandin pathways (Burke et al., 1996). Further, there is direct evidence for the implication of cigarette smoke in the progression of human papillomavirus (HPV)-initiated cervical cancer (Nakao et al., 1996; Nakao et al., 1996).

Environmental Exposure to Polycyclic Aromatic Hydrocarbons and Dioxin

Polycyclic aromatic hydrocarbons (PAHs) are prevalent environmental pollutants formed during the combustion of fossil fuels and the burning of various organic materials. Each year, tens of thousands of PAHs are released into the environment in the United States alone, leading to the contamination of air, water, and soil (Zedeck, 1980). Humans are exposed to PAHs through inhalation of coal and petroleum products, wood smoke and cigarette smoke, as well as through occupational inhalation exposures from tars and fumes (Burchiel and Luster, 2001). Inhalation of PAHs can involve gaseous, or volatilized components, as well as exposure to PAH-coated particles, which are eventually ingested. Exposure to PAHs primarily occurs through ingestion, partly from consumption of charcoal-broiled foods, through which humans are exposed to microgram concentrations of PAHs each day (Burchiel and Luster, 2001). Additional human exposure to PAHs occurs through dermal contact with tars or soot. Since human exposure to PAHs often occurs in combination with exposure to other toxic compounds,
the effects associated with a single exposure to BaP may not directly reflect actual environmental exposures.

Cigarette smoke, a common route of human exposure to PAHs, is a complex mixture containing more than 3,500 particulate and nonparticulate components, at least sixty of which are known toxicants. PAHs, nicotine, cadmium, nitroso compounds, aromatic amines, protein pyrolysates, and carbon monoxide have all been associated with the toxic effects of smoke (Shiverick and Salafia, 1999; Meek and Finch, 1999). Benzo(a)pyrene (BaP), a major component of cigarette smoke, was selected as a target toxicant for study due to its predominance and potency. BaP is present at levels of 20-40 ng/cigarette (Shiverick and Salafia, 1999). The average daily intake of BaP in the United States is approximately 2.2 µg/day, the majority of exposures resulting from ingestion, inhalation, and dermal absorption (Kim et al., 1998; Hattemer-Frey and Travis, 1991). Further, it is estimated that the average intake of BaP in smokers of one pack of unfiltered cigarettes is nearly 0.7 µg/day over background levels, whereas the corresponding intake for filtered cigarettes is 0.4 µg/day over background (Miller and Ramos, 2001b).

Whereas some PAHs are relatively innocuous, BaP is a potent chemical carcinogen. BaP is a highly lipophilic compound that can be readily taken up by cells through the plasma membrane. Once inside the cell, BaP is rapidly distributed throughout the cellular compartments, including the mitochondrion and nucleus, the Golgi, and the cytoplasmic membranes, including the plasma membrane, endoplasmic reticulum, and nuclear envelope (Miller and Ramos, 2001b). Studies from human placentas of women who smoke cigarettes lend support for the study of BaP-mediated
effects on uterine endometrial cancer. It has been reported that EGF-stimulated kinase activity was markedly decreased in placental membrane proteins from women who smoke cigarettes, whereas insulin receptor phosphorylation was unaltered or increased, indicating that maternal smoking was associated with a selective loss of EGF receptor autophosphorylation (Wang et al., 1988). Further studies have shown the induction of placental aryl hydrocarbon hydroxylase (AHH) activity in placental tissue of smokers, an observation associated with the binding of PAH adducts to DNA in vitro (Pelkonen and Saarni, 1980; Berry et al., 1977; Vaught et al., 1979). Studies in human placental cell cultures reflect the dose-dependent decrease of BaP on EGF receptors in early gestation cells (Guyda et al., 1990). Term placental cells, on the contrary, were shown to reflect receptor desensitization upon 10 μM BaP exposure due to a dissociation of EGF binding and EGF receptor protein kinase activity (Guyda et al., 1990). In addition, a dose-dependent induction of AHH by approximately 20- to 150-fold was observed in early placental cells exposed to BaP (Guyda et al., 1990). Furthermore, the dose of 10 μM BaP has been shown to induce CYP1A1 and CYP1B1 in the RL95-2 endometrial cancer line (Charles and Shiverick, 1997).

In consideration of published studies, the concentration of 10 μM BaP was selected for current experiments studying the effects of BaP on uterine cancer cell lines. Since BaP is found in concentrations of 20-40 ng/cigarette, a concentration of 1 μM BaP in cell culture is equivalent to a dose of approximately 250 ng/ml of BaP, or the approximate BaP content of 5 cigarettes. Since BaP is known to bioaccumulate, the actual environmental relevance of the 10 μM BaP dose is somewhat uncertain.
Additional studies investigating dose-response relationships for BaP-mediated effects on attachment, adhesion, and cell cycle effects could prove beneficial.

A genotoxic chemical, BaP significantly alters signal transduction in human and animal cells by binding to the intracellular cytosolic protein aryl hydrocarbon receptor (AhR) and transcriptionally activates cytochrome P450 1A1 (CYP1A1), leading to its own metabolism (Poland et al., 1976; Whitlock, Jr. et al., 1996). The AhR protein, an N-terminal basic helix-loop-helix protein, was found to be expressed in 43 percent of human endometria studied by Kuchenhoff and co-workers, with apical cytoplasmic localization in epithelial cells of endometrial glands (Kuchenhoff et al., 1999). The finding that the PAH BaP is carcinogenic, while the structurally similar PAH benzo(e)pyrene (BeP) is weakly or non-carcinogenic, makes BeP a useful tool in determining specific and nonspecific effects of these chemicals on cell lines (MacLeod et al., 1982) (Figure 1-1).

The cytochrome P450 enzyme CYP1A1, known to function in the bioactivation of procarcinogens, is induced in many tissues by BaP, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the prototype AhR ligand, or other AhR ligands (Omiecinski et al., 1999). Whereas cigarette smoke has been associated with a decreased incidence of endometrial cancer and endometriosis (Baron, 1996), TCDD has been associated with the promotion of uterine disease (Cummings and Metcalf, 1995; Koninckx et al., 1994; Mayani et al., 1997; Rier et al., 1993). In addition, dioxin exposure has been associated with toxic effects such as atrophy, reduced sperm counts, chloracne, teratogenicity, and carcinogenicity (Koninckx et al., 1994; Mayani et al., 1997). Recent data analyzing the twenty year effects on residents from Seveso, Italy after the 1976 massive dioxin exposure indicate a link of dioxin with an overall increased rate of carcinogenesis relative
to unexposed neighboring populations (Bertazzi et al., 2001). However, uterine cancer was not shown among the cancers increased by dioxin exposure after twenty years (Bertazzi et al., 2001). In addition, TCDD responsiveness has been recently been shown in a human, uterine endometrial explant culture model as determined by increased induction of CYP1A1 and CYP1B1 mRNA, protein, and enzymatic activities (Bofinger et al., 2001).

Although the AhR mediates both BaP and dioxin actions, unlike BaP, none of the symptoms from TCDD exposure are believed to result from the metabolism of the parent compound (Figure 1-1) (Kolluri et al., 1999). Aside from the AhR pathway, TCDD further exerts effects through estrogen/estrogen receptor mechanisms and immunologic mechanisms (Hazan and Norton, 1998; Umbreit et al., 1989; Safe et al., 1998).

**Cell Culture Models for Uterine Disease**

Female reproduction is characterized by cyclic transformation of the endometrium mediated by steroids and steroid receptors. During each menstrual cycle, the upper layers of the endometrium, comprising glandular ducts and stroma, are routinely shed, forming a uterine environment in continual flux. Evidence indicates that altered hormonal states are key to the etiology of certain uterine disorders (Hughes and Pfaff, 1998). However, due to the continued flux of the uterine endometrium, careful study of the direct role of cellular adhesion and cytoskeletal proteins proves more variable on primary cultures than on established cell lines.

The cadherins, integrins, and cytoskeletal proteins are key candidates for studies of cell-cell and cell-extracellular matrix adhesion processes involved in endometrial
cancer. Careful determination of their roles as mediators of signal transduction pathways leading to altered cellular attachment requires the use of appropriate cell culture models to control for inherent variability found in primary cultures. Established human endometrial cell lines offer the advantage of providing a homogeneous cell type and a controllable condition for studying the action of environmental chemicals in vitro.

RL95-2 cells have been shown to have a smooth surface structure, a thin glycocalyx, and a non-polarized phenotype which allow them to effectively adhere to trophoblast cells (Thie et al., 1998; Thie et al., 1996). RL95-2 cells express E-cadherin and integrins over their entire surface and represent late stage endometrial cancer (Thie et al., 1996). Further, RL95-2 cells express both cytoplasmic and nuclear estrogen receptors at early passage (subcultured < 30 times), but not at high passage (subcultured > 200 times), thereby providing a useful model for both primary malignancy and metastatic tumor formation (Sundareshan and Hendrix, 1992; Way et al., 1983). The RL95-2 cell line is distinct from others in the adhesiveness of its apical pole for trophoblast cells, thereby serving as an in vitro model of the human uterine epithelium receptive for implantation (Thie et al., 1997). Preliminary data using RL95-2 cell cultures indicate that BaP markedly decreases cellular invasion and attachment (Charles, 1997). The observed alterations in cellular attachment and invasion were hypothesized to result from BaP-mediated altered protein levels or localization of cellular adhesion or cytoskeletal proteins.

HEC-1A and HEC-1B cells, by comparison, have been shown to have a rough surface structure and a thick glycocalyx, and to represent early stage endometrial cancer (Thie et al., 1998). HEC-1A and HEC-1B cells are well established in vitro models often
used to study the effects of hormones and/or growth factors on uterine endometrial cell growth. Both cell lines are characterized by similar proliferative patterns, including doubling time and cell cycle kinetics (Borri et al., 1998). HEC-1A and HEC-1B cells are highly polarized and consequently are non-adhesive upon contact with trophoblast cells (Thie et al., 1998). Further, HEC-1A and HEC-1B cells express E-cadherin and integrins at their lateral membranes. Several reports indicate that HEC-1A cells express an estrogen responsive phenotype with ER-α expression and estrogen-induced cellular proliferation (Castro-Rivera and Safe, 1998), whereas the HEC-1B cell subtype fails to show expression of the ER through RT-PCR analysis (Holsapple et al., 1996). Given recent discoveries of ER subtypes, however, evidence is uncertain over the actual ER status of the HEC-1A and HEC-1B cellular substrains. The studies herein described detail the differences in cellular morphology among the three cell lines likely contributing to their differential cellular responses to chemical insult. The RL95-2, HEC-1A, and HEC-1B cell lines are proposed as an excellent model system for investigations on xenobiotic-mediated cellular adhesion alterations. The three cell lines serve as useful models for the development of biomarkers for the aryl hydrocarbon receptor- (AhR) and oxidative stress-mediated signal transduction pathways for chemical effects. The BaP-mediated dysregulation of cell adhesion molecules has been explored for alterations in uterine epithelial cell polarization that may impact upon cellular attachment and invasion.

**Cell Adhesion Molecules and Uterine Disease**

Cell adhesion molecules play a fundamental role in the determination of tissue architecture and functions of cell assembly and connection to the internal cytoskeleton
and, consequently, are of great significance in the pathophysiology of endometrial cancer. The differential regulation of cadherin, β-catenin, α-catenin, and actin are central to investigations of altered cellular attachment to other cells (Figure 1-2). Cadherins are integral membrane glycoproteins functioning in epithelial cells to form calcium-dependent linkages between cells (Potter et al., 1999; Peralta et al., 1997). Cadherins play a key role in mediating the formation and breakage of cell-to-cell contacts and in maintaining strength in cellular adhesion through homophilic interaction of their extracellular domains, allowing for cellular aggregation to occur (Potter et al., 1999). Classical cadherins, such as E-cadherin found largely in epithelial cells, consist of an extracellular domain, followed by a transmembrane region and highly conserved cytoplasmic domain where cadherins critically interact with β-catenin (Potter et al., 1999). A key membrane-associated protein, β-catenin is responsible for the colocalization of cadherins to sites of cell-cell contact with the actin cytoskeleton. Actin works in conjunction with cytoskeletal microtubules and intermediate filaments in performing essential functions in locomotion and cytokinesis (Hulka and Brinton, 1995). Actin bundles are attached to integral membrane cadherin and integrin proteins, adapter proteins and the contractile bundle. β-catenin forms a signal transduction pathway with actin, the key cytoskeletal player, through α-catenin (Yamada and Geiger, 1997). Both β- and α-catenins are known to associate early with E-cadherin at the endoplasmic reticulum during its synthesis; yet α-catenin is integrated into the adherens complex at the later stage of plasma membrane insertion (Potter et al., 1999). Activation of epidermal growth factor receptor (EGF-R), as well as other tyrosine kinases, has been
shown to directly affect the adhesive function of E-cadherin through catenin alterations (Hayes et al., 1996).

Tumor cell invasion has been highly linked to changes in the integrin family of receptors, as well as to cadherins. Integrins mediate stable adhesion to extracellular matrix (ECM) components; therefore, alterations in integrin expression contribute to tumor cell invasiveness and metastasis (Sanders et al., 1998). Integrins are integral membrane proteins that bind to fibronectin or laminin molecules and are of considerable interest since they not only mediate cell-matrix and cell-cell adhesion, but also function in plasma membrane signaling (Thie et al., 1997). Integrins are composed of two distinct subunits, α and β, which are non-covalently associated with each other (Thie et al., 1997). Unlike the cadherins which produce strong adherence between cells, integrins exhibit relatively low affinities for their ligands, yet allow cells to remain firmly anchored to the matrix due to multiple weak interactions generated by numerous integrins binding to ECM proteins (Hopfer et al., 1996). For cells to successfully migrate, they must be able to make and break numerous, weak integrin contacts. In particular, the disregulation of integrins α5β1, α6β4, α5β3, and α6β1 likely play a significant role in uterine disease pathogenesis, as well as adherence of the trophoblast during implantation (Beliard et al., 1997; Thie et al., 1997). The α5β1 integrin fibronectin receptor has been shown to be lower in endometriotic tissues, suggesting a potential role of the fibronectin receptor in the persistent attachment of endometriotic cells during menstruation (Beliard et al., 1997). Immunolocalized α5β1 function at focal contacts of cells with the substratum keeps the cells tightly bound to the matrix, likely through indirect binding to the actin cytoskeleton. The α5β1 protein has been shown to be reduced in tumor cells, and when
overexpressed, it has been linked with a suppression of cellular growth and tumorigenesis (Plantefaber and Hynes, 1989; Giancotti and Ruoslahti, 1990).

**Oxidative Stress and Toxicology**

Oxidative stress occurs when there is an imbalance in the generation and removal of reactive oxygen species in an organism. Reactive oxygen species (ROS) have the potential to damage tissues and cellular components, including the cell membrane, DNA, and proteins (Guyton and Kensler, 1993). ROS are produced in a cell as a result of normal, physiologic processes, as well as from oxidizing enzymes and xenobiotic metabolism (Guyton and Kensler, 1993; Dalton *et al.*, 1999). Free radicals play a variety of normal physiological roles, including functioning in immune responses, neurotransmission, and muscle relaxation, as well as control of certain transcription factors (Ramos, 1999; Morel and Barouki, 1999). In addition, ROS production can elicit toxic effects in cells, and thereby adversely effect human health (Guyton and Kensler, 1993).

A number of xenobiotics, including BaP and TCDD, can increase ROS production within cells. Benzo(a)pyrene is a carcinogenic polycyclic aromatic hydrocarbon (PAH) known to exert toxic effects on cells through multiple mechanisms. BaP may act directly, initiating tumors through cytochrome P450-mediated metabolic pathways (Miller, 1970; Heidelberg, 1975; Dipple, 1994). BaP may also act indirectly, affecting cell signaling pathways through oxidative and electrophilic signaling pathways, including redox cycling and quinone formation (Bulun *et al.*, 2000). Redox cycling involves the univalent reduction of the xenobiotic to radical intermediates by enzymes
such as NADPH-cytochrome P450 reductase (Kelly et al., 1998). In the process, a radical intermediate transfers an electron to oxygen, producing oxygen radicals (-O\(_2^\cdot\)), and regenerating the parent compound. Multiple oxygen radicals are produced in the process, and NADPH is depleted (Kelly et al., 1998).

Excessive production of oxygen radicals can have toxic effects on cells. ROS can damage lipids, DNA, and proteins. Lipid damage occurs via a chain reaction including initiation, propagation, and termination, thereby altering biological membranes (Guyton and Kensler, 1993). ROS can additionally be damaging to DNA, resulting from hydroxide (HO\(-\)) attack on nitrogenous bases, or the DNA backbone itself. The damage produced can include hydroxylation, ring opening, and DNA fragmentation (Kelly et al., 1998). In particular, BaP exposure is associated with 8-hydroxy-2'-deoxyguanosine (8-OhdG) formation. Typically, DNA damage is repaired by excision repair and postreplication repair enzymes, a process often associated with a prolonged S phase of the cell cycle. In severe cases, inhibition of cell cycle progression occurs to prevent transmission of mutations to daughter cells. ROS can further damage cells by producing protein-DNA crosslinks.

In summary, the present dissertation studies were undertaken to investigate the role of BaP on the initiation of complex AhR- and oxidative stress-mediated signal transduction pathways in three human endometrial cancer cell lines. The differential effects of BaP and TCDD on cellular adhesion, cytoskeleton, and cell cycle checkpoint responses were determined specific to each cell line, thereby providing a model for biomarkers of effect for xenobiotic exposure on the human uterine endometrium, as well as for differential tumor cell response to xenobiotic exposure.
Figure 1-1. Chemical structures of xenobiotics.
The chemical structures of the test compound benzo(a)pyrene (BaP) (A), the structure activity control Benzo(e)pyrene (BeP) (B), and the prototype dioxin 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (C).
Figure 1-2. Cell-cell and cell-extracellular matrix adhesions.
Schematic diagram depicting key cell-cell connections by adherens junctions and cell-extracellular matrix connections by hemidesmosomes and adhesion plaques. Key cellular adhesion molecules involved are depicted, along with their relationship to the actin cytoskeleton.
CHAPTER 2
MATERIALS AND METHODS

Materials

Chemicals and Bioreagents

TCDD was obtained from Midwest Research Institute (Kansas City, MO) through the National Cancer Institute Chemical Carcinogen Reference Repository and benzo(a)pyrene (BaP) was purchased from the Sigma Chemical Co. (St. Louis, MO). Cell culture media was purchased from GIBCO BRL/Life Technologies (Gaithersburg, MD), fetal bovine serum (FBS) from Hyclone Laboratories (Logan, UT), and Matrigel from Collaborative Biomedical Products (Bedford, MA). Penicillin and ampicillin were purchased from the Sigma Chemical Company (St. Louis, MO). [α-\textsuperscript{33}P] dATP was purchased from ICN Biomedicals Inc. (Irvine, CA). The Fisher Leukostat\textsuperscript{TM} stain was from Fisher Scientific (Lexington, MA). LabTec by Nunc\textsuperscript{©} four well chamber slides were purchased from Fisher Scientific (Lexington, MA). 5-(and-6)-chloromethyl-2'7'-dichlorodihydrofluorescein diacetate (CM-H\textsubscript{2}DCFDA) was purchased from Molecular Probes (Eugene, OR). The Human Stress Toxicology Array and the Atlas Human Toxicology 1.2 Array were purchased from Clontech (cat#7747-1). All other chemicals were reagent or molecular biology grade and were obtained from standard commercial sources.
Antibodies

Polyclonal sheep anti-human EGF receptor antiserum was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Polyclonal goat anti-rat CYP1A1 and CYP1B1 antisera were purchased from Gentest (Woburn, MA). Monoclonal mouse anti-human β-catenin, α-catenin, α5 integrin, and β1 integrin antisera were purchased from Transduction Laboratories (Lexington, KY). Monoclonal mouse anti-chicken vinculin antiserum was purchased from Sigma (St. Louis, MO). Monoclonal mouse anti-chicken actin antiserum was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Polyclonal rabbit anti-human PGHS-1 and PGHS-2 antisera were purchased from Oxford Biochemical (Oxford, Michigan) (Tables 2-1 and 2-2).

Methods

Cell Cultures and Chemical Treatments

The human endometrial adenocarcinoma cell lines RL95-2 (passage 127-147), HEC-1A (passage 115-135), and HEC-1B (passage 119-139) were obtained from American Tissue Culture Collection (ATCC). They were maintained in DMEM:HAMS F-12 (1:1), McCoy’s Medium, or Minimum Essential Medium, respectively, supplemented with 10% (w/v) fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO₂ at 37°C. All media contained penicillin and streptomycin at 100 μg/ml. Media were changed every 2-3 days and cells were maintained at a pH of 7.15-7.2. For routine cell culture, cells were rinsed with Hanks Balanced Salt Solution (HBSS) prior to detachment.
All experiments were initiated when cells reached 50-75% confluence. Stock solutions of BaP and TCDD were initially prepared in DMSO and were added to cultures with a final concentration in DMSO of 0.1% (v/v). Unless otherwise indicated, cells were treated with 10 μM BaP and 10 nM TCDD. Tert-butyl hydroperoxide was added to cultures with a final concentration of 200 μM in DMSO. DMSO treated cultures served as vehicle controls for all experiments.

**In Vitro Attachment Assay**

The Matrigel invasion assay was performed using a modified Boyden Chamber apparatus (Grant *et al.*, 1987). RL95-2, HEC-1A, and HEC-1B cell cultures were incubated with 10 μM BaP and 10 nM TCDD for 48 hours, after which cells were trypsinized and collected by centrifugation at 500 x g for 5 minutes. Cells were resuspended in Hank’s buffer, washed twice, and counted using either a hemocytometer or coulter counter. Approximately 30,000 cells treated with respective chemicals were resuspended in 27 μl of serum-free media and were aliquoted into the lower wells of the Boyden chamber. The wells were overlaid with a Matrigel-coated polyvinyl-pyrrolidone-free polycarbonate membrane (Nucleopore, 8 micron diameter pore size for RL95-2 cell experiments, 10 micron diameter pore size for HEC-1A and HEC-1B experiments) and the gasket seal and upper chamber attached. The apparatus was inverted at 37°C/5% CO2 for 2 hours to allow for cell attachment. The apparatus was then disassembled and attached cells stained with Leukostat™ (Fisher Scientific, Lexington, MA) and quantitated by light microscopy. Cells were counted in quadruplicate wells for each treatment regimen. Images of stained cells were digitally captured using a Zeiss
fluorescence microscope under low power magnification and cells quantitated manually using Microsoft Paint software. All data were expressed as the mean ± standard error measurement (SEM) of the number of migrating cells from three separate experiments.

**Immunocytochemistry**

Cells were plated on poly-L lysine-coated four-well chamber slides. Cultures were treated for 48 hours with 10 μM BaP, 10 nM TCDD, or DMSO vehicle for 48 hours. Cells were fixed in freshly prepared 3.7% paraformaldehyde in modified HBSS for 10 minutes at room temperature followed by phosphate buffered saline (PBS) washes (10 mM KPO₄, 150 mM NaCl, pH 7.5). Cells were then blocked in 10% normal serum for 10 minutes at 37°C followed by anti-human EGF-R antibody in PBS/1% BSA (10 μg/ml) overnight at 4°C or for 1 hour at 37°C. In control experiments, the primary antibody was replaced with PBS alone. Slides were washed in PBS and endogenous peroxidase activity blocked by incubation with 0.3% H₂O₂ in PBS for 10 minutes. Cells were incubated with rabbit anti-sheep peroxidase-labeled secondary antibody (1:50 dilution; Southern Biotechnology Associates, Birmingham, AL) for 10 minutes followed by PBS washes. Diaminobenzidine tetrahydrochloride with CoCl₂ enhancement was used as substrate for visualization. Cells were counterstained with hematoxylin and mounted with Fluoromount-G prior to visualization.

For phalloidin immunostaining of actin filaments, fixed and permeabilized cells were incubated with 25 μl of fluorescein-labelled phalloidin (Molecular Probes) in PBS/well. No secondary antibody was necessary for visualization.


**Light and Confocal Microscopy**

Fluorescent light microscopy was utilized to collect images of altered adhesion molecule and cytoskeletal protein alterations, followed by confocal imaging to determine cellular protein variations in alternate focal planes. Confocal microscopy is a specialized technique used to view polarization of cell surface and cytoskeletal proteins in RL95-2 cells by permitting fluorescent molecule visualization in a single plane of focus, thereby creating an immensely sharper image. Through confocal imaging, optical sections (serial sections of fluorescent images at different depths of the sample) are pooled into one three-dimensional image providing information on the distribution of particular protein(s) within a cell. As a result, confocal imaging allows one to create three-dimensional images, see surface contour in minute detail, and accurately measure critical cellular dimensions. Likewise, as compared with standard fluorescence light microscopy, confocal microscopy allows for the acquisition of bright, three-dimensional, high resolution and high contrast images, while overcoming the obstacle of depth penetration which has continually proven a challenge in working with RL95-2 cells.

Fluorescence microscopy was performed on a Zeiss Axiophot microscope equipped with epi-illumination. All confocal microscopy was performed on a Zeiss Inverted Axiovert 100 M BP microscope using a LSM 510 confocal module and Zeiss 2.01 Proprietary software. FITC imaging was performed using a 488 nm excitation argon laser and rhodamine imaging using a 543 nm helium laser. Images were collected through the 40x and 100x power lenses and all image analysis was performed using a Personal Computer with Windows NT software.
**Cellular Protein (Membrane and Lysate) Preparations**

Cells were rinsed three times in ice cold phosphate buffered saline (PBS) solution and collected with a cell scraper in 1 ml PBS containing protease inhibitors (1 mM phenyl-methyl sulfonyl flouride (PMSF), 1 mM Na₃VO₄, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µl/ml pepstatin). Cells were then lysed by three freeze-thaw cycles in liquid nitrogen. The final membrane fractions were obtained by centrifugation at 10,000 x g for 10 minutes at 4°C. Pellets containing cellular membranes were resuspended in 300 µl PBS and protease inhibitors.

Alternately, cells were rinsed three times in ice cold PBS and collected in 1 ml RIPA buffer containing 50 mM Tris, 150 mM NaCl, 1 mM ethylene glycol bis-N,N,N,Tetraacetic acid (EGTA), 1 % Nonidet P-40 (NP-40), deoxycholic acid Na+ salt (0.25%), and sodium fluoride (1 mM), pH 7.4, and protease inhibitors as listed above. Cell solutions were transferred to a 2.0 ml microfuge tube using a 25 gage needle and 1 ml syringe to further lyse cells. Upon 30 minute incubation on ice to ensure lysis, cells were vortexed, and centrifuged at 10,000 x g for 15 minutes at 4°C to separate detergent-extracted cellular lysates. Protein concentrations in supernatants were determined using the BCA protein assay (Pierce #23223).

**Western Immunoblot Analysis**

Cell membrane or detergent-extracted cell lysate protein samples (40 µg) were separated by 7.5% or 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples were then transferred overnight at 4°C to nitrocellulose in 25 mM Tris, 192 mM glycine buffer at pH 8.2, with 20 % methanol according to the method
of Towbin et al. (Towbin et al., 1979). Membranes were blocked in 10% dried milk, tris-buffered saline-Tween (TBS-T) or phosphate buffered saline-Tween (PBS-T) solution for 2 hours with agitation. Membranes were washed, then incubated in primary antibody [See Tables 1 and 2 for respective dilutions] in 5% dried milk and TBS or PBS overnight at 4°C. Following washing, the membranes were incubated in appropriate horseradish peroxidase (HRP)-conjugated or biotinylated secondary antibody [See Tables 1 and 2] in 5% dried milk and TBS or PBS for 2 hours at 37°C with agitation. For experiments using a biotinylated secondary antibody, the membranes were incubated in the diluted biotinylated HRP streptavidin complex subsequent to washing for 45-60 minutes at 37°C. Immunoreactive protein levels were determined using the ECL detection method according to the protocol of Amersham Life Science company.

For EGF-R and CYP1A1 Western immunoblots, immunostaining was performed according to published protocols (Wang et al., 1988). Membranes were incubated in sheep anti-human EGF receptor or goat anti-rat CYP1A1, followed by horseradish-peroxidase conjugated anti-sheep IgG for EGF-R blots and anti-goat IgG for the CYP1A1 blots. Bands were visualized by incubation with 3-amino-9-ethylcarbazole.

For all Western immunoblot experiments, immunoreactive bands were quantitated by scanning on a Microtek ScanMaker II scanner and quantitation was performed using NIH image software. Negative controls were run using preabsorption of antibody or omission of primary antibody to ensure specificity.
Co-Immunoprecipitation of β-catenin with α-catenin

Immunoprecipitation experiments were performed according to the protocols of Dr. Allan Parrish (Parrish et al., 1999), as based on the procedures of Transduction Laboratories (Lexington, KY). Experiments were performed to co-immunoprecipitate proteins under native, non-denaturing, conditions. Given the reactivity of antibodies from the same species, immunoprecipitation experiments were carried using a polyclonal antibody (goat anti-human β-catenin from Santa Cruz, CA), followed by Western immunoblot analysis with the corresponding monoclonal primary antibody for the complexed protein (mouse anti-human α-catenin from Transduction Laboratories, Lexington, KY).

Briefly, cells were rinsed with ice cold PBS (pH 7.4), followed by cell lysis with 500 µl of 1X immunoprecipitation buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM sodium orthovanadate, 0.2 mM PMSF, 0.5% NP-40). Samples were mixed by inversion for 15 minutes at 4°C, followed by centrifugation for 20 minutes at 12,000 rpm. Supernatants were collected and protein concentration determined using the BCA protein assay (Pierce #23223). Cell lysates were then immunoprecipitated by rocking with appropriate soluble antibody (1 µg antibody/100 µg protein) in a total volume of 500 µl immunoprecipitation buffer for 1 hour at 4°C. Twenty µl of Gamma-Bind Plus-Sepharose or Agarose was added to each tube and the incubation continued overnight at 4°C. The samples were centrifuged for 15 minutes at 12,000 rpm and the supernatant removed. The immunoprecipitate (pellet) was washed 3 times in immunoprecipitation buffer, followed by the addition of 30 µl of 2X sample buffer with 10% β-mercaptoethanol, and samples were then boiled for 5 minutes.
Samples were analyzed for co-immunoprecipitation by SDS-PAGE, followed by Western immunoblot analysis for the co-immunoprecipitated proteins.

**Phosphorylation Experiments**

The effects of chemical treatment on β-catenin phosphorylation status were evaluated by immunoprecipitation for β-catenin protein using the polyclonal goat anti-human β-catenin antibody (Santa Cruz, CA), followed by subsequent Western immunoblot analysis for phosphotyrosine residues using a monoclonal α-human phosphotyrosine antibody (cat#P11120, Transduction Laboratories, Lexington, KY). Experiments were carried out according to the immunoprecipitation protocol previously described.

**Flow Cytometry Analysis with Propidium Iodide Fluorescence**

Cells were plated in 10 ml culture dishes and allowed to reach approximately 75% confluency prior to treatment in regular media. Upon 6, 12, 24, and 48 hours treatment, cells were collected and washed in ice-cold PBS and final concentration of cells adjusted to 1 x 10⁶ cells/ml. Cells were then stained with the fluorochrome solution propidium iodide (PI) in sodium citrate buffer using the CycleTest kit (Becton Dickinson, Mountain View, CA). The PI fluorescence of individual nuclei of control and treated samples were measured using a FACSsort cell sorter (Becton Dickinson Immunocytometry Systems, Mountain View, CA) equipped with an ionized argon laser operating at 15 mW output at 488-nm. Red fluorescence due to PI staining of DNA was collected by a 585±21 nm band-pass filter and at least 30,000 cells were analyzed for each sample. The percentages
of cells in G0/G1, S, and G2/M phases were determined by a graphical curve fitting method using ModFit LT 2.0 software (Verity SoftwareHouse, Inc., Topshan, ME).

**Northern Blot Analysis**

Total cellular RNA was isolated from cultured cells by acid guanidium thiocyanate phenol-chloroform extraction according to a published protocol (Xie and Rothblum, 1991). Poly (A)+ RNA was prepared according to the methods of Celano et al. (Celano et al., 1993). For Northern blot analysis, 40 µg samples of total cellular RNA or 10 µg samples of poly (A)+ RNA were denatured and fractioned on a 1.2% formaldehyde RNA gel and transferred to MSI Magnacharge membrane by overnight capillary transfer in 20x SSC solution at room temperature. Upon transfer, the membrane was dried and auto-crosslinked at 1200 µJ/s.

Membrane hybridization with a cDNA probe was accomplished by labeling probes with the Stratagene Prime-it II Random Primer Kit reagents. Upon washing, the membranes were dried and incubated in Clontech ExpressHyb solution in a Kapak/Scotchpak Heat sealable pouch at 68°C. Meanwhile, 20 µl ddH2O, 10 µl random primers, and 4 µl of the cDNA probe were boiled for 5 minutes, then cooled to room temperature, followed by the addition of 10 µl of the 5x dCTP buffer, 5 µl 32P and 1 µl Klenow reagent at 37°C for 10-20 minutes. Upon incubation, Stop solution was added to quench the reaction. The purified probe eluents were collected upon passage through a Stratagene Nuctrap column and 100 µl sonicated salmon sperm DNA was added. Following subsequent boiling and cooling, the probes were incubated with the membranes for 1.5 to 4 hours at 68°C. After hybridization, the membranes were washed
with agitation and the presence of specific RNA was detected on each by autoradiography. All resulting mRNA bands were quantitated by densitometry through the use of NIH image software.

**Ultra-pure RNA Extraction and Poly A+ Purification for cDNA Microarray Analysis**

High purity RNA samples free from DNA contamination were required for cDNA microarray analysis. High purity RNA extraction from confluent cells was carried using the Atlas™ Pure Total RNA Labeling System kit (Clontech laboratories, Palo Alto, CA). All volumes which follow are based on a starting cell number of 1-3 x 10^7 cells (actual volumes used were adjusted accordingly), and all steps were carried out on ice or at 4°C to prevent RNA degradation. Following chemical treatment, cultured cells were scraped in 3 ml of denaturing solution, followed by thorough mixing and centrifugation at 12,000 rpm for 5 minutes. Cellular supernatants underwent two rounds of separate washes in saturated phenol and chloroform, followed by precipitation of RNA from the aqueous phase in 6 ml of isopropanol. Samples were then spun at 12,000 rpm for 15 minutes, pellets washed in 80% ethanol, air dried, and resuspended in RNAse-free water to yield a final concentration of 1-2 μg/μl. Purified samples were then stored at -70°C prior to use.

Subsequently, total RNA samples (0.5 mg) were treated with DNase I and samples extracted in saturated phenol/chloroform prior to purification of poly-A+ (Clontech laboratories, Palo Alto, CA). The overall yield and purity of the total RNA was assessed by measuring absorbance readings at 260 and 280, as well as by comparing intensities of the 28S and 18S ribosomal RNA bands (4.5 and 1.9 kb) after agarose gel
electrophoresis. The ratio of 260 to 280 intensities was determined for RNA purity, with acceptable values for samples in the range of 2:1. Total RNA samples were enriched for Poly A+ RNA using Streptavidin magnetic beads according to the protocol of the Atlas™ Pure Total RNA Labeling System (Clontech laboratories, Palo Alto, CA).

cDNA Microarray Analyses

The RL95-2 microarray experiments were performed using the Clontech Atlas™ Human Stress Array (cat#7747-l) and the HEC-1A and HEC-1B experiments using the Clontech Atlas™ Human Toxicology 1.2 Array (cat# 7859-1). For all experiments, Poly A+ enriched RNA free from DNA contamination was isolated from treated cells at selected time points. $^{33}$P-labelled cDNA probes were generated by reverse transcribing each RNA population to cDNA in the presence of [α-$^{33}$P] dATP using a gene-specific primer mix as described in the Clontech™ protocol. Subsequent purification of the labeled cDNA from unincorporated $^{33}$P-labeled nucleotides and small (<0.1 kb) cDNA fragments was accomplished using column chromatography with NucleoSpin Extraction Spin columns. The resulting activity of purified probes was determined by scintillation counting. Poly A+ RNA-derived probes resulting in the range of 5-20 x $10^6$ cpm were used for analysis.

Each cDNA probe was hybridized overnight to the respective Atlas Array in the presence of C$_{st}$-1 DNA according to instructions outlined in the Clontech User Manual. After a high stringency wash, the hybridized membranes were exposed to a phosphorimager screen for up to two weeks, prior to development. Hybridized arrays were visualized with a Storm Phosphorimager (Molecular Dynamics Inc., Sunnyvale,
CA) at a pixel resolution of 150 microns. Hybridization results were globally normalized against all genes on the membrane. A set of nine housekeeping controls, including ubiquitin and β-actin, as well as negative controls, such as pUC18 and lambda DNA, were present on each membrane. Simultaneous comparison of the expression levels of hundreds of genes was made through side-by-side comparisons of hybridizations from treated and vehicle control cell populations.

**cDNA Microarray Data Analysis**

Digital images of hybridized arrays were quantitated using AtlasImage 1.5 software from Clontech. The data was processed by global normalization, using the value of signal over background for all genes on the arrays for normalization. The AtlasImage 1.5 program was then utilized to generate composite arrays for the three replicate experiments for each designated treatment condition and cell line. The adjusted intensities of genes on each array were normalized when the composite arrays were generated for each treatment group, and then while determining the fold change between treatment groups. Comparison of adjusted average intensity values for each gene to a diagonal line of “identity” using ScatterPlot analysis in the Prism 2.01 software program resulted in determination of the fold change cut-off for altered genes in each experiment.

Also, in HEC-1A and HEC-1B experiments, average intensity values were compared for each gene in control cell lines prior to treatment to determine basic differences in cellular metabolism enzymes prior to chemical challenge. Genes which showed an average fold change of 2-fold or greater in their expression were identified along with functional categories. Subsequently, GenBank and SwissProt, genomic
information public databases, were used to determine function of alternately expressed genes (Bassett, Jr. *et al.*, 1999).

Since knowledge of where and when a gene is expressed can provide insight into gene function, the genes analyzed using microarrays were organized on the basis of similarities in their expression profiles (Bassett, Jr. *et al.*, 1999). The adjusted average intensity values prior to normalization for each gene were analyzed for functional significance by Cluster and TreeView Software programs developed by Dr. Michael Eisen (http://RANA.stanford.edu/software, Stanford University) (Eisen *et al.*, 1998). The means and standard deviations were calculated across experiments for each individual gene on each array, and the half of the total population of genes showing the highest standard deviations, or greatest deviation from the mean, were selected for analysis. Variance normalizations were then calculated for the selected half of array genes according to the following equation:

\[
\text{Variance Normalization} = \frac{(\text{Normalized Intensity for each gene} - \text{Mean value})}{\text{Standard Deviation}}
\]

The resulting variance normalization values were then analyzed by binary, agglomerative, heiralichal clustering using the Cluster and TreeView software programs (Eisen *et al.*, 1998). Genes were clustered according to common expression patterns across treatments and cell lines using Average Linkage Clustering, and the clusters were visualized using interactive TreeView Software. The expression profiles for the top 50 percent of genes were plotted in a hierarchical tree graph according to common
expression patterns, with genes expressing strong similarity being joined by very short branches, and increasingly dissimilar genes joined by longer branches. Genes with a log ratio of 0 (unchanged genes) were colored black, genes with increasingly positive log ratios red (increase) and genes with decreasing log ratios green (decrease) (Eisen et al., 1998). Through organizing data using inherent, orderly features and graphically representing the data in a naturalistic manner, the resulting patterns of gene expression provided indication of the activities of signal transduction pathways within the cells (Brown and Botstein, 1999). Human toxicology and stress array analysis generated a unique fingerprint of genetic alterations due to chemical exposure, providing a useful tool in classifying toxicants based upon their characteristic expression profiles and mechanisms of action (Bartosiewicz et al., 2001a; Bartosiewicz et al., 2001a).

**Quantitation of Intracellular Oxidative Stress by Dichlorofluorescein Assay**

RL95-2 cells were plated into 96-well plates (Corning) 1 day prior to experiments. The following day, cells at approximately 70% confluence were treated with respective chemicals (10 μM and 20 μM BaP, 10 μM and 20 μM BeP, 10 nM TCDD, 1 μM, 10 μM, and 200 μM T-butyl hydroperoxide, and 0.1 % v/v DMSO) in regular, 10% serum-containing medium. Following 6, 24, and 48 hours treatment, the cells were washed twice in PBS and were incubated with the identical chemical treatments in PBS in the presence of 10 μM 5-(and-6)-chloromethyl-2′7′-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) for 30 minutes at 37°C.
The fluorescence of the cells from each well was measured and recorded using a FL600 Microplate fluorescence reader (Bio-Tek Instruments) with constant temperature at 37°C. The excitation filter was set at 485 ± 10 nm and the emission filter was set at 530 ± 12.5 nm. The fluorescence from each well was captured, digitized, and stored on a computer using KC4 software (Bio-Tek Instruments). Data points were taken every 5 minutes for 30 minutes and the data were exported to Excel spreadsheet software (Microsoft, Seattle, WA) for analysis. The net fluorescence was calculated for each treatment relative to background controls (chemicals in the presence or absence of CM-H$_2$DCFDA). Data were expressed as the net change in fluorescence for each treatment.

**Data Analysis**

All quantitative experiments were performed in triplicate for each experiment. For scanned image analysis, control lanes were standardized to 100% and treatments were assessed relative to controls for each. The paired Student’s t-test was used to analyze numerical data averaged over several experiments. All statistical analyses were performed using Microsoft Excel software, with the exception of cDNA microarray experiments, which were analyzed using Clontech® AtlasImage software, as well as Cluster and Treeview interactive software.

**Potential Hazards and Precautions**

The experiments herein described contain numerous potential hazards which could pose a risk for personnel involved. Such hazards include the use of dangerous chemicals, cancer cell lines, and radioactivity. All chemicals used were handled with
extreme care and disposed of as described by the Chemical Waste Management Guide of the University of Florida. Cancer cell lines were treated as biohazards and were handled with extreme precaution and disposed in bleach/water solution. Radioactivity was similarly handled according to the methods outlined by the University of Florida Division of Environmental Health and Safety Department of Radiation Control and Radiological Services. All laboratory personnel were certified in the use of hazardous chemicals and radioactivity by the University of Florida.
<table>
<thead>
<tr>
<th>Target Protein/source</th>
<th>Molecular Weight</th>
<th>Western Blot: Primary/Secondary Antibody</th>
<th>Immunocytochemistry: Primary/Secondary Antibody</th>
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<tr>
<td>Santa Cruz, #sc-1499</td>
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<td></td>
<td></td>
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<tr>
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<td>10 μg/ml / 1:2000</td>
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<tr>
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**TABLE 2-2:** Antibody Sources and Dilutions for Cellular Enzyme Western Blots

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<th>Western Blot: Primary Antibody</th>
<th>Western Blot: Secondary Antibody</th>
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CHAPTER 3
EFFECTS OF BAP AND TCDD ON CELL ATTACHMENT, INVASION, AND EXPRESSION OF ADHERENS JUNCTION, CYTOSKELETAL, AND GROWTH FACTOR RECEPTOR PROTEINS IN RL95-2 CELLS

Introduction

Evidence from epidemiological and laboratory studies support a role for several environmental toxicants in the etiology of endometrial cancer and endometriosis. The environmental chemicals benzo(a)pyrene (BaP) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) have been shown to produce adverse reproductive effects in animals. Studies in human populations indicate that women who smoke cigarettes have less than half the risk of developing endometrial cancer of non-smokers, and maintain similar decreased incidence rates for endometrial hyperplasia, uterine fibroids and endometriosis (Baron et al., 1990; Cramer et al., 1986; Matorras et al., 1995). In contrast, recent studies in rhesus monkeys and rodents indicate that TCDD may promote endometriosis (Cummings et al., 1996; Cummings and Metcalf, 1995; Rier et al., 1993). In addition, human epidemiological studies provide some evidence for an association between dioxin exposure and the promotion of uterine disease, including carcino genesis (Koninckx et al., 1994; Bertazzi et al., 2001; Mayani et al., 1997). Uterine cancer, however, was not shown to be increased by dioxin exposure in human epidemiological studies up to twenty years from the time of exposure (Bertazzi et al., 2001).
The present studies were conducted to test the hypothesis that BaP, and not TCDD, decreases cellular attachment and invasion through alterations in membrane adherens junction and cytoskeletal proteins, thereby producing an anti-adhesive phenotype in RL95-2 cells. Benzo(a)pyrene, a major toxicant of cigarette smoke, was selected as a target for investigation due to its predominance and potency. A genotoxic chemical, BaP is recognized to significantly alter signal transduction in human and animal cells by binding to the intracellular cytosolic protein aryl hydrocarbon receptor (AhR). BaP transcriptionally activates cytochrome P450 1A1 (CYP1A1), leading to its own metabolism (Poland et al., 1976; Whitlock, Jr. et al., 1996). TCDD, a classical, non-genotoxic carcinogen, binds to the AhR in affecting cellular proliferation and division.

Current studies were undertaken to evaluate the effects of the AhR-ligands BaP and TCDD on the ability of RL95-2 cells to attach to matrigel-coated membranes, and on key adherens junction, cytoskeletal, and growth factor proteins involved in these processes. Cell adhesion mechanisms play a fundamental role in the determination of tissue architecture and the functions of cell assembly and connection to the internal cytoskeleton and, consequently, are of great significance to the pathophysiology of endometrial cancer. The well-studied human endometrial RL95-2 cell line was selected as a model for these studies because of its relevance as an in vitro model for endometrial cancer (Way et al., 1983). In addition, the RL95-2 cell line has been shown to exhibit adhesiveness of its apical pole for the trophoblast, thereby also serving as a model for human uterine epithelium receptive for implantation (Thie et al., 1997).
Results

Cellular Attachment on Matrigel

Cells were cultured in the presence of 10 μM BaP, 10 nM TCDD, or DMSO vehicle for 48 hours, then trypsinized and applied to Matrigel-coated porous membranes. All treated cells exhibited greater than 95% viability as determined by tryphan blue dye exclusion. Pretreated cells were applied to the Matrigel surface for 2 hours, after which the membranes were stained for adherent nuclei. Figure 3-1 shows that BaP-pretreated cells exhibit minimal attachment, whereas TCDD-exposed cells show a high level of attachment comparable to DMSO controls.

Effects on Epidermal Growth Factor Receptors (EGF-R)

EGF-R immunoreactivity was detected as brown immunostaining primarily localized in intercellular regions of adjacent cells and associated with the plasma membrane, with a lesser amount detected in the cytoplasm (Figure 3-2A). Treatment with 10 μM BaP for 48 hours resulted in a marked decrease in EGF-R staining along cell membranes, whereas cytoplasmic staining was still present (Figure 3-2B). In contrast, 10 nM TCDD treatment resulted in no apparent effect in EGF-R localization, relative to control (Figure 3-2C). Replacement of the primary antibodies with PBS showed a complete lack of EGF-R immunostaining (Figure 3-2D).

Western immunoblot data showed the expression of EGF-R as a 170 kDa protein in RL95-2 cells in both cell membrane and cell lysate fractions. Data further indicated a selective loss of plasma membrane EGF-R following 10 μM BaP treatment, with no change in EGF-R protein levels in detergent extracted cell lysates (Figure 3-2E).
Treatment with 10 nM TCDD failed to show an effect on EGF-R expression levels. In data not shown, 10 μM BaP caused a substantial loss of membrane EGF-R protein levels by 7 hours treatment.

**Effects on Cadherin and β-catenin Cellular Adhesion Molecules**

Studies have been conducted on the cell adherens junction proteins cadherin, β-catenin, and vinculin. Data in Figure 3-3A show that cadherin is expressed in RL95-2 cells as a 120 kDa protein. Treatment with 10 μM BaP, but not 10 nM TCDD, produced a significant 38% decrease in cadherin levels in RL95-2 cell lysates (p < 0.005). β-Catenin, a second adherens junction protein, is detected as a 92 kDa protein present in both cell membrane and detergent-extracted cell lysate preparations of RL95-2 cells. Treatment with 10 μM BaP is associated with a selective loss of β-catenin in cell membrane fractions, a significant 80% decrease as compared to control, whereas cell lysate protein levels remain unchanged. In contrast, treatment with 10 nM TCDD had no effect on β-catenin levels in either preparation (Figure 3-3B). In comparison, 10 μM BaP and 10 nM TCDD treatments had no effect on vinculin protein levels in cell membrane and lysate fractions as determined by Western immunoblot analysis (Figure 3-3C).

**Effects on Actin Cytoskeletal Protein**

Subsequent immunocytochemical studies were performed investigating BaP-mediated effects on actin cytoskeletal localization in RL95-2 cells. Experiments utilized the fluorescently-labelled peptide phalloidin to selectively visualize filamentous actin using fluorescence light microscopy. Results indicate that filamentous actin is localized
in a subcortical layer in control cells (Figure 3-4A). In contrast, 10 μM BaP treatment is associated with the formation of subcortical actin aggregates in RL95-2 cells (Figure 3-4B). At the same time, however, overall actin levels in cell membrane and triton X-100 fractions remain unaltered as demonstrated by Western immunoblot analysis of the 42 kDa actin protein in RL95-2 cells (Figure 3-4C).

Discussion

Endometrial cancer is the most common gynecologic malignancy in the United States. Due to recent evidence implicating environmental agents like TCDD and cigarette smoke in the etiology of endometrial cancer and endometriosis (Matorras et al., 1995; Cummings et al., 1996; Rier et al., 1993; Mayani et al., 1997), current studies investigated roles of these environmental agents in altering cellular attachment of endometrial cells.

The present study demonstrates that BaP produced a significant decrease in the cell attachment of the RL95-2 endometrial cell line. In contrast, TCDD exposure did not significantly affect the attachment of RL95-2 cells. The observation that BaP inhibits endometrial cancer cell attachment is potentially indicative of a conversion to a more metastatic phenotype. Since increased metastasis is associated with a poorer prognosis in endometrial cancers (Wronski et al., 1993), the decreased cellular attachment associated with BaP exposure cannot account for the seemingly “protective” effect of cigarette smoke (Cramer et al., 1986; Matorras et al., 1995). The reported effects of BaP on inhibiting cellular invasion and proliferation could, however, potentially account for the seemingly protective effects against endometrial cancer (Charles, 1997). These data are
strengthened by reports of BaP having independent effects on growth-related gene expression and signaling compared with effects on acute liver cytotoxicity (Parrish et al., 1999).

Studies indicate that BaP might act to alter cellular attachment through a change in the expression of cell adhesion molecules. The BaP-mediated alterations in endometrial cancer cell attachment appear to be linked with a loss of cell surface expression of adherens junction and cytoskeletal proteins, such as cadherin, β-catenin, and actin. Cadherins are integral membrane glycoproteins which function in epithelial cells to form calcium-dependent linkages between cells (Potter et al., 1999; Knudsen and Soler, 2000). Cadherins play a key role in mediating the formation and breakage of cell-to-cell contacts and in maintaining strength in cellular adhesion through homophilic interaction of their extracellular domains, allowing for cellular aggregation to occur (Potter et al., 1999). Cadherins connect to β-catenin, a key membrane-associated protein responsible for the colocalization of cadherins to sites of cell-cell contact with the actin cytoskeleton (Potter et al., 1999). The BaP-mediated alterations in the membrane associated adherens junction proteins indicate the likely role of BaP in decreasing cellular attachment in RL95-2 cells. A phenotype with decreased attachment has been associated with increased cellular metastasis (Wronski et al., 1993).

Although the present studies provide useful information about BaP-mediated membrane β-catenin effects, they do not address whether there is a true compartmentalization of membrane-associated β-catenin after BaP treatment since cell lysate preparations currently described have centrifuged out detergent insoluble proteins. Future experiments should address BaP-mediated effects on cell membrane proteins from
whole cell lysate preparations prepared from lysed cells without centrifugation to determine whether the loss of membrane-associated β-catenin is compensated for by increases in cytoplasmic β-catenin. It can be speculated that if BaP treatment is causing a redistribution of β-catenin to the cytoplasmic and nuclear fractions, where β-catenin can initiate signal transduction pathways leading to carcinogenesis, BaP exposure is thereby not protective against endometrial cancer.

The actin cytoskeleton was additionally investigated for BaP effects due to its fundamental role in cellular adhesion. Actin works in conjunction with cytoskeletal microtubules and intermediate filaments in performing essential functions in locomotion and cytokinesis (Hulka and Brinton, 1995). Actin bundles are attached to integral membrane cadherin and integrin proteins, adapter proteins and the contractile bundle. Recent studies have shown that treatment of MCF-10A nontransformed human mammary epithelial cells with BaP and UV light resulted in the reorganization of actin filaments into substrate-associated aggregates (Seagrave and Burchiel, 2000). Similar alterations in filamentous actin conformation following BaP, yet not TCDD treatment, were observed in RL95-2 cells, reflecting another fundamental structural alteration with BaP exposure.

Activation of epidermal growth factor receptor (EGF-R), as well as other receptor tyrosine kinases, has been shown to directly affect the adhesive function of cells (Hazan and Norton, 1998). In particular, EGF-R has been shown to directly regulate cell-cell adhesion through effects on E-cadherin interactions with actin in a human breast cancer cell line (Hazan and Norton, 1998). Cigarette smoking has been consistently associated with a decrease in placental EGF receptors (Shiverick and Salafia, 1999). It has also been reported that both BaP and TCDD mimic growth factor signaling pathways in
human mammary epithelial cells, as shown by increased tyrosine phosphorylation of insulin-like growth factor (IGF-I) receptor beta, IRS-1 and Shc (Tannheimer et al., 1998). Studies in three endometrial cancer cell lines indicate an inverse relationship between cellular EGF-R levels and the grade of the tumor (Lelle et al., 1993). The marked loss of cell surface-EGF-R at the cell-cell interface of RL95-2 cells in the present study represents the dramatic alterations in the adherens complex upon BaP treatment. The BaP-specific loss of expression of EGF-Rs in RL95-2 cells is in agreement with previously published reports of BaP-, yet not TCDD-mediated loss of EGF-Rs in human placental choriocarcinoma JEG-2 cells (Zhang and Shiverick, 1997).

Published studies indicate that the first step of invasion and metastasis is the detachment of cancer cells from the primary tumor, a process mainly controlled by the adherens junction proteins, consisting of E-cadherin, α- and β-catenins, vinculin, and actin. Consequently, it can be inferred that the decreased cellular attachment of the RL95-2 cells upon BaP exposure is consistent with promotion of a more metastatic phenotype which would actually promote, rather than protect against, endometrial cancer. This view is supported by studies from human breast cancer patients which show that tumors negative for either α- or β-catenin expression demonstrate a higher incidence of distant metastasis than those expressing both catenins (Yoshida et al., 2001). Further, a reduction or loss of E-cadherin expression has been associated with lymph node metastases and poor prognosis of invasive breast cancers (Yoshida et al., 2001). Additional studies from colorectal cancer patients indicate an association between decreased α-catenin expression and increased metastasis (Gofuku et al., 1999). However, a conflicting report from pancreatic cancer patients found an association
between an intact E-cadherin/catenin complex and increased liver metastasis (Gunji et al., 1998).

The RL95-2 endometrial carcinoma cell line, which has characteristics distinct from other endometrial cell lines, exhibits an adhesiveness of its apical pole for trophoblast cells and thereby serves as an *in vitro* model for the human uterine epithelium receptive for implantation (Thie et al., 1997). BaP exposure therefore likely inhibits attachment in benign uterine disorders, while additionally proving unfavorable for implantation and the establishment of pregnancy.
Figure 3-1. Effects of BaP and TCDD on RL95-2 cell attachment to membranes. Cells were pre-treated for 48 hours with 10 μM BaP, 10 nM TCDD, or 0.1% DMSO vehicle (control). Cells (30,000 per well) in serum-free media were aliquoted into the lower wells of the Boyden Chamber apparatus and were allowed to attach to a Matrigel-coated membrane for 2 hours. Upon attachment, cells were stained with Leukostat and counted by light microscopy.
Figure 3-2. Effects of BaP and TCDD on the localization and protein levels of EGF-R in RL95-2 cells.
A-D: Immunocytochemical localization of EGF-R in cells following 48 hours treatment with DMSO vehicle control (A and D), 10 μM BaP (B), and 10 nM TCDD (C). Cells were incubated with sheep anti-human EGF-R antiserum (A-C) or PBS (D). E: Western Immunoblot analysis of cell membrane preparations compared with detergent-extracted cell lysates of cells following BaP and TCDD treatment. Samples containing 100 μg protein were electrophoresed, transferred to nitrocellulose, and immunostained with anti-EGF-R antibody.
Figure 3-3. Western Immunoblot analysis of the effects of BaP and TCDD on Cadherin, β-Catenin, and Vinculin Levels.

RL95-2 cells were treated with 10 μM BaP, 10 nM TCDD, or 1% v/v DMSO control for 48 h. Membrane and detergent-extracted cell lysate preparations were electrophoresed, transferred to nitrocellulose, and immunostained with antibodies to cadherin, β-catenin, or vinculin, followed by horseradish peroxidase conjugated IgG. A. Results from cadherin Western immunoblot analysis represents the mean ± SE of three experiments (p<0.005).
**Figure 3-3-continued.** B. Data for β-catenin Western Immunoblot analysis represents the mean ± SE of three experiments (p<0.05).
**Figure 3-3-continued.** C. Data for vinculin Western immunoblot analysis represents the mean ± SE of three experiments.
Figure 3-4. Effects of BaP on the localization of actin filaments and protein levels in RL95-2 cells.
A-B: Immunocytochemical localization of actin in RL95-2 cells following 48 hours treatment with DMSO vehicle control (A) and 10 μM BaP (B). Cells were incubated with fluorescently labeled phalloidin to visualize altered actin structure upon BaP treatment. C: Western Immunoblot analysis of cell membrane and cell extract preparations following DMSO, BaP, and TCDD treatment. Samples containing 40 μg of protein were electrophoresed, transferred to nitrocellulose, and immunostained with anti-actin antibody.
<table>
<thead>
<tr>
<th>Actin 42 kDa</th>
<th>Membranes</th>
<th>Triton-X 100 Extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMSO</td>
<td>BaP</td>
</tr>
<tr>
<td></td>
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*HEW*
CHAPTER 4
SIGNAL TRANSDUCTION PATHWAYS FOR BAP AND TCDD EFFECTS ON RL95-2 CELLS: THE AHR, CELL CYCLE, AND OXIDATIVE STRESS

Introduction

BaP and its metabolites are known to modulate mammalian gene expression through alterations in cell cycle and epigenetic mechanisms involving the AhR signaling pathway, oxidative stress, and altered mitogenic signaling. In particular, oxidative metabolism of BaP is associated with most adverse health effects of BaP. Further, DNA adducts can result in deleterious genetic mutations, leading to disruption of gene expression.

The Arylhydrocarbon Receptor

Benzo(a)pyrene (BaP) is a ubiquitous environmental pollutant heavily implicated in human cancer etiology. Whereas the parent compound BaP is not directly biologically active, BaP is readily metabolized to mutagenic and carcinogenic metabolites (Kim et al., 1998). Consequently, BaP is considered a procarcinogen because it requires metabolic activation to reactive intermediates to elicit toxic effects. The enzymes involved in BaP metabolism include Phase I enzymes, such as cytochrome P450s, epoxide reductases, and epoxide hydrolases, as well as Phase II enzymes, such as glutathione transferases, UDP-glucuronyl transferases, and suflotransferases (Miller and Ramos, 2001a). The primary
products of BaP metabolites include epoxides, dihydrodiols, phenols, and quinones (Miller and Ramos, 2001a).

Xenobiotics such as BaP and the dioxin TCDD bind to the cytosolic arylhydrocarbon receptor (AhR) protein in mediating gene transcription. The AhR is a member of the bHLH/PAS transcription factor family characterized by the basic helix-loop-helix DNA binding domain and a Per/AhR/Arnt/Sim homology region (Burbach et al., 1992). In the process of binding to xenobiotics, the AhR dissociates two heat-shock 90 proteins and the BaP-AhR complex translocates to the nucleus where it binds to the Ah receptor nuclear translocator (Arnt) protein. Subsequent binding of the xenobiotic-AhR-Arnt complex to the xenobiotic response elements (XRE) on the CYP1A1 gene promoter locally modifies the chromatin structure and activates gene transcription (Whitlock, Jr. et al., 1996).

*In vivo* studies of AhR gene expression in the human uterine endometrium indicate that AhR mRNA levels were significantly lower in women who smoke cigarettes than in non-smokers (Igarashi et al., 1999). However, studies indicate that no significant difference was observed in the AhR mRNA levels in human uterine endometrium in women with or without endometriosis (Igarashi et al., 1999). Further, the levels of CYP1A1 transcripts have been shown to be a marked 8.7 times higher in endometriotic tissues than in the eutopic endometrium (Bulun et al., 2000).

Cytochrome P450 (CYP) enzymes including CYP1A1 catalyze the reactions leading to the oxidation of endogenous or xenobiotic substrates. Among the CYP enzymes, numerous genes are highly inducible by xenobiotics, including CYP1A1, CYP1A2, and CYP1B1. CYP1A1 is expressed in numerous tissues after induction by
polycyclic aromatic hydrocarbons (PAHs) such as BaP, and polyhalogenated hydrocarbons such as TCDD (Barouki and Morel, 2001; Omiecinski et al., 1999). CYP1A2 is expressed constitutively in the liver, and is induced through the AhR pathway (Omiecinski et al., 1999). The enzyme CYP1B1 is constitutively expressed in many tissues and is inducible through the AhR pathway (Omiecinski et al., 1999). CYP1B1 has been shown to be an important activator of PAHs in tissues such as the mammary gland when environmental chemical exposures minimally induce CYP1A1 (Larsen et al., 1998). Further, CYP1B1 has been shown to carry out the metabolism of BaP to BPDE at higher rates than CYP1A2, but slower than CYP1A1 (Kim et al., 1998).

Whereas both BaP and the dioxin TCDD are potent AhR ligands known to induce CYP1A1 and CYP1B1, TCDD, yet not BaP, exerts toxic effects on cells in the absence of prior metabolism of the parent compound. Recent knock-out studies indicate that AhR activation is essential to TCDD-mediated toxicity. Data show that in the absence of a functional AhR, most toxicity associated with TCDD is not observed (Fernandez-Salguero et al., 1996). BaP, on the contrary, can exert toxic effects through both AhR-dependent and AhR-independent pathways (Kim et al., 1997; Marnett, 1990).

**Oxidative Stress and Cell Cycle**

In 1953, Howard and Pelc demonstrated that DNA synthesis occurs at a precise period during the cell cycle, designated the S phase (Howard and Pelc, 1953). The period before the S phase became known as the Gap 1 (G1) phase, whereas the period subsequent to the S phase and before mitosis became known as the Gap 2 (G2) phase. The modern concept of the cell cycle emerged with identification of distinct G1, S, G2,
and M (mitosis) phases. Subsequently, it became known that dividing cells contained factors which ensure orderly progression and timing throughout the cell cycle, and that a lack of genomic integrity can result in cell death or the accumulation of deleterious mutations.

While cells typically proceed throughout the cell cycle without interruption, DNA damaged cells have the ability to pause in the G1, S, or G2 phase to allow time for DNA repair. In cases of severe DNA damage, cells may undergo apoptosis or enter into an irreversible G0 state (Shackelford et al., 2000). Cell cycle checkpoints are crucial to allow cells more time to pause and repair DNA damage before progression through the more critical phases of DNA replication and mitosis. Although cells are highly sensitive to DNA damage throughout the cell cycle, once they pass the restriction point, their checkpoint pause is delayed until the subsequent phase of the cell cycle (Shackelford et al., 2000). Defects in cellular checkpoints have been observed in hereditary cancer syndromes and in early stages of cellular transformation (Kaufmann and Paules, 1996).

Regulation of cell cycle progression is accomplished through complex pathways involving cyclin accumulation and degradation, phosphorylation of cyclin dependent kinases (Cdks), cyclins, and other proteins, regulation of cyclin/Cdk dimerization, and the binding of Cdk inhibitory proteins (Shackelford et al., 2000). Unrepaired DNA damage from the G1 phase, as well as S phase damage, can cause a checkpoint response which lowers the rate of DNA synthesis in cells. The S phase checkpoint response is known to be a rapid biological response, similar to the G2 checkpoint, and to be far more sensitive to DNA damage than the G1 checkpoint (Kaufmann and Paules, 1996). Cyclin A / Cdk2 activity is known to be a requirement for S phase progression and DNA synthesis, and
consequently functions in protecting cells against DNA replication errors (Shackelford et al., 2000).

Certain DNA damaging agents have been shown to induce the S-phase checkpoint response in cells, including infrared (IR), ultraviolet (UV-B and UV-C), methylmethane sulfonate, and benzo(a)pyrene diolepoxide (BPDE) (Shackelford et al., 2000). Evidence demonstrates that the BPDE metabolite of BaP forms a DNA adduct with the N2 nitrogen on a guanine base in DNA, causing cells to accumulate in the S phase of the cell cycle (Gezer et al., 1988). Whereas some adducts can be removed during transcription, unpaired adducts in the coding strand of transcriptionally active genes can block RNA polymerase II and may interfere with gene synthesis (Kaufmann and Paules, 1996). The toxicity of adducts progresses as they persist throughout a cell’s progression through the cell cycle. For example, adducts formed during the G1 phase typically lack permanent genotoxicity provided they are repaired before the S phase, whereas S phase adducts have been shown to block DNA synthesis, leaving gaps in daughter DNA (Kaufmann and Paules, 1996). As a result, base substitution errors can occur during postreplication repair and damage can proceed to a double strand break in the DNA. Incorrect rejoining of the double strand break can produce genetic deletion, translocation, or amplification through chromosomal aberrations (Kaufmann and Paules, 1996). In brief, errors in repair of DNA damage at any stage can produce far more permanent damage than errors at a preceding stage.

Studies indicate that increases in oxygen pressure or reactive oxygen species (ROS) can cause mutations, chromosomal and DNA damage, inhibition of cellular division, and tumor promotion (Figure 4-1) (Shackelford et al., 2000). Further, the AhR
battery of genes have been shown to be capable of both producing and preventing oxidative stress through stress-detoxifying enzymes, as well as to assist cells in choosing between apoptosis and continuation throughout the cell cycle (Nebert et al., 2000). It is believed that dioxin, the most potent AhR ligand, activates oxidative stress genes primarily through the AhR-mediated aromatic hydrocarbon response element (AhRE) DNA motif, whereas electrophiles and certain BaP metabolites additionally activate genes through the electrophile response element (EpRE) (Nebert et al., 2000).

Although BaP, an AhR ligand, is a relatively unreactive 5-ring polycyclic planer hydrocarbon, this compound is readily metabolized to toxic or tumorigenic metabolites. BaP contains the structural motif known as a “bay region” which is responsible for steric hindrance in the molecule, thereby increasing metabolism through oxidation and radical formation, while decreasing detoxification and conjugation. Through metabolic transformation, BaP is converted to a variety of products which can exert cellular effects by directly binding DNA, perhaps the most noted being benzo(a)pyrene 7,8-diol-9,10-epoxide (BPDE) (Miller and Ramos, 2001a), as well as the various quinone and semiquinone metabolites formed during redox cycling (Joseph and Jaiswal, 1994). Studies in a non-human primate model indicate high levels of BaP-induced adduct formation in maternal and fetal organs throughout gestation (Lu et al., 1993). Furthermore, human epidemiological studies indicate the presence of BaP-adducted bases in the urine of three of seven cigarette smokers and three of seven women exposed to coal smoke, whereas no BaP-adducted bases were observed in non-smoking women (Casale et al., 2001). TCDD, on the contrary, has been associated with cell cycle arrest in the G1
phase of the cell cycle in human hepatoma cells through a mechanism known to involve the AhR (Ge and Elferink, 1998).

**Prostaglandin H-Synthase**

Prostaglandin H-synthase (PGHS) is a membrane protein localized in the endoplasmic reticulum, the nuclear membrane, and the plasma membrane which functions in the oxidation of xenobiotics in the presence of arachadonic acid or lipid peroxides (Degen, 1993). PGHS consists of two primary components, a cyclooxygenase which generates the peroxide PGG2 and a peroxidase that reduces PGG2 to PGH2. The PGHS-mediated oxidations can occur by three mechanisms: direct oxidation of the carcinogen by the PGHS peroxidase, oxidation from peroxyl radicals generated during prostaglandin biosynthesis, and bioactivation through a second oxidant species formed from the metabolite derived by the peroxidase (Eling and Curtis, 1992). PGHS plays a crucial role in cellular metabolism, complementing the cytochrome P450 isoenzymes in metabolic bioactivation reactions, as well as providing an alternate metabolic pathway for xenobiotic metabolism from the P450-mediated pathways (Degen, 1993). Specifically, PGHS has been shown to contribute to the bioactivation of various procarcinogens, including benzo(a)pyrene-7,8-diol, in extrahepatic tissues where the P-450 monooxygenases have low activity (Marnett, 1990; Kelley *et al.*, 1997). Consequently, PGHS likely does not play a role in systemic drug metabolism, but is substantially involved in organ and cell specific toxicities (Degen, 1993).
Oxidative Stress and Cellular Adhesion

It has been shown that oxidative injury in cultured cells is capable of markedly impeding cellular adhesion, in part through alterations in cellular membranes, mitochondria, and the cytoskeleton (Bellomo et al., 1990). The mechanism for oxidative stress-mediated cellular alterations is believed to involve oxidative modification of cytoskeletal protein sulfhydryl groups, thereby impairing the cytoskeletal network (Bellomo and Mirabelli, 1992). In particular, studies have shown that cells treated with the oxidative-stress-inducing compounds have markedly disrupted cytoskeletal structures (Bellomo et al., 1990). Since the cytoskeleton is crucial to the structural and metabolic function of the cell, disruptions in the cytoskeleton can have a profound impact on cellular response to toxicants. Cytoskeletal structures such as actin microfilaments, microtubules, and intermediate-size filaments are considered significant targets in quinone-induced oxidative stress (Bellomo et al., 1990). Current studies were undertaken to determine the role of the various BaP metabolism enzymes on the observed cellular adhesion and cytoskeletal effects of BaP.

Results

Cell Cycle Phase Distribution

Cells were cultured in the presence of 10 μM BaP, 10 nM TCDD, or 0.1% v/v DMSO vehicle control for 48 hours and then trypsinized, washed, and stained with propidium iodide prior to analysis for alterations in cell cycle phase distribution. Data in Figure 4.2 indicate that control RL95-2 cells have a large percentage of cells in the G0-G1 phases of the cell cycle (74%), with considerably less cells in S (20%) and G2-M
phases (6 %). BaP treatment produces a 2 fold increase in the percentage of cells in the S phase, as compared to DMSO vehicle controls, with a marked 50% decrease in the percentage of cells in the G0-G1 phases (Figure 4-2). In contrast, TCDD-treated cell cultures appeared similar to controls after 48 hour treatment (Figure 4-2). Data from a representative experiment indicating BaP and TCDD effects on cell cycle phase distribution are shown in Figure 4-3. Results indicate that treatment with BaP, but not TCDD, altered cell cycle phase distribution in the direction of increased S and G2M phase accumulation, with a corresponding marked decrease in the G0-G1 phases.

Effects on CYP1A1 and CYP1B1 mRNA Levels

Since both BaP and TCDD have been shown to act through the aryl hydrocarbon (Ah) receptor, initial studies were performed investigating the induction of CYP1A1 and CYP1B1 mRNA. Studies evaluated the effects of 48 hour treatment with 10 µM BaP and 10 nM TCDD on cytochrome P450 CYP1A1 and CYP1B1 mRNA levels. Northern blot data indicate that control RL95-2 cells express low levels of a 3.0 kb CYP1A1 mRNA transcript (Figure 4-4A). BaP and TCDD treatment of RL95-2 cells results in a strong induction of CYP1A1 mRNA. Further, data indicate that RL95-2 cells express a constitutively low level of a 5.2 kb CYP1B1 mRNA transcript, which is markedly induced by both BaP and TCDD (Figure 4-4 B).

Effects on CYP1A1, PGHS-1, and PGHS-2 Protein Levels

Experiments investigating the effects of BaP- and TCDD-treatment on CYP1A1, PGHS-1 and PGHS-2 protein levels were performed in RL95-2 cells after 48 hour
treatment. Western immunoblot analysis indicates that CYP1A1 protein is not detected in control cells, but is expressed as a 55 kDa protein with 10 μM BaP and 10 nM TCDD treatment (Figure 4-5). It is noteworthy that TCDD produces a considerably higher level of CYP1A1 protein expression than BaP treatment.

In addition to cytochrome P450 proteins, it is proposed that the enzyme prostaglandin H-synthase (PGHS-1) may be involved in the biotransformation of BaP in RL95-2 cells. Data in Figure 4-6 show the constitutive expression of PGHS-1 in RL95-2 cells as a 70 kDa protein by Western immunoblot analysis. Data further indicate that PGHS-1 is significantly increased by 2.2 fold (p<0.005) following 10 μM BaP exposure. On the contrary, treatment with 10 nM TCDD did not alter PGHS-1 levels (Figure 4-6). In data not shown, PGHS-2 protein was not detected in control RL95-2 cells, as determined by comparison with positive PGHS-2 control (Cayman), and PGHS-2 expression was not induced by either BaP or TCDD treatment at 48 hours.

cDNA Microarray Analysis for Induction of Stress-related Genes

Further studies were performed to determine whether BaP or TCDD was causing transcriptional alterations in stress-related genes, as compared to an overall cellular response as described above. Experiments were conducted using Clontech Human Stress microarrays to determine changes in 236 genes known to function in stress response regulation, including genes involved in DNA damage response, repair, and recombination, base excision repair, nucleotide excision repair, mismatch repair, and drug and xenobiotic metabolism. Functionally, the cDNA microarray procedure involves reverse transcription of mRNA to radiolabelled cDNAs, which are then hybridized to
nylon membrane arrays containing 236 gene sequences. The radioactive signals bound to each cDNA on the array represent relative expression levels of each gene as detected by phosphorimaging. Each array contains 600 to 2400 base pairs of cDNA per gene, spotted in duplicate for each gene represented, thereby providing information about differential gene induction profiles.

The rationale for the present gene array experiments was to profile mRNA expression after a 6 hour exposure to BaP, TCDD, or t-butylhydroperoxide. It was expected that the non-metabolized AhR ligand TCDD would activate primarily the AhR-mediated battery of genes, whereas the classic oxidative stress inducer t-butyl peroxide would induce genes characteristic of a cellular oxidative stress response. It was further expected that BaP would activate oxidative stress-response genes at an earlier time point than AhR-regulated genes; however, since BaP has been shown to exert effects through both pathways, it was expected that both batteries of genes would likely be activated by BaP.

The pattern of gene induction by BaP at 6 hours was compared with patterns induced by TCDD and t-butyl peroxide to provide some insight into which signal transduction pathways were being activated by cellular exposure to BaP. AtlasImage 1.5 software with global normalization was utilized to generate composite arrays for each experiment. The subsequent comparison of composite arrays provided overall fold change inductions for each gene. ScatterPlot analysis was used to determine the distribution of all globally normalized genes relative to a diagonal line of identity (Figure 4-7). Genes distributed outside the two diagonal lines have a greater than two-fold change in intensity relative to control. Genes from composite arrays which showed an
average fold-change of two-fold or greater between control and treated samples or between the two untreated control cell lines were included in the final report only if their adjusted intensities after global normalization had a defined ratio (a ratio not equal to zero). However, exclusive consideration of genes with Clontech defined ratios of two-fold or greater may neglect to acknowledge a number of additional genes which are significantly altered and thus should be open for future consideration.

Results indicate four genes had a two-fold or greater down-regulation in RL95-2 cells following 6 hour treatment with 10 μM BaP (Table 4-1), whereas 10 genes were up-regulated two-fold or more (Table 4-2). The genes altered by BaP include stress-response regulators, DNA damage, repair, and recombination, and xenobiotic metabolism genes. Similarly, 6 hour treatment of RL95-2 cells with 10 nM TCDD resulted in the down-regulation of 10 genes (Table 4-3), whereas 5 genes were up-regulated with TCDD relative to control (Table 4-4). TCDD-altered genes include primarily stress-response regulators and xenobiotic metabolism enzymes, with some effect on DNA damage response and one housekeeping gene.

Treatment of RL95-2 cells with 200 μM t-butylhydroperoxide for 6 hours, a concentration known to produce a strong oxidative stress response in cell cultures, resulted in the two-fold or greater down-regulation of 5 genes (Table 4-5), whereas 10 genes were up-regulated (Table 4-6). Tert-butylhydroperoxide treatment was largely responsible for alterations in genes associated with stress response, with some effect on genes for base and nucleotide excision repair, xenobiotic metabolism, as well as two housekeeping genes.
Comparison of differential gene induction across BaP, TCDD, and t-butylhydroperoxide treatments is shown in the Venn diagram (Figure 4-8). As illustrated, no genes were similarly regulated by the three chemical treatments. However, BaP and TCDD treatments affected the same two genes, BaP and t-butylhydroperoxide the same one gene, and TCDD and t-butylhydroperoxide the same four genes.

**Cluster and TreeView Analysis of Common Gene Expression Patterns**

Unlike the array analysis previously described, current experiments utilized the raw, non-globally normalized adjusted intensities for all genes on the membrane in the analysis. All intensities were individually normalized, including the intensities which fell below the background level for individual experiments. The net intensities from each gene across all three experiments were transformed using variance normalization calculations. The data were normalized manually using Microsoft Excel software and were analyzed using ScatterPlot analysis to represent the distribution of genes around a diagonal line of identity (Figure 4-9). As compared to the ScatterPlot analysis in Figure 4-7, the actual values for all genes were included in the analysis. The data were then analyzed using Cluster and Treeview software for common expression patterns across three separate experiments and differential chemical treatments (Figure 4-10).

**Quantitation of Intracellular Oxidative Stress by Dichlorofluorescein Assay**

Xenobiotic-mediated induction of intercellular oxidative stress in RL95-2 cells was determined by monitoring the oxidation of dichlorofluorescein using a microplate reader. 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-
H2DCFDA) (Molecular Probes, Cat. #C-6827), a chloromethyl derivative of H2DCFDA that is believed to exhibit much better retention in live cells, was added to buffer containing xenobiotics. It is understood that CM-H2DCFDA passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases and a thiol-reactive chloromethyl group reacts with intracellular glutathione and other thiols. Subsequent oxidation yields a fluorescent adduct that is trapped inside the cell, thus facilitating the monitoring of intracellular stress over time.

Results shown in Table 4-7 indicate that no definite conclusions may be drawn regarding the relative levels of oxidative stress in RL95-2 cells upon differential chemical treatment. Tert-butylhydroperoxide (TBH), the positive control for oxidative stress, did not display a dose-response relationship for oxidative stress as measured by the DCF assay. Significance was shown for 20 μM BeP, 10 nM TCDD, and 10 μM TBH treatments at 6 hours, with all three treatments producing a decreased level of intracellular oxidative stress relative to DMSO vehicle control (Figure 4-7). Results are speculative, at best, due to the inconsistencies in establishing a dose-response relationship for TBH using the DCF fluorescence assay.

**Discussion**

Cancerous neoplastic progression has been strongly associated with increasing genetic instability (Shackelford *et al.*, 1999). Since DNA mutations have been associated with alterations in cell cycle progression, the present experiments were carried out to determine the effect of the xenobiotics BaP and TCDD on cell cycle phase distribution. Flow cytometry analysis with propidium iodide fluorescence was used to measure
relative percentages of cells in each phase of the cell cycle at specified time periods.

RL95-2 cells, which are reported to have a doubling time of 22-34 hours, have presumably undergone a minimum of one cell cycle by 48 hours (Way et al., 1983). Data in Figures 4-2 and 4-3 show a prolonged S phase response of RL95-2 cells following 48 hour BaP treatment. In contrast, TCDD, a non-genotoxic AhR ligand, did not elicit an increased percentage of cells in the S phase, indicating that ligand activation of the AhR alone is not sufficient to block cell cycle progression.

Present findings of a specific enhanced S phase response in RL95-2 cells upon BaP treatment complement prior studies of human MCF-7 breast cancer cells treated with BaP (Khan and Dipple, 2000). This study found that that cells treated with low, non-cytotoxic doses of the genotoxic BaP metabolite BPDE result in an accumulation of cells in the S phase of the cell cycle (Khan and Dipple, 2000). Further, MCF-7 cells failed to show an accumulation in the G1 phase, as would be expected from an early G1 checkpoint response (Khan and Dipple, 2000). The investigators therefore hypothesized that the “stealth characteristic” of BaP enables cells to evade G1 arrest and likely increases the probability of malignant change because DNA replication with BaP treatment subsequently occurs on a damaged template (Khan and Dipple, 2000). Similar studies in human lymphoblast cells further reflect the prolonged S phase response of cells upon BPDE treatment (Black et al., 1989). Alterations in RL95-2 cell cycle phase distribution upon BaP treatment are therefore likely the result of a mechanism preventing the replication of BaP-adducted, damaged DNA.

Many carcinogens exert their effects on cellular growth and differentiation by perturbing the signal transduction pathways involved in normal cell cycle control. BaP
specifically is known to modulate mammalian gene expression through epigenetic mechanisms involving the AhR signaling pathway, oxidative stress, and altered mitogenic signaling.

BaP has been demonstrated to undergo different multi-step pathways in its conversion to toxic or tumorigenic metabolites (Kim et al., 1997; Wells et al., 1997; Ramos, 1999). The predominant pathway utilizes the cytochrome P450 isozyme CYP1A1 as an initial step in converting BaP to oxide-intermediates, followed by the enzyme epoxide hydrolase, which results in dihydrodiol formation. The present study found that BaP and TCDD treatment induced CYP1A1 mRNA and protein levels in RL95-2 cells, as well as increased CYP1B1 mRNA levels (Figures 4-4 and 4-5). The observed effects of BaP and TCDD on CYP1A1, and additionally on CYP1A2 mRNA levels were further validated by cDNA microarray data, indicating enhanced mRNA of both enzymes upon 6 hour treatment (Tables 4-2 and 4-4). CYP1B1 was not included on the Human Stress array, and therefore could not be validated by this method. Since CYP1A1 and CYP1B1 are both induced by BaP and TCDD, whereas cellular adhesion, cytoskeleton, and cell cycle effects are specific to BaP treatment alone, factors beyond AhR control likely account for the specific BaP effects on cellular attachment and morphology.

In addition to AhR-mediated effects, BaP has been shown to be metabolized in AhR-independent pathways. The pathway leading to the well-characterized toxic metabolite of BaP, (+) 7,8-dihydrodiol-9,10 epoxide (BPDE), utilizes CYP1A1 and prostaglandin H-synthase (PGHS) in the conversion of the dihydrodiol to its bioactive form (Degen, 1993). PGHS is an enzyme that co-oxidizes some xenobiotics and
carcinogens in vitro in the presence of arachadonic acid or lipid peroxides (Degen, 1993). PGHS probably does not play a major role in systemic drug metabolism, but may be involved in organ and cell specific toxicities. PGHS has been localized in the uterus primarily in the surface endometrial epithelium, although some localization was evident in glandular epithelium (Van Voorhis et al., 1990).

PGHS has been shown to occur in two isoforms, namely PGHS-1 and PGHS-2. PGHS-1 is the constitutive form of PGHS, which supplies prostanoids crucial for basal cellular functions and appears to be regulated developmentally (Smith and DeWitt, 1996). PGHS-2, on the contrary, is expressed in extremely low levels in most cells, but is rapidly induced by stimuli such as mitogens, tumor promoters, and cytokines, maintaining important roles in cellular inflammation and proliferation (Smith and DeWitt, 1996; Kulmacz, 1998). The present study shows that constitutive PGHS-1 protein levels are increased 2.2 fold by BaP treatment, with no significant change in PGHS-1 levels with TCDD treatment (Figure 4-6). PGHS-2 expression, in contrast, was not detected in control RL95-2 cells, and was not induced upon BaP or TCDD treatment, evidence that PGHS-2 is not involved in the specific BaP effects. The lack of expression of PGHS-2 in uterine RL95-2 cells is in contrast to published results from oral epithelial cells which indicate that BaP induces PGHS-2 (Kelley et al., 1997). Interestingly, benzo(e)pyrene, a non-carcinogenic congener of BaP, did not effect PGHS-2 levels (Kelley et al., 1997). The sensitivity of PGHS levels to transcriptional and translational stimulation by growth factors such as epidermal growth factor (EGF) can potentially impact on BaP effects (Marnett, 1990). Overall, it is likely that PGHS-1 plays a key role
in the pathogenesis of BaP-mediated effects on uterine endometrial cells through its dual role in carcinogen activation and catalysis of prostaglandin biosynthesis.

Whereas CYP1A1 and PGHS have been classically implicated in toxic metabolite formation, a few recent studies have provided evidence that BaP can be metabolized to potentially toxic quinone metabolites through PGHS alone (Degen, 1993; Sivarajah et al., 1981). In particular, BaP can be metabolized to 1,6-, 3,6-, and 6,12- quinones (BPQs), which are able to undergo redox cycling and thereby produce reactive oxygen species (ROS) such as O$_2^-$, OH, OH$, and$ H_2$O$_2$ (Liu et al., 1998; Mauthe et al., 1995). Although quinones themselves are not considered mutagenic or carcinogenic in animal models, quinone redox cycling leading to the generation of reactive oxygen species (ROS) is responsible for marked cellular toxicity and mutagenicity (Miller and Ramos, 2001a). Moreover, BaP metabolism has been associated with oxidative stress by the depletion of reduced cellular glutathione (GSH) and ATP levels (Romero et al., 1997). Consequently, BaP may have effects due to metabolites generated at the cellular level through cytochrome P450, prostaglandin H-synthase (PGHS), lipoxygenase, and/or lipid peroxidation.

TCDD, as well as BaP, has been reported to be associated with oxidative stress in cells, although to a lesser extent than BaP (Morel and Barouki, 1999; Yoshida and Ogawa, 2000; Radjendirane and Jaiswal, 1999). In particular, dioxin exposure has been associated with a sustained oxidative stress response in the mouse (Shertzer et al., 1998). It has been reported that mice exposed to 5 $\mu$g TCDD/kg for 3 consecutive days had oxidized hepatic glutathione levels and elevated urinary 8-hydroxydeoxyguanosine levels, a product of DNA base oxidation (Shertzer et al., 1998).
Due to the instability and high reactivity of ROS and their low steady state levels, quantitation of ROS is a difficult task. In the current study, two main approaches were taken to evaluate the oxidative stress response of RL95-2 cells to BaP and TCDD treatments. First, experiments utilizing cDNA microarrays were performed to determine changes in the transcriptional expression of the genes for enzymes involved in the production and removal of ROS at 6 hours. Subsequently, a biochemical assay based on oxidation of dichlorofluorescin was employed to estimate the overall levels of intracellular ROS production.

Microarray technology has emerged as a cutting-edge tool for investigating normal biological and disease processes, profiling differential gene expression, and discovering potential therapeutic and diagnostic drug targets. Data obtained using the Human Stress/Toxicology Array indicate that a total of 4 genes were down-regulated and 10 genes were up-regulated by BaP relative to DMSO vehicle control by a two-fold or greater magnitude. All of the genes had defined ratios, meaning the intensities of both genes were above background. Further, two of the genes regulated by BaP were similarly regulated by TCDD, namely CYP1A1 and CYP1A2, thereby serving as a control for AhR induction.

Interestingly, cyclic AMP response element binding protein 1 (CREB 1), a transcription factor for cAMP-regulated genes, was shown to be down-regulated by BaP relative to control. Cyclic AMP second messenger pathways are important to cellular growth, differentiation, and function through the regulation of extracellular signals. It could be speculated that a down-regulation of CREB 1 could prevent key signal transduction pathways involved in cellular growth. Further, results indicate a down-
regulation of cyclin-dependent kinase inhibitor 1 (or p21), known to associate with cyclins A, D, and E in the control of the G1 to S phase transition. P21 is known to inhibit cyclin dependent kinase proteins (cdks), as well as to inhibit the phosphorylation of the retinoblastoma protein.

Whereas 2 stress response-regulator genes were down-regulated by BaP in RL95-2 cells, 7 stress-response regulator genes were up regulated (Tables 4-1 and 4-2). Results are potentially indicative of BaP producing a low level of oxidative stress in cells. Among the genes up-regulated by BaP relative to control was the 150 kDa oxygen-regulated protein, shown to play an important role in protein folding and secretion in the endoplasmic reticulum, likely acting as a molecular chaperone protein to cope with environmental stress. In addition, the UDP-glucuronosyltransferase 1-6 precursor protein was up-regulated in RL95-2 cells with BaP treatment, indicating the induction of secondary metabolism pathways for BaP. As expected, results indicate the induction of CYP1A1 and CYP1A2 mRNA by BaP.

TCDD treatment, by contrast, was shown to effect the regulation of 15 genes, two of which were similarly regulated by BaP and 4 other genes similarly regulated by t-butylhydroperoxide (Tables 4-3 and 4-4). The genes for heat shock cognate 71-kDa protein and peptidyl-propyl cis-trans isomerase were down-regulated by both TCDD and t-butylhydroperoxide, whereas the gene heat shock 70-kDa protein 4 was up-regulated by both chemicals. In contrast, the gene for glutathione S-transferase theta 1 was down-regulated by TCDD treatment, whereas it was up-regulated upon treatment with t-butylhydroperoxide. Interestingly, 5 stress-response regulator genes were down-regulated in response to TCDD treatment, whereas only 2 were up-regulated, a trend
opposite to that of BaP which predominantly up-regulated stress response genes. Interestingly, the gene NADH-cytochrome B5 reductase (DT diaphorase) was shown to be down-regulated by TCDD in RL95-2 cells. Diaphorase is known to function in the disassociation and elongation of fatty acids, cholesterol biosynthesis, and drug metabolism.

Finally, a total of 15 genes were perturbed by t-butylhydroperoxide treatment, including 5 stress-response regulator genes which were down-regulated and 3 stress response regulators which were up-regulated (Tables 4-5 and 4-6). Since TBH is known to elicit a strong oxidative stress response in cells, the seeming contradiction of a predominantly down-regulated stress response set of genes is believed to result from the cells experiencing severe DNA damage at the 200 μM concentration. Evidence for DNA damage is shown in the up-regulation of various base-excision and nucleotide-excision repair genes upon TBH treatment. Interestingly, endonuclease III homolog 1, which excises damaged pyrimidines, was shown to be up-regulated with t-butylhydroperoxide treatment, as well as the genes for UV excision repair protein RAD23A and DNA excision repair protein ERCC2. Glutathione S-transferase theta 1, which functions in secondary detoxification metabolism, was similarly up-regulated, as well as glutathione peroxidase-related protein 2, which catalyzes the reduction of H₂O₂, organic and lipid peroxides by reduced glutathione.

Further studies were employed to estimate the total levels of intracellular ROS production upon BaP, TCDD, and TBH treatment. It has been reported that increased 5-(and-6)-chloromethyl-2'7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) oxidation is a useful assay for oxidative stress (Jakubowski and Bartosz, 2000; Xie et al.,
Wang and Joseph, 1999). CM-H2DCFDA has been used successfully to measure intracellular reactive oxygen species in cardiac myocytes (Xie et al., 1999). Studies in cultured monocytes indicate a significantly enhanced production of superoxide ions upon treatment with BaP, whereas treatment with benzo(e)pyrene (BeP), a relatively noncarcinogenic congener of BaP, failed to detect superoxide at significant levels (Xie et al., 1999).

The present study used the DCF assay failed to demonstrate a dose-response relationship for intracellular oxidative stress using the positive control t-butylhydroperoxide. Consequently, current results from the DCF assay do not adequately assess the effects of BaP or TCDD on intracellular oxidation. Likely flaws in the experimental design include unequal detachment of cells from the 96-well plates after repeated PBS washes and difficulties in uniformly removing all traces of phenol red from samples prior to absorbance readings. It is speculated that pre-treatment of the wells with collagen may alleviate the unequal cellular attachment, and enable the acquisition of more reproducible data. Further development of a biochemical measure for oxidative stress will need to be made in future studies.

In conclusion, the development of an increased understanding of the role of signal transduction pathways involved in cell-cycle checkpoint responses to environmental exposures holds great promise in disease prevention, as well as in development of efficacious therapeutic strategies for the treatment of environmentally-linked cancers. Current data provide unique molecular fingerprints for xenobiotic effects on RL95-2 cells and indicate that BaP may produce a low level of oxidative stress in cells. Further, data suggest that inducible CYP1A1, CYP1B1 and/or PGHS-1 may mediate a pathway for
BaP bioactivation and toxicity. It is hoped that such information will prove useful in the assessment of cancer risk for the whole population, as well as subpopulations of individuals with particular genetic susceptibilities.
Figure 4-1. Intermediates from normal metabolism of atmospheric oxygen by successive 1-electron reductions.
Figure 4-2. Graphical Representation of BaP and TCDD Effects on Cell Cycle Phase Distribution. Graphical representation of BaP and TCDD effects on RL95-2 cell cycle phase distribution at 48 hours. Data represent the mean ± SEM for four separate experiments (p<0.05).
Figure 4-3. Representative Experiment Showing the Differential Effects of BaP and TCDD on RL95-2 Cell Cycle Phase Distribution. Flow cytometry analysis with propidium iodide fluorescence was performed to evaluate the effects of 10 μM BaP and 10 nM TCDD on cell cycle phase distribution in RL95-2 cells.
Figure 4-4. Induction of CYP1A1 and CYP1B1 mRNA by BaP and TCDD in RL95-2 cells. Northern blot analysis was performed to analyze CYP1A1 and CYP1B1 mRNA in RL95-2 cells. Results are representative blots for two separate experiments.
Figure 4-5. Western immunoblot analysis for the effects of BaP and TCDD on CYP1A1 protein level.
RL95-2 cells were treated with 10 μM BaP or 10 nM TCDD for 48 hours, and the detergent-extracted cell lysates were electrophoresed, transferred to a nitrocellulose membrane, and immunostained with anti-human CYP1A1 followed by horseradish peroxidase conjugated IgG. Results were consistent among four replicates.
Figure 4-6. Western immunoblot analysis of the effects of BaP and TCDD on PGHS-1 protein levels. RL95-2 cells were treated with 10 μM BaP or 10 nM TCDD for 48 hours, and detergent-extracted cell lysates were electrophoresed, transferred to a nitrocellulose membrane, and immunostained with anti-human PGHS-1 followed by horseradish peroxidase conjugated IgG. Data represent the mean ± SE for 5 experiments (p<0.005).
Figure 4-7. RL95-2 Microarray experiment ScatterPlot Analysis.
The Clontech Atlas Human Stress array with AtlasImage 1.5 software was used to analyze overall intensities of gene expression for the 236 genes known to modulate toxic cellular responses. Total RNA was recovered from control, BaP, TCDD, and t-butylhydroperoxide-treated RL95-2 cells and a cDNA target prepared with incorporation of $^{33}$P. Values for gene expression were obtained for composite arrays from three separate experiments per treatment condition. Intensities for gene expression were globally normalized to all genes on the membrane using AtlasImage 1.5 and Excel software. Globally normalized (AtlasImage 1.5) values were calculated for xenobiotic-treated samples relative to control, and were analyzed using ScatterPlot analysis for each gene relative to a two-fold or greater change. Genes lying close to the diagonal line of identity show similar gene expression, whereas those lying farther away have greater variation.
Clontech Globally Normalized Intensities in Untreated RL95-2 cells

Clontech Globally Normalized Intensities in T-butylhydroperoxide Treated RL95-2 cells (200 μM)

Clontech Globally Normalized Intensities in TCDD-Treated RL95-2 cells (10 nM)

Clontech Globally Normalized Intensities in BaP-Treated RL95-2 cells (10 mM)
### Table 4-1. Stress-related genes with defined ratios down-regulated by benzo(a)pyrene in RL95-2 cells.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Access. #</th>
<th>BaP</th>
<th>BaP:DMSO</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAMP-response element binding protein</td>
<td>M34356</td>
<td>-</td>
<td>0.38</td>
<td>Stress-response regulator</td>
</tr>
<tr>
<td>C-jun N-terminal kinase 3 alpha 2</td>
<td>U34819</td>
<td>-</td>
<td>0.42</td>
<td>DNA damage response</td>
</tr>
<tr>
<td>Purine-rich single-stranded DNA-binding protein alpha</td>
<td>M96684</td>
<td>-</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td>Cyclin-dependent kinase inhibitor 1</td>
<td>U09579</td>
<td>-</td>
<td>0.49</td>
<td></td>
</tr>
</tbody>
</table>

### Table 4-2. Stress-related genes with defined ratios up-regulated by benzo(a)pyrene in RL95-2 cells.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Access. #</th>
<th>BaP</th>
<th>BaP:DMSO</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondrial 10-kDa heat shock protein</td>
<td>U07550</td>
<td>+</td>
<td>2.03</td>
<td>Stress-response regulator</td>
</tr>
<tr>
<td>Protein disulfide isomerase-related protein ERP72 precursor</td>
<td>J05016</td>
<td>+</td>
<td>2.79</td>
<td></td>
</tr>
<tr>
<td>51-kDa fK506-binding protein</td>
<td>U42031</td>
<td>+</td>
<td>2.42</td>
<td></td>
</tr>
<tr>
<td>94-kDa glucose-regulated protein</td>
<td>X15187</td>
<td>+</td>
<td>5.00</td>
<td></td>
</tr>
<tr>
<td>NADP-regulated thyroid hormone-binding protein</td>
<td>L02950</td>
<td>+</td>
<td>4.11</td>
<td></td>
</tr>
<tr>
<td>Probable protein disulfide isomerase P5 precursor</td>
<td>D49489</td>
<td>+</td>
<td>3.00</td>
<td></td>
</tr>
<tr>
<td>150-kDa oxygen-regulated protein</td>
<td>U65785</td>
<td>+</td>
<td>2.47</td>
<td></td>
</tr>
<tr>
<td>Cytochrome P450 1A2</td>
<td>Z00036</td>
<td>+</td>
<td>5.38</td>
<td>Xenobiotic metabolism</td>
</tr>
<tr>
<td>Cytochrome P450 1A1</td>
<td>K03191</td>
<td>+</td>
<td>4.60</td>
<td></td>
</tr>
<tr>
<td>Microsomal UDP-glucuronosyltransferase 1-6 precursor</td>
<td>J04093</td>
<td>+</td>
<td>2.52</td>
<td></td>
</tr>
</tbody>
</table>
TABLE 4-3. Stress-related genes with defined ratios down-regulated by TCDD in RL95-2 cells.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Access. #</th>
<th>TCDD</th>
<th>TCDD:DMSO</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor necrosis factor type 1 receptor associated</td>
<td>U12595</td>
<td>-</td>
<td>0.38</td>
<td>Stress-response regulator</td>
</tr>
<tr>
<td>protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor metastatic process-associated protein</td>
<td>X17620</td>
<td>-</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Heat shock cognate 71-kDa protein</td>
<td>Y00371</td>
<td>-</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>Peptidyl-prolyl cis-trans isomerase</td>
<td>M34539</td>
<td>-</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>T-complex protein 1 eta subunit</td>
<td>U83843</td>
<td>-</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>FLAP endonuclease 1</td>
<td>L37374</td>
<td>-</td>
<td>0.41</td>
<td>DNA damage response</td>
</tr>
<tr>
<td>Multidrug resistance-associated protein 1</td>
<td>L05628</td>
<td>-</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>Glutathione S-transferase theta 1</td>
<td>X79389</td>
<td>-</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>Microsomal UDP-glucuronosyltransferase 1-1 precursor</td>
<td>M57899</td>
<td>-</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>NADH-cytochrome B5 reductase</td>
<td>Y09501</td>
<td>-</td>
<td>0.42</td>
<td></td>
</tr>
</tbody>
</table>

TABLE 4-4. Stress-related genes with defined ratios up-regulated by TCDD in RL95-2 cells.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Access. #</th>
<th>TCDD</th>
<th>TCDD:DMSO</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat shock 70-kDa protein 4</td>
<td>L12723</td>
<td>+</td>
<td>3.06</td>
<td>Stress-response regulator</td>
</tr>
<tr>
<td>Calreticulin precursor</td>
<td>M84739</td>
<td>+</td>
<td>2.96</td>
<td></td>
</tr>
<tr>
<td>Cytochrome P450 1A2</td>
<td>Z00036</td>
<td>+</td>
<td>2.94</td>
<td>Xenobiotic metabolism</td>
</tr>
<tr>
<td>Cytochrome P450 1A1</td>
<td>K03191</td>
<td>+</td>
<td>10.60</td>
<td></td>
</tr>
<tr>
<td>60S ribosomal protein L13A</td>
<td>X56932</td>
<td>+</td>
<td>2.32</td>
<td>Housekeeping genes</td>
</tr>
</tbody>
</table>
TABLE 4-5. Stress-related genes with defined ratios down-regulated by tert-butylhydroperoxide in RL95-2 cells.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Access. #</th>
<th>T-but</th>
<th>T-but:DMSO</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>27-kDa heat shock protein</td>
<td>X54079</td>
<td>-</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>47-kDa heat shock protein precursor</td>
<td>X61598</td>
<td>-</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>Heat shock cognate 71-kDa protein</td>
<td>Y00371</td>
<td>-</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>Peptidyl-propyl cis-trans isomerase</td>
<td>M34539</td>
<td>-</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td>FK506-binding protein 13 precursor</td>
<td>M65128</td>
<td>-</td>
<td>0.35</td>
<td></td>
</tr>
</tbody>
</table>

TABLE 4-6. Stress-related genes with defined ratios up-regulated by tert-butylhydroperoxide in RL95-2 cells.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Access. #</th>
<th>T-but</th>
<th>T-but:DMSO</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-jun N-terminal kinase 3 alpha 2</td>
<td>U34819</td>
<td>+</td>
<td>2.59</td>
<td>Stress-response regulator</td>
</tr>
<tr>
<td>Nck, ash &amp; phospholipase C gamma-binding protein</td>
<td>AB005216</td>
<td>+</td>
<td>2.61</td>
<td></td>
</tr>
<tr>
<td>Heat shock 70-kDa protein 4</td>
<td>L12723</td>
<td>+</td>
<td>3.66</td>
<td></td>
</tr>
<tr>
<td>Endonuclease III homolog 1</td>
<td>U79718</td>
<td>+</td>
<td>2.00</td>
<td>Base excision repair</td>
</tr>
<tr>
<td>UV excision repair protein RAD23A</td>
<td>D21235</td>
<td>+</td>
<td>2.71</td>
<td>Nucleotide excision repair</td>
</tr>
<tr>
<td>DNA excision repair protein ERCC2</td>
<td>X52221</td>
<td>+</td>
<td>2.59</td>
<td></td>
</tr>
<tr>
<td>Glutathione S-transferase theta 1</td>
<td>X79389</td>
<td>+</td>
<td>2.26</td>
<td>Xenobiotic metabolism</td>
</tr>
<tr>
<td>Glutathione peroxidase-related protein 2</td>
<td>X53463</td>
<td>+</td>
<td>2.04</td>
<td></td>
</tr>
<tr>
<td>Phospholipase A2</td>
<td>M86400</td>
<td>+</td>
<td>4.63</td>
<td>Housekeeping genes</td>
</tr>
<tr>
<td>HLA class I histocompatibility antigen C-4 alpha subunit</td>
<td>M11886</td>
<td>+</td>
<td>3.14</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4-8. Venn diagram for BaP, TCDD, and t-butylhydroperoxide effects on RL95-2 cells. Venn diagram depicts sets of genes perturbed by at least a factor of two with defined ratios for 10 μM benzo(a)pyrene (BaP), 10 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and 200 μM t-butylhydroperoxide (T-butyl) treatment as determined by cDNA array analysis. The Clontech Human Stress array with AtlasImage 1.5 software was used to analyze overall fold-change alterations in gene expression upon treatment relative to control for composite arrays from three separate experiments per treatment condition. Genes perturbed by sets of two (pair intersections) or three (global intersections) of these drugs are also shown with the total number of genes affected for each treatment given in parentheses. For example, the regulation of 14 genes is affected by BaP, of which 1 is similarly affected by T-butyl, whereas two are similarly affected by TCDD. No general toxicity genes (genes affected by all three treatments) were evident in this analysis.
Figure 4-9. RL95-2 ScatterPlot for TreeView Analysis.
The Clontech Atlas Human Stress array with AtlasImage 1.5 software was used to analyze overall intensities of gene expression for the 236 genes known to modulate toxic cellular responses. Values for gene expression were obtained for composite arrays from three separate experiments per treatment condition. Intensities for gene expression were globally normalized to all genes on the membrane using Excel software. Adjusted intensities for normalized BaP-treated samples relative to control and were analyzed using ScatterPlot analysis with Prism 2.01 software for each gene relative to a two-fold or greater change.
FIGURE 4-10. RL95-2 cDNA Microarray analysis Treeview Diagram.
The Clontech Human Stress array with AtlasImage 1.5 software was used to analyze overall intensities of gene expression for the 236 genes known to modulate cellular responses to various stressors. Values for gene expression were obtained for composite arrays from three separate experiments per treatment condition. Raw intensities for gene expression were imported into Excel, from which variance normalizations for the top 50% of genes with the highest standard deviations were plotted in a horizontal hierarchical tree graph, separated into clusters based upon the similarities in their gene expression profiles. Genes expressing strong similarity are joined by short branches, whereas increasingly dissimilar genes are joined by longer branches. Genes with a log ratio of 0 (unchanged genes) are black, genes with increasingly positive log ratios (increased genes) are red, and genes with decreasing log ratios (decreased genes) are green.
**TABLE 4-7.** Biochemical Measure of Oxidative Stress in RL95-2 cells – DCF Fluorescence using a Microplate Reader.

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>10 μM BaP</th>
<th>20 μM BaP</th>
<th>10 μM BeP</th>
<th>20 μM BeP</th>
<th>10 nM TCDD</th>
<th>1 μM T-butyl</th>
<th>10 μM T-butyl</th>
<th>200 μM T-butyl</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>6 hours</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>100±0</td>
<td>112.4±10.9</td>
<td>97.8±12.9</td>
<td>90.4±6.2</td>
<td>78.3±2.8</td>
<td>75.9±1.6</td>
<td>160.3±78.1</td>
<td>82.4±3.9</td>
<td>123.1±12.6</td>
</tr>
<tr>
<td>P value</td>
<td>0.32</td>
<td>0.87</td>
<td>0.20</td>
<td>0.001</td>
<td>0.0001</td>
<td>0.48</td>
<td>0.01</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td><strong>24 hours</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>100±0</td>
<td>105.7±3.7</td>
<td>112.2±4.6</td>
<td>95.8±6.5</td>
<td>91.4±5.4</td>
<td>99.9±5.4</td>
<td>139.0±30.6</td>
<td>103.4±3.4</td>
<td>110.3±35.9</td>
</tr>
<tr>
<td>P value</td>
<td>0.20</td>
<td>0.06</td>
<td>0.56</td>
<td>0.19</td>
<td>0.99</td>
<td>0.27</td>
<td>0.38</td>
<td>0.79</td>
<td></td>
</tr>
<tr>
<td><strong>48 hours</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>100±0</td>
<td>112.6±8.1</td>
<td>106.1±13.4</td>
<td>103.3±2.4</td>
<td>97.2±7.5</td>
<td>98.5±9.8</td>
<td>150.4±44.5</td>
<td>100.3±13.1</td>
<td>102.4±26.9</td>
</tr>
<tr>
<td>P value</td>
<td>0.20</td>
<td>0.68</td>
<td>0.25</td>
<td>0.72</td>
<td>0.88</td>
<td>0.32</td>
<td>0.99</td>
<td>0.93</td>
<td></td>
</tr>
</tbody>
</table>

RL95-2 cells were treated with various xenobiotics in regular, serum-containing media, followed by multiple washings and subsequent treatment of cells with xenobiotics in PBS in the presence of CM-H2DCFDA. Absorbance measurements of resulting fluorescent adduct formation was monitored over time using a microplate reader for three separate experiments. P values were calculated for each treatment relative to DMSO control. All values shown indicate the absorbance of cells treated with respective chemicals minus the raw absorbance of the chemicals alone in the presence of DCF.
Oxidative Stress

DNA Damage

Cell cycle effects

Activation or Repression of Signal Transduction Pathways

Cytoskeletal reorganization

Cell surface alterations

Figure 4-10. Cellular effects of oxidative stress. Oxidative stress produces initial events which trigger apoptosis in cultured cells, leading to an altered cellular phenotype.
CHAPTER 5
DIFFERENTIAL EFFECTS OF BAP ON CELL ATTACHMENT AND ADHERENS JUNCTION AND CYTOSKELETAL COMPLEX INTEGRITY, EXPRESSION, AND PHOSPHORYLATION IN HEC-1A AND HEC-1B CELLS

Introduction

An estimated 36,000 new cases of cancer of the uterine corpus, usually of the endometrium or uterine lining, are diagnosed each year, with an estimated 6,500 deaths, making endometrial cancer the most common form of gynecological cancer in the United States (American Cancer Society, 1998). Further, endometriosis, a particularly painful and chronic uterine disease often associated with infertility, has been found in 10 to 15 percent of all premenopausal women undergoing gynecological surgeries (Haney, 1990) and in 25 percent of all women in their thirties and forties (Chalmers, 1980). Epidemiological studies indicate that cigarette smoking in women has been linked with a substantially decreased incidence of benign and malignant uterine disease, including endometrial cancer and endometriosis (Baron, 1996; Burke et al., 1996). Studies show that women who smoke cigarettes have less than half the risk of developing endometriosis of nonsmokers, and maintain similar decreased incidence rates for endometrial hyperplasia, uterine fibroids, and endometrial cancer (Baron, 1996; Cramer et al., 1986; Matorras et al., 1995).

The stage of tumor differentiation of endometrial cancers has been shown to be a major indicator of prognosis. Patients with well differentiated (Grade I) tumors have a five year greater survival rate (85%) than patients with poorly differentiated endometrial
tumors (Grade III) (58%) (Tornus and Elvio, 1993). Likewise, cellular adhesion molecules have been shown to play diverse and essential roles in cancer biology, including the provision of attachment and structural support for cells, as well as the mediation of key signal transduction pathways. It is widely accepted that epithelial cells form calcium-dependent cellular linkages through cadherins, integral membrane glycoproteins which critically connect to β-catenin at their cytoplasmic domains (Potter et al., 1999; Peralta et al., 1997). The key membrane-associated protein β-catenin is responsible for the colocalization of cadherins to sites of cell-cell contact with the actin cytoskeleton through the key adherens junction protein, α-catenin (Potter et al., 1999). α-catenin has been recently shown to play a central role in the recruitment of actin-binding proteins during the formation of cell-cell contacts, and therefore is essential to actin polymerization and the sealing of cellular borders (Vasioukhin et al., 2000). In addition, α-catenin has been shown to help stabilize already formed adherens complexes and to function in the rearrangement of actin stress fibers in cells (Vasioukhin et al., 2000). Whereas both α- and β-catenins are known to associate early with E-cadherin at the endoplasmic reticulum during its synthesis, α-catenin is integrated into the adherens complex at the later stage of plasma membrane insertion (Potter et al., 1999). The significance of α- and β-catenins is further enhanced by their interaction with epidermal growth factor receptor (EGF-R), as well as other tyrosine kinases, whereby they directly affect the adhesive function of E-cadherin through changes in phosphorylation status (Hayes et al., 1996; Hazan and Norton, 1998).

The actin cytoskeleton is also known to play a crucial role in cellular adhesion. Actin works in conjunction with cytoskeletal microtubules and intermediate filaments in
performing the essential cellular functions of locomotion and cytokinesis (Hulka and Brinton, 1995). Actin bundles are attached to integral membrane cadherin and integrin proteins, adapter proteins and the contractile bundle. Actin stress fibers are characteristic of differentiated stationary cells and confer anchorage dependence, contact inhibition, and reduced cellular motility (Kolega, 1986). Studies in RL95-2 cells have shown that inhibition of protein tyrosine kinases has been associated with actin filament reorganization (Carter and Bellido, 1999).

The present studies were undertaken to define key morphological differences between two human uterine endometrial cancer cell lines, HEC-1A and HEC-1B, and to examine the differential effects of the aryl hydrocarbon receptor ligand benzo(a)pyrene (BaP) on both cell lines with respect to cell morphology, protein phosphorylation, and adherens complex integrity. The hypothesis tested in this chapter is that BaP alters cellular attachment and adherens junction protein levels and localization in HEC-1A, but not HEC-1B, cells through alterations in β-catenin phosphorylation levels, resulting in the disassociation of the adherens complex.

Results

Differential Localization of Actin Cytoskeletal Proteins

Initial studies were performed to characterize basic morphological differences between HEC-1A and HEC-1B endometrial carcinoma subtypes prior to xenobiotic treatment. Initial studies investigated the differences in actin cytoskeletal conformation in HEC-1A and HEC-1B cells. Cells were cultured in regular media on glass chamber slides and all experiments were performed when cells reached approximately 70%
confluence. The fluorescently-labelled peptide phalloidin was used to selectively visualize filamentous actin by epi-fluorescent confocal microscopy. Results indicate that HEC-1A cells had filamentous actin localized in a thick, cortical rim along intercellular boundaries (Figure 5-1A). Further, HEC-1A cells tend to pile up in culture and form continuous cell-cell boundaries. In contrast, HEC-1B cells display a distribution of fluorescence intensity which correlates with the position of the nucleus (Figure 5-1B). HEC-1B cells remain more spread out and diffuse under standard culture conditions, likely due to the collapse of the filament network, resulting in a perinuclear distribution of actin.

**Levels of β-catenin, α-catenin, and EGF-R Proteins**

Further experiments characterized the basic differences between the HEC-1A and HEC-1B sublines using Western immunoblot analysis to determine the relative levels of the adherens junction proteins β-catenin, α-catenin, and EGF-R. Data in Figure 5-2A indicate that β-catenin was detected as a 92 kDa protein which was expressed at comparable levels in both HEC-1A and HEC-1B cell membranes. α-catenin, a second adherens junction protein, was expressed as a 102 kDa protein in both cell lines. α-catenin was found to be expressed at 59% lower levels in the membrane fractions of HEC-1B cells than HEC-1A cells (p<0.05) (Figure 5-2B). EGF-R was detected in HEC-1A and HEC-1B cells as a 170 kDa protein. Further, EGF-R was expressed at significantly 126% higher levels in HEC-1B compared with HEC-1A cells (p<0.05) (Figure 5-2C). In data not shown, no differences in overall protein levels of β-catenin, α-catenin, or EGF-R were observed in cell lysate samples from HEC cells.
Effects of BaP on HEC-1A and HEC-1B Cell Attachment and Invasion

HEC-1A and HEC-1B cells were cultured in the presence of 10 μM BaP or 0.1% v/v DMSO vehicle control for 48 hours, then trypsinized and applied to Matrigel-coated porous membranes. All treated cells exhibited greater than 95% viability as determined by trypan blue dye exclusion. In the attachment experiment, 2 x 10^4 cells in serum-free or regular media were aliquoted into the lower wells of the Boyden Chamber apparatus. The wells were overlaid with a Matrigel-coated polyvinyl-pyrrolidone-free polycarbonate membrane (10 micron pore size) and the gasket seal and upper chamber attached. The apparatus was inverted for 2 hours to allow for cell attachment. The apparatus was then disassembled and the attached cells stained with Leukostat and quantitated by light microscopy. Figure 5-3A shows that HEC-1A cells in serum-free media pretreated with BaP exhibited minimal attachment, an 86% decrease relative to DMSO vehicle controls (P<0.0005). In data not shown, BaP-exposed HEC-1A cells in regular 10 percent serum-containing media showed a 77% decrease in attachment relative to control (p<0.005). In contrast, no effect was observed on cellular attachment for BaP-treated HEC-1B cells in either serum-free or regular media conditions.

Subsequent studies evaluated the effects of BaP on cell invasion through a Matrigel-coated porous membrane. In the invasion experiment, cells in serum-free media were allowed to attach to the membrane for 90 minutes, after which the upper wells of the Boyden chamber were loaded with complete media. Cells were then allowed to invade the porous membrane for 24 hours, after which invaded cells were stained with Leukostat and counted by light microscopy. Data indicate that BaP-pretreatment of HEC-1A cells resulted in an 86% decrease in overall cellular invasion as compared to
DMSO vehicle control cells (p<0.0005) (Figure 5-3B). In contrast, HEC-1B cells treated with BaP showed no significant changes in ability to invade the Matrigel-coated membrane.

**Effects of BaP on α-catenin, β-catenin, and EGF-R Protein Levels**

Western immunoblot analysis was performed for α-catenin and β-catenin proteins to determine whether the BaP-mediated effects on cellular attachment were due to alterations in adhesion complex protein expression. Results indicate that treatment with 10 μM BaP produced a significant 31% decrease in cell lysate α-catenin levels in HEC-1A cells (p<0.05), whereas cell membrane α-catenin levels were unchanged (Figure 5-4A). In contrast, BaP treatment of HEC-1B cells had no effect on either cell lysate or cell membrane α-catenin fractions (Figure 5-4B). Further studies indicate that 48 hour treatment with 10 μM BaP resulted in a significant 26% decrease in membrane β-catenin levels in HEC-1A cells (p<0.05) (Figure 5-5A). In contrast, BaP did not effect cell lysate β-catenin levels in HEC-1A cells (Figure 5-5B). Further, BaP treatment did not alter cell membrane or lysate β-catenin protein levels in HEC-1B cells (Figure 5-5B).

In addition, studies were performed to evaluate the effects of BaP treatment on cellular EGF-R levels. Results indicate that BaP treatment caused a significant 45% decrease in HEC-1A cellular membrane EGF-R levels (p<0.05) (Figure 5-6A), whereas HEC-1B membrane EGF-R levels were unaltered (Figure 5-6B). Effects on HEC-1A and HEC-1B cell lysate EGF-R levels were not investigated.
Effects of BaP on β-catenin and α-catenin Protein Interactions

Co-immunoprecipitation experiments were performed to determine the effect of BaP on adhesion complex integrity of α- and β-catenins in HEC-1A and HEC-1B cell lines (Figure 5-7). Cells treated with 10 μM BaP for 48 hours were lysed at 4°C in an immunoprecipitation buffer containing Triton x-100, NP-40, and various protease and phosphatase inhibitors. A polyclonal anti-human β-catenin antibody was used to immunoprecipitate β-catenin protein from total cellular protein preparations. Subsequently, immunoprecipitated protein samples were analyzed by Western immunoblot analysis using a monoclonal α-catenin antibody to determine the levels of association of the two proteins.

Results shown in Figure 5-8 indicate that both HEC-1A and HEC-1B cells have β-catenin protein complexed to α-catenin. Whereas BaP treatment results in a HEC-1A cell type-specific decrease in both β-catenin membrane and α-catenin lysate proteins, upon immunoprecipitation, a decrease in the protein levels of the α-catenin/β-catenin protein complex was observed in both the HEC-1A and HEC-1B cell lines in two separate experiments (Figure 5-8A).

Effects of BaP on β-catenin Phosphorylation

Additional immunoprecipitation experiments were performed to determine the effects of BaP on the phosphorylation status of β-catenin phosphotyrosine residues in HEC-1A and HEC-1B cells. A polyclonal β-catenin antibody was used to immunoprecipitate β-catenin protein from both cell lines. β-catenin immunoprecipitates were subsequently analyzed by Western immunoblot analysis for changes in
phosphorylation levels using a monoclonal phosphotyrosine antibody. Results from three separate experiments indicate that there was no significant change in β-catenin phosphorylation status upon BaP treatment in either the HEC-1A or HEC-1B cell line (Figure 5-9).

**Discussion**

Cellular adhesion mechanisms play a fundamental role in the determination of tissue architecture and functions of cell assembly and connection to the internal cytoskeleton and, consequently, are of great significance to the pathophysiology of endometrial cancer. The differential regulation of cadherin, β-catenin, α-catenin, and actin are central to investigations of altered cellular attachment to other cells.

The present chapter further investigates endometrial carcinoma cell response to BaP exposure as discussed in Chapter 3, using the HEC-1A and HEC-1B endometrial cancer cell lines as models for BaP effectiveness or ineffectiveness, respectively. HEC-1A and HEC-1B cells are polarized cells with a rough surface structure and a thick glycocalyx, thereby representing early stage endometrial cancer (Thie et al., 1998). The RL95-2 cells, on the contrary, are non-polarized and represent late stage endometrial cancer (Thie et al., 1996).

Whereas HEC-1A cells exhibit a thick rim of cortical filamentous actin along intercellular boundaries, HEC-1B cells display nuclear-localized actin and exhibit non-continuous cellular boundaries. Further, α-catenin, a key cell adhesion protein, is expressed significantly less in HEC-1B cells than HEC-1A cells, whereas both cell lines have comparable levels of β-catenin. The fundamental and crucial differences in cellular
morphology between the HEC-1A and HEC-1B cell lines make their differential responsiveness to BaP treatment particularly intriguing and strengthen their role in the development of useful biomarkers for BaP-mediated effects.

Results indicate a differential inhibitory effect of BaP on endometrial carcinoma cell attachment and adhesion complex protein expression in the two cell lines. Whereas 48 hour treatment of HEC-1A cells with 10 μM BaP resulted in a marked loss of cellular attachment and invasion, treatment of HEC-1B cells showed no effect compared to control. It was further shown that treatment of HEC-1A cells with BaP resulted in a significant decrease in membrane β-catenin and total cellular α-catenin protein levels relative to control, whereas BaP-treatment of HEC-1B cells had no effect on either adhesion complex protein. The key cellular adhesion protein β-catenin is believed to be intricately involved in the process of carcinogenesis. Evidence supporting the role of β-catenin on signal transduction pathways in human cancer include its down-regulation by the tumor suppressor APC, its transduction of the oncogenic wnt growth factor signal to the nucleus, and its high mutation frequency in human cancers (Orford et al., 1999).

Studies have shown that the first step of invasion and metastasis is the detachment of cancer cells from the primary tumor, a process mainly controlled by the adherens junction proteins, consisting of E-cadherin, α- and β-catenins, vinculin, and actin. Consequently, it can be inferred that the decreased cellular attachment of the HEC-1A cells upon BaP exposure is consistent with promotion of a more metastatic phenotype which would actually promote, rather than protect against, endometrial cancer. This view is supported by studies from human breast cancer patients which show that tumors negative for either α- or β-catenin expression demonstrate a higher incidence of distant
metastasis than those expressing both catenins (Yoshida et al., 2001). Further, a reduction or loss of E-cadherin and α-catenin expression has been associated with lymph node metastases and poor prognosis of invasive breast cancers (Yoshida et al., 2001). Additional studies from colorectal cancer patients indicate an association between decreased α-catenin expression and increased metastasis (Gofuku et al., 1999). However, a conflicting report from pancreatic cancer patients found an association between an intact E-cadherin/catenin complex and increased liver metastasis (Gunji et al., 1998).

The adhesion complex protein β-catenin has been shown to become phosphorylated in response to growth factors such as EGF-R (Hazan and Norton, 1998). Enhanced phosphorylation of β-catenin has been associated with decreased cell adhesion by dissociation of α-catenin, actin, and vinculin from the adhesion complex in MDA-MB-468 breast cancer cell lines (Hazan and Norton, 1998). These changes were accompanied by dissociation of actin from the membrane and rounding up of the apical surfaces (Hazan and Norton, 1998). Further studies have shown that mutations in the phosphorylated region of β-catenin result in a lack of cellular invasiveness (Shibata et al., 1996).

In order to determine whether the BaP-mediated loss of HEC-1A cell attachment was due to a loss of membrane-localized α- and β-catenin proteins, or whether the effects were specific for their decreased interaction, co-immunoprecipitation experiments were performed in HEC-1A and HEC-1B cells upon BaP treatment. Results indicate that β-catenin protein is complexed to α-catenin in HEC-1A and HEC-1B cells prior to BaP treatment. Upon treatment with 10 μM BaP for 48 hours, both HEC-1A and HEC-1B
cells show a decrease in co-immunoprecipitated β- and α-catenin protein levels. The BaP-mediated decrease in the complexed catenin proteins in HEC-1B cells is likely the direct result of the dissociation of the two proteins since both proteins were previously shown to be present at control levels after BaP treatment. Definitive conclusions cannot be drawn under the conditions of the current study regarding the effects of BaP on α- and β-catenin protein association in HEC-1A cells due to prior independent Western immunoblot experiments indicating decreased protein levels of membrane β-catenin and total cellular α-catenin after BaP exposure. The lower levels of complexed α- and β-catenin protein upon BaP treatment could reflect independent decreases in either α- or β-catenin, or both, and not necessarily represent a dissociation of the complex.

Additional experiments were performed investigating the role of BaP treatment on the phosphorylation of β-catenin. It was speculated that enhanced phosphorylation of β-catenin could account for its decreased overall association with α-catenin and cellular attachment effects. Results indicate that there was no major variation in the overall phosphorylation levels of HEC-1A and HEC-1B cells upon BaP treatment. Consequently, phosphorylation of β-catenin cannot account for the observed cellular attachment effects. Under conditions used in the present study, data are not in agreement with the hypothesis that β-catenin is exerting its effects by enhanced phosphorylation. Results are consistent with published studies using chimeric cadherin/α-catenin molecules, indicating that β-catenin phosphorylation may not necessarily be responsible for the modulation of cellular adhesion (Takeda et al., 1995).

In summary, results demonstrate a differential inhibitory effect of BaP on endometrial cancer cell attachment and adhesion complex protein expression in the HEC-
1A and HEC-1B substrains. Data are inconclusive on the role of dissociation of β-catenin and α-catenin proteins in HEC-1A specific loss of cellular attachment, and further evidence does not support the role of β-catenin phosphorylation changes in mediating this effect. It is proposed that the observed loss of cellular attachment in HEC-1A cells directly results from altered protein levels or localization of adherens junction proteins, in particular α-catenin and β-catenin, as well as altered actin cytoskeletal conformation upon BaP treatment. It is further proposed that the basic differences in cellular morphology between the HEC-1A and HEC-1B cell lines plays a key role in mediating the differential cellular responsiveness to toxic insult. The BaP-mediated dysregulation of cell adhesion molecules in the HEC-1A and HEC-1B cells serves as a useful biomarker for alterations in uterine epithelial cell polarization which may impact upon cellular attachment and invasion. The endometrial carcinoma cell lines HEC-1A and HEC-1B serve as useful models for BaP-mediated effects in differential endometrial tumor cell response.
Figure 5-1. Differential localization of filamentous actin in HEC-1A and HEC-1B cells. HEC-1A (A) and HEC-1B (B) cells were fixed with paraformaldehyde and permeabilized with acetone. They were then incubated with fluorescently labelled phalloidin to visualize filamentous actin structure. Slides were mounted with Fluoromount G and viewed under epi-fluorescent confocal light microscopy.
Figure 5-2. Western Immunoblot profiles of \( \beta \)-catenin, \( \alpha \)-catenin, and EGF-R protein levels in HEC-1A and HEC-1B cell lines.

HEC-1A and HEC-1B cell membranes were immunostained with antibodies to \( \beta \)-catenin (A), \( \alpha \)-catenin (B), and EGF-R (C). Results indicate that HEC-1B cells have \( \alpha \)-catenin, a key adhesion complex protein, in significantly lower levels and EGF-R in significantly higher levels than HEC-1A cells, whereas both cell lines have comparable levels of \( \beta \)-catenin (p<0.05). Results represent the mean ± SE for 3 experiments (p<0.05).
Figure 5-3. Effects of BaP on HEC-1A and HEC-1B cell attachment and invasion.

Cells were pre-treated for 48 hours with 10 μM BaP or 0.1% DMSO vehicle (control). A and B) Attachment Assay: 2 x 10^4 cells in serum-free or regular media, respectively, were aliquoted into lower wells of the Boyden Chamber apparatus and were allowed to attach to a Matrigel-coated membrane for 2 hours, followed by cell staining with Leukostat. C) Invasion Assay: Cells were allowed to attach to a Matrigel-coated membrane for 90 minutes. Upon attachment, upper wells of the Boyden Chamber were loaded with complete media and cells were allowed to invade for 24 hours, after which cells were stained with Leukostat and counted by light microscopy. Data are expressed as the mean ± SEM for three separate experiments (p<0.0005).
Figure 5-4. Western Immunoblot analysis of the effects of BaP on α-Catenin Protein in HEC-1A and HEC-1B cells.

HEC-1A and HEC-1B cells were treated with 10 μM BaP for 48 hours and whole cell lysate preparations were electrophoresed, transferred to nitrocellulose, and immunostained with an antibody to α-Catenin. Results indicate the mean ± SE for three experiments (p<0.05).
Figure 5-5. Western Immunoblot analysis of the effects of BaP on β-Catenin Protein in HEC-1A and HEC-1B cells.
HEC-1A (A) and HEC-1B (B) cells were treated with 10 μM BaP for 48 hours and whole cell lysate preparations were electrophoresed, transferred to nitrocellulose, and immunostained with an antibody to β-Catenin. Results indicate the mean ± SE for three experiments (p<0.05).
Figure 5-6. Western Immunoblot analysis of the effects of BaP on EGF-R Protein in HEC-1A and HEC-1B cells.
HEC-1A and HEC-1B cells were treated with 10 \( \mu \text{M} \) BaP for 48 hours and membrane preparations were electrophoresed, transferred to nitrocellulose, and immunostained with an antibody to EGF-R. Results indicate the mean \( \pm \) SE for 3 experiments (\( p<0.05 \)).
HEC-1A and HEC-1B cells were lysed using a Triton X-100 and NP-40 containing Immunoprecipitation buffer.

β-catenin protein was complexed to an antigen-specific antibody.

The β-catenin-antibody complex was incubated with Protein A Agarose (α-catenin co-IP) or Protein G Sepharose (phosphotyrosine IP) beads overnight at 4°C.

The complexes were then washed 3X in IP buffer.

Co-immunoprecipitated complexes were dissociated by centrifugation.

Samples were analyzed for co-immunoprecipitated α-catenin protein or β-catenin phosphotyrosine residues using SDS-PAGE, followed by Western Immunoblot analysis.

Figure 5-7. Schematic representation of immunoprecipitation protocol.
Figure 5-8. β-catenin/α-catenin complex co-immunoprecipitates from HEC-1A and HEC-1B cells following BaP treatment.

HEC-1A (A) and HEC-1B (B) cells were treated with 10 μM BaP for 48 hours. Whole cell lysate proteins (100 μg) were immunoprecipitated with a polyclonal β-catenin antibody (1 μg) at 4°C overnight, and the immunoprecipitates were further probed with α-catenin antibody by Western immunoblot analysis. A representative blot indicating a decrease in β-catenin-associated α-catenin in both cell lines and densitometric analysis indicating the average of two independent experiments are shown. Bars indicate range of values for two experiments.
Figure 5-9. Tyrosine phosphorylation of β-Catenin protein in HEC-1A and HEC-1B cells upon BaP treatment.

HEC-1A (A) and HEC-1B (B) cells were treated with 10 μM BaP for 48 hours. Whole cell lysate proteins (100 μg) were immunoprecipitated with a polyclonal β-catenin antibody (1 μg) and the immunoprecipitates assessed by Western immunoblot analysis for phosphorylation status using a monoclonal phosphotyrosine antibody. A representative blot from three separate experiments indicates the mean ± SE for β-catenin phosphorylation status upon BaP treatment for 3 separate experiments.
CHAPTER 6
ALTERNATE ENZYMATIC PATHWAYS AND CELL CYCLE EFFECTS
OF BAP ON HEC-1A AND HEC-1B CELLS.

Introduction

Evidence indicates a strong association between cigarette smoking and the decreased risk of endometrial cancer in women (Baron, 1996). Further, the cigarette smoke component, BaP, has been shown to induce an anti-proliferative, anti-attachment phenotype with corresponding alterations in membrane-associated cellular adhesion proteins in the HEC-1A, but not the HEC-1B, endometrial carcinoma cell line. Current studies were undertaken to determine the differential effects of BaP on cell cycle phase distribution and key metabolic enzyme pathways potentially involved in the differential cellular response of the HEC-1A and HEC-1B substrains to BaP treatment.

Metabolic Enzymes and Cell Cycle

The xenobiotic BaP is known to direct its own metabolism through the induction of gene transcription (Poland et al., 1976; Whitlock, Jr. et al., 1996; Kim et al., 1998). BaP is a genotoxic chemical recognized to alter signal transduction in human and animal cells by binding to the intracellular cytosolic protein aryl hydrocarbon receptor (AhR) and activating the transcription of the Phase I cytochrome P450 drug metabolizing enzymes, CYP1A1, CYP1A2, and CYP1B1.
BaP exposure has been associated with the formation of cellular DNA damage through the generation of bulky adducts and apurinic sites in DNA which are degraded to DNA strand breaks (Shackelford et al., 1999). As a result, BaP has been shown to inhibit DNA synthesis and induce S phase cell cycle arrest and the S phase checkpoint response (Shackelford et al., 1999; Shackelford et al., 2000). In addition, BaP has been shown to undergo metabolism prior to or independent of the AhR pathway, including PGHS-induced formation of quinone metabolites, a process often associated with cellular oxidative stress (Degen, 1993; Sivarajah et al., 1981).

Whereas phase I metabolic enzymes are associated with bioactivation of xenobiotics, phase II enzymes are primarily responsible for xenobiotic elimination. In particular, sulfation and glucuronidation pathways may act to reduce the bioavailability of a xenobiotic and the overall resulting level of toxic insult. A recent study indicates that cells having a glutathione S-transferase mu (GSTM1) null phenotype have significantly more chromosomal damage, and a higher level of polycyclic aromatic hydrocarbon-DNA adducts than cells positive for GSTM1, thereby lending support for the important detoxification role of GSTM1 in BaP metabolism (Salama et al., 2001). Consequently, the overall levels of Phase I and Phase II metabolic enzymes are critical to observed cell-type specific toxicity of BaP (Barouki and Morel, 2001).

**Gene Expression Profile Analysis**

Over a quarter of a century ago, Edward Southern reported that labeled nucleic acid molecules could be used to probe other nucleic acid molecules attached to a solid support, thereby designing the first Southern blot (Southern, 1975). Nearly 25 years
later, cDNA expression arrays have emerged as a valuable tool in monitoring the simultaneous expression of thousands of genes, and in the classification of toxicants based upon their unique patterns of gene expression (Bartosiewicz et al., 2001a). Microarrays are solid supports to which hundreds or thousands, even ten-thousands, of cDNAs have been covalently attached at high density. cDNAs on microarrays typically span 600 to 2400 base pairs in length, thereby providing a wealth of information about differential gene expression profiles. Functionally, cDNA microarray procedures involve reverse transcription of mRNA to radiolabelled cDNAs, which are then hybridized to separate, identical, nylon membrane arrays. The radioactive signals bound to each cDNA on the array represent relative expression levels of each gene as detected by phosphorimaging.

DNA microarrays provide a systematic and comprehensive means for exploring the human genome, based upon the extreme specificity and affinity of complementary base pairing of DNA and RNA (Brown and Botstein, 1999). They serve as a vital tool to enable researchers to measure the transcripts for hundreds of genes simultaneously under a variety of treatment conditions. Since genes with similar expression patterns are likely to share common molecular control processes, microarrays enable the accumulation of a large body of data and the integrated assessment of cellular response through evaluation of gene expression patterns. Further, they provide both static information about gene expression profiles in tissues or cells, as well as dynamic information about the relationship of gene expression for one particular gene to that of others (Duggan et al., 1999). Microarrays currently hold tremendous promise in toxicological screening of chemicals, as well as in cancer diagnosis and determination of treatment responsiveness.
Since most toxicologically relevant outcomes require the differential expression of multiple genes, by studying the patterns of gene expression using microarrays, researchers can gain insight into the fundamental mechanisms of xenobiotic toxicity (Farr and Dunn, 1999).

However, despite their many applications, there is currently debate over their use in particular experimental applications. One group believes that exploring the genome using cDNA microarrays should not be driven by hypotheses and actually should be “as model-independent as possible” (Brown and Botstein, 1999), whereas others believe that arrays are most effectively used when answering specific biological questions. Both hypothesis-driven and hypothesis-independent approaches have been utilized in the current array experiments. The Clontech Atlas Human Toxicology 1.2 microarray was used to determine changes in gene expression in 1,176 key toxicology-related genes functioning in xenobiotic metabolism, DNA damage repair, oncogenesis, tumor suppression, basic transcription factors, cellular adhesion and the cytoskeleton, the kinase network, apoptosis, stress-response, and cellular growth and signaling. The data obtained from BaP treatment, relative to control, were then profiled for patterns of gene expression that were associated with the cellular outcome of BaP action on the HEC-1A, yet not HEC-1B, cell line.

**Results**

**Cell Cycle Phase Distribution**

HEC-1A and HEC-1B human uterine endometrial carcinoma cell lines were cultured in the presence of 10 μM BaP, 10 nM TCDD, a non-metabolized AhR ligand, or
0.1% v/v DMSO vehicle control for 48 hours. Cells stained with propidium iodide were analyzed by flow cytometry analysis to analyze alterations in cell cycle phase distribution. Data in Figure 6-1 (A) indicate that control HEC-1A cells treated with DMSO have a large percentage of cells in the G0-G1 phases of the cell cycle (66.7%), with considerably less cells in S phase (20.5%) and G2-M phases (12.9%) after 48 hours. Flow cytometry analysis indicates that BaP-treatment of cells produced a significant 79 percent increase in the percentage of cells in the S phase (36.7%), with a corresponding decrease in the percentage of cells in the G0-G1 phases (51.3%), and no change in the G2-M phase (Figure 6-1A). The observed increase in S phase was not evident in TCDD-treated HEC-1A cell cultures, which appeared identical to controls after 48 hours treatment (Figure 6-1A).

In contrast, the HEC-1B substrain showed no effect of BaP or TCDD treatment on cell cycle phase distribution (Figure 6-1B). HEC-1B cells upon 48 hours treatment are primarily in the G0-G1 phase of the cell cycle (81.4%), with the remainder in S (13.0%) and G2-M (5.5%) phases. The results of BaP and TCDD treatment on HEC-1A and HEC-1B cell cycle phase distributions were repeated twice. Thus, treatment with BaP, but not TCDD, altered cell cycle phase distribution in the direction of an increased S phase in HEC-1A cells, with no corresponding effect on the HEC-1B substrain.

**Time Course of Effects on HEC-1A and HEC-1B Cell Cycle Phase Distribution**

Further studies were carried out to determine the temporal relationship of BaP-mediated effects on HEC-1A and HEC-1B cell cycle phase distribution. Data in Figure 6-2 show the differential effects of BaP on HEC-1A and HEC-1B cell cycle phase over 6
hours, 12 hours, 24 hours, and 48 hours treatment. BaP treatment appears to effect the cell cycle of HEC-1A cells by 12 hours, causing a decrease in the percentage of cells in G2-M and S phase with corresponding increases in the G0-G1 phase. The observed BaP treatment effect on HEC-1A cells reverses between 24 and 48 hours, the later exhibiting the most dramatic deviation from control. In contrast, BaP treatment of HEC-1B cells appears to have little or no effect on cell cycle phase distribution as compared to vehicle control at all time points investigated. Control and BaP-treated HEC-1B cells exhibit parallel shifts in cell cycle phase distribution throughout the 48 hour time period studied.

Effects on CYP1A1 and CYP1B1 Levels

Since both BaP and TCDD have been shown to act through the aryl hydrocarbon (Ah) receptor, initial studies were performed to determine whether there is induction of CYP1A1 and CYP1B1 mRNA in both HEC-1A and HEC-1B cell lines. Northern blot data indicate CYP1A1 mRNA is not present in either HEC-1A or HEC-1B control cell lines. BaP treatment of both HEC-1A and HEC-1B cells results in the induction of a 3.0 kb CYP1A1 mRNA transcript (Figure 6-3A). Further data indicate that both HEC-1A and HEC-1B cells express a constitutive level of a 5.2 kb CYP1B1 mRNA transcript (Figure 6-3B). CYP1B1 mRNA levels appear to be decreased in HEC-1A cells relative to control, whereas HEC-1B CYP1B1 was not shown to be effected by BaP treatment compared to vehicle control (Figure 6-3B).

Experiments investigating the effects of BaP-treatment on CYP1A1 protein levels were performed in HEC-1A and HEC-1B cells upon 48 hour BaP treatment. Western immunoblot analysis indicates the presence of CYP1A1 protein in both HEC-1A and
HEC-1B cultures (Figure 6-4). In contrast, the 55 kDa CYP1A1 protein band is absent in both HEC-1A and HEC-1B control cells (Figure 6-4).

cDNA Microarray Analysis to Profile Changes in Toxicology Gene Expression

Studies were performed to profile transcriptional level changes in mRNA for key enzymatic and cellular adhesion genes between the HEC-1A and HEC-1B cell lines, as well as BaP-mediated effects specific to each cell line using cDNA microarray analysis. The Atlas Human Toxicology 1.2 microarray (Clontech laboratories) was used to identify differentially expressed genes in HEC-1A and HEC-1B cells treated for 48 hours with 10 μM BaP or 0.1% v/v DMSO vehicle control. The array contains 1,176 sequences, each 200 to 600 base pairs in length, which have been amplified from a region of mRNA lacking the poly-A tail, repetitive elements, or other highly homologous sequences. Each spot on the membrane contains a full-length cDNA containing the 3' end of a gene, cross-linked to a positively charged nylon membrane.

Studies determining alternate effects of BaP in HEC-1A and HEC-1B cells were performed using P³²-labelled cDNA probes reverse transcribed from poly A+ enriched mRNA. Analysis of genes was performed using AtlasImage software to define which genes varied between control and BaP-treated samples (Figure 6-5). ScatterPlot analysis was performed on AtlasImage globally-normalized data to represent gene expression around a diagonal line of identity, with genes lying farthest from the line of identity having the greatest variance in gene expression (Figure 6-6). Genes from composite arrays which showed an average fold-change of two-fold or greater between control and treated samples or between the two untreated control cell lines were included in the final
report only if their adjusted intensities after global normalization had a defined ratio (a ratio not equal to zero). However, exclusive consideration of genes with Clontech defined ratios of two-fold or greater may neglect to acknowledge a number of additional genes which are significantly altered and thus should be open for future consideration.

Data from HEC-1A arrays indicate that 6 genes were down regulated in BaP-treated samples, as compared to DMSO controls. No genes were increased in HEC-1A cells upon BaP treatment (Table 6-1). The genes regulated by BaP include heat shock proteins, xenobiotic transporters, stress response proteins, ribosomal proteins, growth factors/cytokines, and cytoskeleton proteins. In contrast, HEC-1B arrays indicate that 4 genes were down-regulated with BaP treatment and 28 genes were up-regulated as compared to control (Tables 6-2 and 6-3). Among the genes down-regulated were a cell surface antigen, a xenobiotic metabolism gene, an extracellular matrix protein, and a DNA damage repair protein (Table 6-2). Increased expression of the following categories of genes were observed upon BaP treatment of HEC-1B cells: basic transcription factors, cell cycle proteins, oncogenes and tumor suppressors, heat shock proteins, extracellular matrix proteins, energy metabolism, chaperones, ribosomal proteins, histones, growth factors, kinase network members, and cytoskeletal proteins (Table 6-3). The largest variation was evident when comparing control HEC-1A and HEC-1B cell lines: 25 genes were higher and 39 genes were lower in HEC-1B, as compared to HEC-1A cell lines. The categories of altered gene expression are listed in Tables 6-4 and 6-5. Comparison of differential gene induction across chemical treatments is shown in the Venn diagram (Figure 6-7).
Cluster and TreeView Analysis of Common Expression Patterns of Genes

The larger variability between HEC-1A and HEC-1B cell lines than between control and treated samples from the same cell line was similarly reflected in a separate analysis. The net intensities from each gene across all three experiments were transformed using variance normalization calculations. Unlike the data described in the previous section, these data were not transformed using AtlasImage 1.5 global normalization prior to analysis. The raw intensity data adjusted for background were normalized separately using Microsoft Excel software and were plotted using ScatterPlot analysis to show the distribution of genes around a diagonal line of identity (Figure 6-8). In contrast to the analysis in Figure 6-6, all genes were included in the final analysis shown in Figure 6-8. The data were then analyzed using Cluster and Treeview software for common expression patterns across three separate experiments and differential chemical treatments.

Results indicate the presence of 28 defined gene clusters, labelled A-AB with a high correlation for similar gene expression (Figure 6-9). In general, it appears that both the HEC-1A and HEC-1B cells showed a decrease in gene induction upon BaP treatment, although the patterns for affected genes were markedly different between the two cell lines. Enlarged views indicating the genes from selected clusters (Clusters B, K, AB, and T) are also shown, along with their correlation coefficients (Figure 6-10).

The accuracy of all cDNA fragments on Clontech microarray membranes were confirmed by the company. Quality control measures utilized by Clontech include the use of gene specific primers for amplification, gel electrophoresis for size verification, PCR screening using gene-specific primers and/or vector specific universal primers,
isolation and sequencing of the candidate clone using universal primers, additional PCR, and re-purification by gel electrophoresis.

**Discussion**

The present study finds that 48 hour exposure to 10 μM BaP results in marked alterations in RL95-2 and HEC-1A cellular attachment, as well as profound alterations in cellular membrane-associated adhesion proteins. Data further indicate that HEC-1B cells fail to show any appreciable response to BaP treatment on the endpoints of cellular attachment and adhesion protein levels or localization. The cell-type specific susceptibility of HEC-1A and RL95-2 cells to BaP-mediated attachment and adherens complex protein effects seem to reflect an intrinsic property shared by the two cell lines which is absent in HEC-1B cells. Since BaP must be metabolized prior to exerting cellular effects, the underlying differences between the cell lines could involve a differential presence or induction of alternate metabolic enzyme pathways involved in primary or secondary BaP metabolism, resulting in a differential activation of BaP.

In previous chapters, RL95-2 cells were shown to have an increased percentage of cells in the S-phase after BaP, but not TCDD treatment. Similar experiments were carried out to determine the effect of BaP exposure on the cell cycle phase distributions of the HEC-1A and HEC-1B substrains. Flow cytometry analysis indicates that BaP produces an enhanced percentage of cells in the S phase in HEC-1A relative to control, but HEC-1B cells remain unaffected. Similarly, TCDD treatment did not alter cell cycle phase distribution in either HEC-1A or HEC-1B cells. The present study confirms published studies indicating a BaP-specific effect on cell cycle progression. It has been
reported that 72 hour exposure of MCF-7 breast cancer cells to 1-5 µM BaP results in enhanced S and G2/M phases of the cell cycle (Jeffy et al., 2000). The BaP-mediated disruption of the cell cycle was reportedly inhibited by co-treatment of the MCF-7 cells with α-naphthoflavone, thereby implicating the AhR in the BaP-mediated growth arrest (Jeffy et al., 2000; Vaziri and Faller, 1997). Current studies likewise confirm published accounts that TCDD treatment does not mediate cellular growth arrest, indicating that ligand activation of the AhR is insufficient to inhibit progression throughout the cell cycle (Vaziri and Faller, 1997). Particular studies in human lymphoblastoid cells indicate that the BaP metabolite BPDE is responsible for BaP-mediated growth arrest (Black et al., 1989). BPDE is formed in BaP-exposed cells in response to induction of CYP1A1 and epoxide hydrolase enzymes.

HEC-1A and HEC-1B cell cycle data further provide evidence of the presence of intrinsic differences between the two cell lines. Reports of HEC-1A and HEC-1B cell lines cultured with leuproleline, a luteinizing hormone-releasing hormone (LHRH) agonist, show a HEC-1A-specific inhibition of cell proliferation and [3H] thymidine incorporation and a slight accumulation of cells in the G0/G1 phase of the cell cycle in leuproleline-exposed cells (Borri et al., 1998). Evidence further indicates that HEC-1B cells fail to demonstrate an inhibition of proliferation, DNA synthesis, or cell cycle distribution upon leuproleline exposure (Borri et al., 1998). Therefore, the HEC-1A and HEC-1B endometrial cancer cell lines serve as models for in vivo responsive or unresponsive endometrial to xenobiotic exposure.

It has been reported that metabolism of substrates by CYP1A1 and CYP1A2 causes reactive oxygen metabolite-mediated oxidative stress (Nebert et al., 2000). BaP
has been shown to activate genes through both the AhR mediated aromatic hydrocarbon response element (AHRE) as well as through the electrophile response element (EPRE) of cells, whereas dioxin is only known to activate genes via the AhRE (Nebert et al., 2000). Further studies were carried out to determine the effects of BaP exposure on HEC-1A and HEC-1B metabolic enzyme levels of CYP1A1 and CYP1B1. Whereas both HEC-1A and HEC-1B cells show a BaP-mediated induction of CYP1A1 mRNA and protein, CYP1B1 mRNA was shown to be constitutively present in both cell lines. Since a variety of xenobiotic metabolism enzymes are known to be polymorphic, including the enzyme CYP1A1, it is possible that genetic polymorphisms between the two cell lines in CYP1A1 or the presence of alternate levels of secondary metabolism enzymes could account for the observed cell cycle and attachment differences. In addition to the wild-type CYP1A1*1A, six additional CYP1A1 alleles have been identified. It has been reported that there is a strong association between the CYP1A1*4 allele and uterine endometrial cancer risk in humans (Schwarz et al., 2001).

In order to more fully explore fundamental differences between the two cell lines prior to, and subsequent to, BaP exposure, cDNA microarray analysis was performed to evaluate global and gene-specific differences in enzyme induction in both HEC-1A and HEC-1B cell lines. Microarray experiments indicate that 6 toxicologically-relevant genes were shown to be down-regulated by BaP in HEC-1A cells (Table 6-1). Surprisingly, however, no genes were shown to be up-regulated by BaP in the HEC-1A cells. The genes down-regulated included genes involved in heat shock, xenobiotic transport, stress response, ribosomal protein, and cytoskeleton and motility. Among the genes down-regulated in HEC-1A cells by BaP were the 27 kDa heat shock protein, which has been
shown to function in thermotolerance and drug resistance, and microsomal glutathione S-transferase mu 1, which catalyzes the reaction of glutathione to electrophilic compounds in detoxification reactions (Table 6-1). In addition, cytoplasmic beta-actin as shown to be down-regulated, indicating possible significance for cellular motility, structure, and integrity.

HEC-1B cells, however were shown to down-regulate 4 genes, whereas they up-regulated 28 genes (Tables 6-2 and 6-3). Among the more prevalent categories of induced genes were oncogenes and tumor suppressors, ribosomal proteins, growth factors and cytokines, and cytoskeleton and cellular motility genes. Included among the down-regulated genes were glutathione S-transferase mu 5, involved in detoxification, as well as soluble galactose-binding lectin 3, which is involved in cellular motility. BaP treatment also resulted in a down-regulation of an x-ray repair gene known to provide efficient repair of DNA single strand breaks from exposure to ionizing radiation and alkylating agents involved in base excision repair. Among the genes increased by BaP treatment in HEC-1B cells were the early growth response protein 1, known to play a role in transcriptional regulation, as well as prothymosin alpha, known to be associated with cellular proliferation. Also increased was the 27-kDa heat shock protein, known to be involved in thermotolerance and drug resistance. The gene for defender against cell death, a negative regulator of programmed cell death, was also increased in HEC-1B cells in response to BaP treatment.

Interestingly, trends in both Tables 6-1 and 6-3 reflect a significant impact of BaP on cytoskeletal genes. Further, similar trends are shown for the genes thymosin beta 10, profilin, and cytoplasmic beta-actin, all of which are known to be coordinately regulated.
in the cells. Table 6-4 compares the base levels of gene expression in control HEC-1A and HEC-1B cells. Data indicate that there is a substantially higher level of the genes thymosin beta 10, profilin, and cytoplasmic beta-actin in the HEC-1A, as compared with the HEC-1B cells, potentially accounting for the altered cytoskeletal structure and the different phenotype of the HEC-1B cells.

Data from present studies differ from published accounts from BaP-exposed mice, indicating the induction of CYP1A1, CYP1B1, and GST-mu1 using array analysis (Bartosiewicz et al., 2001a; Bartosiewicz et al., 2001b). The array studied did not contain the CYP1B1 sequence. In addition, the present array studies did not detect CYP1A1 induction, which was shown by Northern and Western blot analyses. GST-mu1 was shown to be higher in control HEC-1A than HEC-1B cells (Table 6-4), and was shown to be down-regulated in response to BaP treatment in HEC-1A cells (Table 6-1), while HEC-1B cells were unaffected (Tables 6-2 and 6-3). The difference between present results and those reported by Bartosiewicz, et al. may reflect species- and tissue-specific differences in gene expression.

Results indicate that HEC-1A cells have higher levels of 38 toxicologically-relevant genes when compared with the HEC-1B cell line, including oncogenes and tumor suppressors, ribosomal proteins, interleukin and interferon receptors, growth factors & cytokines, DNA damage repair proteins, and cytoskeleton and motility proteins, among various others (Table 6-4). HEC-1B cells, by contrast, have higher levels of 25 genes as compared with HEC-1A cells. Among the genes higher in HEC-1B cells are cell-cell adhesion receptors, oncogenes and tumor suppressors, xenobiotic metabolism genes, hormones, intracellular kinase network members, as well as various others (Table
Interestingly, the enzyme Diaphorase 1, involved in quinone reduction, was shown to be higher in control HEC-1B cells as compared with control HEC-1A cells, and could prove an interesting avenue for future investigations. In addition, the HEC-1A and HEC-1B cell lines were shown to have different levels of various secondary metabolism enzymes prior to treatment, thereby potentially altering the capability of the cells to detoxify BaP upon exposure.

Since the location and timing of gene expression provides key information on gene function, one can gain insight into the functional significance of a particular gene or group of genes by clustering genes according to common expression patterns (Bassett, Jr. et al., 1999). Treeview analysis, involving hierarchical clustering to determine inherent order, followed by graphical representation with the abundance of each transcript represented by color, powerfully depicts the relationships among genes whose tree branch lengths reflect the degree of similarity (Eisen et al., 1998). Treeview thereby allows clustering results to be viewed in a simple graphical display providing an overall image of transcriptional response, without distorting the primary data (Bassett, Jr. et al., 1999). Consequently, patterns in genome-wide expression can provide comprehensive representation of the status of cellular processes (Eisen et al., 1998). Likewise, through consideration of clusters of known genes, relationships of uncharacterized and novel genes can be considered in respect to possible functional categories (Brown and Botstein, 1999; Eisen et al., 1998).

The data herein described reflect the emergence of microarrays as a useful tool for obtaining the molecular fingerprints of gene expression patterns and the generation of biomarkers of exposure for xenobiotic-mediated toxicity. Molecular profiling is a useful
technique of analyzing global gene expression patterns to identify individual genes or groups of genes that mediate a particular cellular effect. There is a multitude of possible patterns of gene expression for cellular expression of chemically-induced toxicity. Since each gene can be either altered or not altered by chemical exposure, for an array of 1176 genes, there are $2^{1176}$ possible patterns of gene expression (Farr and Dunn, 1999). However, evidence is beginning to show that the number of toxicologically relevant patterns of gene expression is quite small compared to the total patterns conceivably possible (Farr and Dunn, 1999). Certain groups of genes, known as damage-specific genes, respond particularly to DNA, protein, cell membrane, and the cytoskeleton damage (Farr and Dunn, 1999). The present data indicate the presence of 28 distinct clusters of genes expressing a high degree of correlation. Future studies confirming the differential gene induction pathways will prove useful in determining the mechanisms for the differential responsiveness of the HEC-1A and HEC-1B cell lines to BaP exposure.

However, despite its many advantages, microarray analysis has several limitations, one being the determination of the extent to which genes are altered in each cell (Farr and Dunn, 1999). Determination of partial versus total gene induction will provide further insight into the mechanism of genetic regulation, in particular by determining whether genes are regulated in an on-off or continuous fashion by chemical treatment. A further limitation of microarray analysis is its inability to determine whether the up- or downregulation of particular genes is the result of a particular phenotypic change, or the cause of the changes. Additional studies using antisense mRNA or function-blocking antibodies can help in the determination of the causative mechanism for observed cellular protein effects. In addition, there is an inherent limitation from
clustering by positive correlation of gene expression. For example, a particular process might activate the expression of one gene while inhibiting another. Therefore, negative correlation should be taken into account in consideration of microarray data as well (Somogyi, 1999).

In conclusion, developing an increasing understanding of the role of cell-cycle checkpoint responses to environmental exposures holds promise in disease prevention, as well as in development of efficacious therapeutic strategies for the treatment of environmentally-linked cancers. It is hoped that such information will help in the assessment of cancer risk for the whole population, as well as subpopulations of individuals with particular genetic susceptibilities to particular genetic mutations. Current studies provide correlational data between the observed genotoxicity of BaP and the induction and repression of key xenobiotic metabolism enzymes in a BaP-responsive (HEC-1A), and a BaP-unresponsive (HEC-1B) cell line. The current study lends support for the use of adhesion, cytoskeletal, and cell cycle biomarkers to characterize the susceptibility of cells to toxic insult and to enhance overall knowledge regarding individual susceptibility to the development and treatment of environmentally-derived uterine endometrial cancer.
Figure 6-1. Differential Effects of BaP and TCDD on Cell Cycle Phase Distribution in HEC-1A and HEC-1B Cell Lines.

Flow cytometry analysis with propidium iodide fluorescence was performed to evaluate the effects of BaP and TCDD on cell cycle phase distribution in HEC-1A and -1B cell lines. A) Treatment of HEC-1A cells for 48 hours with 10 μM BaP results in a nearly two-fold increase in the percentage of cells in the S and G2M phases of the cell cycle as compared to DMSO control, while 10 nM TCDD had no effect. B) Neither BaP nor TCDD showed any effect on HEC-1B cell cycle phase distribution.
A. BaP Effects on G0-G1 Phase in HEC-1A cells

B. BaP Effects on G0-G1 Phase in HEC-1B cells

Figure 6-2. BaP differentially regulates HEC-1A and HEC-1B cell cycle phase distribution over time.

HEC-1A and HEC-1B cells were treated with 10 μM BaP or 0.1% v/v DMSO vehicle control for 6, 12, 24, and 48 hours and then analyzed for alterations in cell cycle phase distribution by flow cytometry with propidium iodide fluorescence.
Figure 6-3. Induction of CYP1A1 and CYP1B1 mRNA by BaP in HEC-1A and HEC-1B cells.

Northern blot analysis was performed to analyze mRNA levels of CYP1A1 and CYP1B1 in HEC-1A and HEC-1B cells upon BaP chemical treatment. Clontech Poly A+ enriched RNA (40 micrograms per lane) was run on an agarose gel, and was probed with cDNA for CYP1A1 and CYP1B1. Results shown indicate representative blots for each gene from two separate experiments.
Figure 6-4. Western Immunoblot analysis of the effects of BaP on CYP1A1 in HEC-1A and HEC-1B cells.
HEC-1A and HEC-1B cells were treated with 10 μM BaP for 48 hours and membrane and whole cell lysate preparations were electrophoresed, transferred to nitrocellulose, and immunostained with antibody to CYP1A1. Results shown indicate a representative blot from three separate experiments.
Isolate mRNA and prepare cDNA probes labeled with $^{33}$P.

Hybridize and Wash gene expression arrays. Detect by phosphorimaging.


Figure 6-5. Illustration representing generalized protocol for cDNA microarray analysis.

As shown, High quality RNA is isolated from differentially treated cells, and cDNA probes are prepared using a gene-specific primer mix. Probes are simultaneously hybridized to separate identical membranes, membranes are washed, and signal is detected by phosphorimaging. Differential gene expression is then analyzed by AtlasImage 1.5, Cluster, and Treeview Software.
Figure 6-6. HEC-1A and HEC-1B Microarray experiment ScatterPlot Analysis.
The Clontech Atlas Human Toxicology 1.2 array with AtlasImage 1.5 software was used to analyze overall intensities of gene expression for the 1,176 genes known to modulate toxic cellular responses. Total RNA was recovered from control and BaP-treated HEC-1A and HEC-1B cells and a cDNA target prepared with incorporation of $^{33}$P. Values for gene expression were obtained for composite arrays from three separate experiments per treatment condition. Intensities for gene expression were globally normalized to all genes on the membrane using Clontech AtlasImage 1.5 software. Adjusted intensities for normalized BaP-treated samples relative to control were analyzed using ScatterPlot analysis for each gene relative to a two-fold or greater change.
Clontech Globally Normalized Intensities in Untreated HEC-1A cells

Clontech Globally Normalized Intensities in BaP-Treated HEC-1A cells (1 μg/ml)

Clontech Globally Normalized Intensities in Untreated HEC-1B cells

Clontech Globally Normalized Intensities in BaP-Treated HEC-1B cells (10 μg/ml)
**TABLE 6-1.** Human toxicology genes down-regulated by benzo(a)pyrene in HEC-1A cells. No genes were up-regulated by benzo(a)pyrene in the HEC-1A cell line.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Access. #</th>
<th>BaP:DMSO</th>
<th>Function</th>
</tr>
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<tbody>
<tr>
<td>27-kDa heat shock protein</td>
<td>X54079</td>
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<td>Heat shock proteins</td>
</tr>
<tr>
<td>Microsomal glutathione S-transferase 1</td>
<td>J03746</td>
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<td>Xenobiotic Transporters</td>
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<td>Glutathione S-transferase mu 1</td>
<td>X68676</td>
<td>0.20</td>
<td>Stress Response Proteins</td>
</tr>
<tr>
<td>60S ribosomal protein L3</td>
<td>X73460</td>
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<td>Ribosomal Proteins</td>
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<tr>
<td>Thymosin beta 10</td>
<td>M92381</td>
<td>0.47</td>
<td>Cytoskeleton &amp; Motility</td>
</tr>
<tr>
<td>Cytoplasmic beta-actin</td>
<td>X00351</td>
<td>0.46</td>
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</tr>
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</table>

**TABLE 6-2.** Human toxicology genes down-regulated by benzo(a)pyrene in HEC-1B cells.

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<th>Access. #</th>
<th>BaP:DMSO</th>
<th>Function</th>
</tr>
</thead>
<tbody>
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<td>Cell surface antigens</td>
</tr>
<tr>
<td>Glutathione S-transferase mu 5</td>
<td>L02321</td>
<td>0.45</td>
<td>Xenobiotic metabolism</td>
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<td>Tenascin</td>
<td>X78565</td>
<td>0.46</td>
<td>Extracellular matrix proteins</td>
</tr>
<tr>
<td>X-ray repair-complem. defect. repair in Chinese hampster cells</td>
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<td>DNA damage repair protein / ligase</td>
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<tr>
<td>Gene Name</td>
<td>Access. #</td>
<td>BaP:DMSO</td>
<td>Function</td>
</tr>
<tr>
<td>--------------------------------------------------------------------------</td>
<td>-----------</td>
<td>----------</td>
<td>----------------------------------------------------------------</td>
</tr>
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<td>Basic transcription factors</td>
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<td>Early growth response protein 1</td>
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<td>Transcription factors and repressors</td>
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<td>Prothymosin alpha</td>
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<td>Oncogenes and tumor suppressors</td>
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TABLE 6-4. Human toxicology genes higher in control HEC-1A cells than HEC-1B cells.

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<th>Function</th>
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<td>Transcription factors &amp; Repressors</td>
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<td>CDK Inhibitors</td>
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<td>Oncogenes &amp; Tumor suppressors</td>
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<td>CBS inhibitors</td>
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<td>Chaperones &amp; Heat shock proteins</td>
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TABLE 6-5. Human toxicology genes higher in control HEC-1B cells than HEC-1A cells.

<table>
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<th>Function</th>
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<td>Carcinoembryonic antigen-related cell adhesion molecule 1</td>
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<td>Oncogenes &amp; Tumor suppressors</td>
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<td>U11292</td>
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<td>Other trafficking &amp; targeting protein</td>
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Figure 6-7. Venn diagram depicting genes regulated by BaP in HEC-1A and HEC-1B cell lines.

Venn diagram showing sets of genes perturbed by at least a factor of two with defined ratios for 10 µM benzo(a)pyrene (BaP) treatment in HEC-1A and HEC-1B cells as determined by cDNA array analysis. The Clontech Atlas Toxicology 1.2 array with Atlas Image 1.5 software was used to analyze overall fold-change alterations in gene expression upon treatment relative to control for composite arrays from three separate experiments per treatment condition. Genes perturbed by sets of two (pair intersections) with BaP in each cell lines are also shown with the total number of genes affected for each treatment given in parentheses. For example, the regulation of 6 genes is affected by BaP in HEC-1A cells, all of which result in a downregulation. None of these genes are similarly regulated by BaP in the HEC-1B cell line.
Figure 6-8. HEC-1A and HEC-1B ScatterPlot for TreeView Analysis.
The Clontech Atlas Human Toxicology 1.2 array with AtlasImage 1.5 software was used to analyze overall intensities of gene expression for the 1,176 genes known to modulate toxic cellular responses. Values for gene expression were obtained for composite arrays from three separate experiments per treatment condition. Intensities for gene expression were globally normalized to all genes on the membrane using Excel software. Adjusted intensities for normalized BaP-treated samples relative to control were analyzed using ScatterPlot analysis for each gene relative to a two-fold or greater change.
Figure 6-9. HEC-1A and HEC-1B cDNA Microarray analysis Treeview Diagram. The Clontech Atlas Human Toxicology 1.2 array with AtlasImage 1.5 software was used to analyze overall intensities of gene expression for the 1,176 genes known to modulate toxic cellular responses. Values for gene expression were obtained for composite arrays from three separate experiments per treatment condition. Raw intensities for gene expression were imported into Excel, from which variance normalizations for the top 50% of genes with the highest standard deviations were plotted in a horizontal hierarchical tree graph, separated into clusters based upon the similarities in gene expression profiles. The genes are shown ordered according to their expression patterns. The abundance of each transcript is shown relative to a reference specific for each experiment, with red representing relative increased expression and green representing relative decreased expression. Overall color saturation reflects the magnitude of difference in expression level of each gene. The rows of the table correspond to each of the 1,176 genes and the columns represent each experimental measurement of transcript levels for each of the genes.
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Figure 6-10. Selected Clusters from the HEC-1A and HEC-1B Microarray TreeView Diagram.

Selected clusters from the TreeView analysis for the Human Toxicology 1.2 array are illustrated. A. Cluster B is comprised of 18 genes with a correlation coefficient of 0.96. B. Cluster K is comprised of 31 genes with a correlation coefficient of 0.97.
Figure 6-10 continued.
C. Cluster AB is comprised of 25 genes with a correlation coefficient of 0.88. B. Cluster T is comprised of 23 genes with a correlation coefficient of 0.86.
CHAPTER 7
CONCLUSIONS AND FUTURE DIRECTIONS

The current studies were undertaken in consideration of the epidemiological findings linking cigarette smoking in women and a decreased incidence of endometrial cancer. The environmental contaminant benzo(a)pyrene (BaP), a major toxicant in cigarette smoke, has been shown to inhibit the growth of human uterine endometrial carcinoma RL95-2 and HEC-1A cell lines in culture. Exposure to the dioxin 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), on the contrary, has been associated with an increased incidence of endometriosis in animal models, and possibly women as well. The objectives of the present studies were to evaluate the usefulness of three human, uterine endometrial cancer cell lines, RL95-2, HEC-1A, and HEC-1B as in vitro models to determine the cellular and molecular mechanisms of BaP and TCDD actions on attachment, adhesion, and cytoskeletal conformation of endometrial cancer cells. Additional studies were conducted to investigate the influence of AhR, cell cycle, and oxidative stress as potential mechanisms in the BaP-mediated loss of cellular attachment.

The overall goal of determining whether BaP causes decreased cellular attachment by decreasing cellular adhesion molecules was tested using human, uterine RL95-2, HEC-1A, and HEC-1B endometrial cancer cell lines as a model system. Initial experiments characterizing the effects of BaP and TCDD treatment on cellular attachment and invasion indicate that BaP produces a significant decrease in RL95-2 and
HEC-1A, yet not HEC-1B, endometrial cancer cell lines. Further, TCDD exposure was shown to have no effect on the attachment of RL95-2, HEC-1A, or HEC-1B cells. The present observation that BaP inhibits attachment and invasion in endometrial cancer cells is consistent with epidemiological findings that cigarette smoking is correlated with a significantly reduced incidence of endometriosis and endometrial cancer (Baron et al., 1990; Baron, 1996; Matorras et al., 1995).

Further studies were performed to characterize BaP effects on cellular adhesion molecule and cytoskeletal protein levels and localization in relation to decreased cellular attachment and invasion. BaP treatment has been shown to cause a loss or significant decrease in plasma membrane expression of several adherens junction proteins in RL95-2 and HEC-1A cell lines, including cadherin, β-catenin, and α-catenin, as well as epidermal growth factor receptor. However, there was no change in expression of any of these proteins in total cellular lysates, implying that the effect was membrane-specific. In contrast, the HEC-1B cell line failed to show a loss of the membrane-associated adhesion proteins, implying a fundamental cellular difference among the cell lines.

The effects of BaP on membrane β-catenin levels are fascinating in light of the diverse functions of β-catenin within the cell. β-catenins are well known to unite the adherens complex with the actin cytoskeleton through α-catenin, acting as prominent players in cellular adhesion. In addition, β-catenins bind to nuclear transcription factors in stimulating gene expression, and have been shown to interact with axin and adenomatous polyposis coli (APC) proteins in the cytoplasm, leading to their degradation (Ben Ze'ev and Geiger, 1998). Further, β-catenin is known to be the target of signal transduction pathways mediated by the src and wnt-1 oncogenes, and to play essential
roles in development (Hinck et al., 1994; Haegel et al., 1995). In addition to β-catenin, studies from deletion mutants of E-cadherin-α-catenin fusion molecules lacking various domains of α-catenin show that α-catenin is essential for cadherin-mediated cellular adhesion (Imamura et al., 1999). BaP-mediated effects on cadherin and α-catenin are of particular interest since a reduction in their expression is frequently associated with an increased metastasis of tumor cells (Ghadimi et al., 1999).

Future studies should determine the resulting down-stream effects of the BaP-mediated alterations on β-catenin utilizing function blocking antibodies or antisense for the binding sites of β-catenin and α-catenin. Furthermore, the BaP-mediated absence or loss of the various cellular adhesion molecules in the plasma membrane could signify a change in compartmentalization of the proteins or a decrease in their formation along with a mechanism of receptor breakdown. If the cellular adhesion and EGF receptors are being compartmentalized by BaP, it is reasonable to assume that they may be stored cytoplasmically under chemical treatment and then restored to the plasma membrane under more favorable conditions. Additional recovery studies removing BaP from the culture media of treated cells and monitoring membrane levels of cellular adhesion proteins over several cell cycles should be performed to determine whether the BaP-mediated protein effects are transient.

A potential pathway for BaP-mediated effects on the cellular membrane could involve alterations in the actin cytoskeleton. BaP treatment was shown to profoundly alter actin structure in RL95-2 cells from subcortical filaments in control cells to subcortical aggregates upon BaP treatment. Alterations in actin are associated with malignant transformation of cultured cells, and occur with a high frequency in naturally
occurring tumors (Rao and Cohen, 1991; Rao et al., 1991). The BaP-mediated alterations on cellular adherens junction and cytoskeletal proteins indicate the likely role of BaP on the decreased cellular attachment of RL95-2 and HEC-1A cells. Due to evidence linking microtubule disruption with an inhibition of cell growth (Aizu-Yokota et al., 1994), future studies should be undertaken to characterize the effects of BaP on cellular tubulin levels and structure in relation to cellular attachment and adhesion complex alterations.

In addition to investigations into differential cellular responsiveness of RL95-2, HEC-1A, and HEC-1B cells to BaP treatment, the key morphological differences among the cell lines were exploited for their predictive values in the determination of molecular and cellular biomarkers for BaP-mediated effects. Studies have shown that α-catenin, a key cell adhesion protein, is expressed significantly less in HEC-1B cells than HEC-1A cells, whereas both cell lines have comparable levels of β-catenin. Consequently, data support the potential role of α-catenin levels and actin conformation on susceptibility of endometrial cancer cells to BaP-mediated attachment alterations. The fundamental morphological differences between the two cell lines are further reflected in actin conformation, with HEC-1A cells exhibiting a thick rim of cortical filamentous actin along intercellular boundaries and HEC-1B cells displaying nuclear-localized actin and exhibit non-continuous cellular boundaries. The striking differences between HEC-1A and HEC-1B substrains make their differential response to BaP treatment particularly intriguing and strengthen their role in the development of useful biomarkers for BaP-mediated effects.

Since initial results indicated that BaP profoundly effects cellular attachment, as well as invasion, studies need to be performed to investigate the effects of BaP on the
α5β1 integrin fibronectin receptor, a membrane-associated protein responsible for cellular attachment to the extracellular matrix. In particular, the α5β1 integrin fibronectin receptor is a key integrin shown to be lower in endometrial glands, as compared with endometriotic glands (Beliard et al., 1997). It has been shown to be reduced in tumor cells, and when overexpressed, α5β1 has been linked with a suppression of cellular growth and tumorigenesis (Plantefaber and Hynes, 1989). Additional studies investigating the role of BaP on altering levels or localization of focal adhesion kinase (FAK) would additionally prove useful.

Further experiments were conducted to evaluate the effects of BaP on the integrity of the adhesion complex. Co-immunoprecipitation experiments using HEC-1A and HEC-1B cell lines indicate that β-catenin is complexed to α-catenin prior to BaP treatment. Upon BaP exposure, both HEC-1A and HEC-1B cells showed an initial trend towards a decrease in co-immunoprecipitated β- and α-catenin protein levels. Additional experiments will need to be completed to determine significance. The lower levels of complexed catenin protein could reflect independent or integrative decreases in either α-catenin or β-catenin proteins, and therefore conclusions cannot be drawn regarding the role of BaP on altering adherens complex integrity. Additional co-immunoprecipitation experiments for E-cadherin with β-catenin, as well as for α-catenin with actin, will provide further insight into BaP-mediated effects on adherens complex integrity.

It has been shown that epithelial cell migration is accompanied by tyrosine phosphorylation of beta-catenin and an increase of its free cytoplasmic pool (Muller et al., 1999). Since enhanced phosphorylation of β-catenin has been shown to account for its decreased association with α-catenin, the effects of BaP treatment on phosphorylation
levels of β-catenin were determined. Results indicate that there was no variation in the overall phosphorylation levels of β-catenin protein in HEC-1A or HEC-1B cells upon BaP treatment. Results are therefore inconclusive on the role of the dissociation of β-catenin and α-catenin proteins in the HEC-1A specific loss of cellular attachment, and evidence does not support the role of β-catenin phosphorylation changes in mediating this effect.

Exciting research in cellular adhesion is currently clarifying the cross-talk between individual adhesion complex components and the cytoskeleton with cell cycle and metabolic endpoints. Studies were conducted to determine the effects of BaP and TCDD on cell cycle phase alterations in all three cell lines by flow cytometric analysis. Results indicate that BaP exposure results in an enhanced percentage of cells in the S and G2-M phases of the cell cycle in both RL95-2 and HEC-1A cells, whereas BaP fails to significantly alter cell cycle phase distribution in HEC-1B cells. Further, TCDD treatment has been shown to have no effect on the cell cycle phase distribution of any of the three cell lines studied. Future investigations into the association between BaP-mediated cell cycle effects and adhesion molecule alterations will prove useful in light of recently published studies. It has been shown that β-catenin specifically functions in altering cell cycle progression and enhancing growth of cells in soft agar, a biomarker for tumorigenicity (Orford et al., 1999). Further, β-catenin has been shown to confer resistance to anoikis and radiation-induced cell cycle arrest in MDCK normal epithelial cells, thereby functioning as an oncogene (Orford et al., 1999).

Additional studies were performed to characterize the molecular mechanisms linking cellular adhesion receptors and the cellular cytoskeleton to their function in signal
transduction. The effects of BaP and TCDD on gene expression in uterine cancer cells were determined through two separate signal transduction pathways, the AhR and oxidative stress. Whereas BaP and TCDD have been reported to activate AhR-mediated genes, studies were performed to determine the role of xenobiotic metabolism enzymes and the induction of an oxidative stress response in cells on the observed altered cellular phenotype. Results have shown that both BaP and TCDD treatment result in an induction of CYP1A1 in all three cell lines. CYP1B1 was induced in RL95-2 cells upon BaP and TCDD treatment, whereas HEC-1A and HEC-1B cells were shown to express a constitutive level of CYP1B1 upon BaP exposure. Protein expression of PGHS-1, involved in the non-AhR-mediated pathway for the biotransformation of BaP, was markedly increased upon BaP, but not TCDD treatment in RL95-2 cells, whereas PGHS-2 protein was absent in all cell lines studied. Future studies should utilize gas chromatography – mass spectroscopy to monitor the differential formation of BaP metabolites in the three cell lines, in particular exploring the role of quinone production. In addition, radiolabelled incorporation studies should be performed to investigate changes in BaP diol epoxide and quinone adduct incorporation in DNA in the three cell lines and data correlated with cellular adhesion effects.

The remarkable effects of BaP on RL95-2 and HEC-1A, but not HEC-1B cellular attachment, adhesion, and cell cycle phase distribution prompted investigations into differences in cellular metabolism. Since BaP has been shown to exert marked cellular alterations through oxidative stress and quinone formation, as well as through the AhR-mediated pathways, subsequent experiments were carried out to compare the major gene induction pathways of BaP with TCDD, a non-metabolized AhR ligand, and t-
butylhydroperoxide, a classic oxidative stress inducer. Results from cDNA microarray technology indicate demonstrated alternate patterns of gene expression for all three chemical exposures. Whereas the genes induced by t-butylhydroperoxide and not BaP were characteristic of a massive oxidative stress response, it can be speculated that BaP produces a low level of oxidative stress in RL95-2 cells which is not characterized by marked gene induction at the 6 hour time point. Further array studies at later time points will prove useful for determining BaP influences on cellular oxidative stress.

A biochemical measure for oxidative stress, the oxidation of dichlorodihydrofluorescein diacetate, was further employed to characterize the xenobiotic effects on intracellular oxidative stress in RL95-2 cells. Results were inconclusive due to the large variability among experimental replicates likely due to cellular detachment during washing. Additional DCF experiments will need to be performed to investigate intracellular oxidative stress, possibly by pre-treating the wells with collagen to enhance attachment of cells to the substrate.

Additional cDNA microarray experiments were carried out in HEC-1A and HEC-1B cells to examine the genetic profiles for the alternate cellular responses to 48 hour BaP treatment. Resulting data were analyzed for fold-change induction of individual genes, as well as for common patterns of gene expression across multiple experiments and chemical treatments. Data demonstrate key xenobiotic metabolism and toxicology genes requiring further investigation for roles in the alternate signal transduction pathways of the two cell lines. The genes of interest demonstrating a two-fold or greater induction will need to be confirmed by RT-PCR analysis prior to the determination of signal transduction pathways effected by BaP exposure.
In addition to determining fold change alterations in gene induction, microarray data was also used in determining patterns of gene expression and resulting functional clusters across the cell lines and treatment conditions. Results from TreeView analysis of HEC-1A and HEC-1B cell lines indicate a tight correlation of gene expression among the 28 indicated clusters. Furthermore, interesting genes were shown to be co-clustered among the experiments described. In the future, BaP-treatment of RL95-2 cells should be explored using the Atlas Human Toxicology 1.2 array, and results compared with those from the HEC cell lines for functional significance.

Since immortal cancer cell lines may differ from their normal cell of origin in unpredictable ways, it is recognized that biological understandings gained from cancer cell lines may not directly apply to primary culture. Additional development of in vitro assays using primary and secondary uterine endometrial cultures should be pursued to provide insight into normal tissue homeostasis. Since BaP is an environmental carcinogen acting on biological systems in the environment in conjunction with other potent endocrine disrupters, additional future studies are needed to determine potential additive, synergistic, and antagonistic effects of BaP with other potent and prevalent environmental chemicals. Studies are also needed on the altered susceptibility to BaP-mediated effects in the population due to genetic polymorphisms in metabolic enzymes, as well as determination of genetic biomarkers which could confer resistance to BaP- and other endocrine disrupter-mediated effects.

Additional future directions for study include molecular profiling in human endometrial cancer patients to determine the effects of cigarette smoke on gene expression patterns in the uterine endometrium. In addition, since there is often a lack of
correlation between mRNA and protein levels, knowledge of protein expression upon xenobiotic exposure will provide necessary information in determining the signaling pathways involved in eliciting the particular cellular responses. The ability to effectively combine RNA and protein expression data to profile transcriptional and post-transcriptional changes in cells holds tremendous promise for future research.

Further studies investigating the role of BaP on estrogen receptor and estrogen metabolism-mediated pathways will prove useful. It has been recently reported that BaP at concentrations greater than 1 µM has been shown to elicit responses comparable to that of 0.1 nM 17β-estradiol (E2) (Charles et al., 2000). This effect was completely inhibited by the ER antagonist ICI 182,780 (ICI), indicating that the responses were ER-mediated, possibly through the generation of the 3-OH metabolite (Charles et al., 2000).

In summary, BaP has been shown to alter a complex array of signal transduction pathways in human endometrial adenocarcinoma cell lines, resulting in profound alterations in cellular adhesion, cytoskeleton, and cell cycle checkpoint responses. The differential response of the various cell lines to BaP treatment serves as a predictive model for biomarkers of effect for xenobiotic exposure on the human uterine endometrium, as well as differential tumor cell response to chemical exposure. The present investigations provide useful information on the complex relationships of cellular adhesion proteins, cytoskeleton, cell cycle, and metabolism on cellular susceptibility to environmental insult. It is hoped that through understanding the mechanisms by which xenobiotics alter cellular adhesion and cell cycle, we may come to better understand the etiology of certain environmentally associated cancers.
Although epidemiological evidence to date suggests a seeming protective role of cigarette smoking in uterine disease prevention, it is essential to realize that such benefits do not outweigh the risks associated with smoking, nor do they provide a rationale for smoking. The data obtained from experiments herein proposed are intended to help clarify mechanisms of disease pathogenesis, and to point to productive treatment or preventative options from elucidating potential mechanisms involved in the enhancement or diminution of endometrial cancer. It is hoped that research findings on the cellular and molecular markers of endometrial cancer altered by benzo(a)pyrene will provide useful information in the selection of effective treatment modalities for women with high-risk early stage tumors and assist in the development of new therapy options for women with recurrent cancer.
Table 7-1. Summary of BaP Effects on Three Endometrial Cancer Cell Lines: RL95-2, HEC-1A, and HEC-1B

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**Cellular Morphology**

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**BaP Effects on Attachment & Adherens Complex Proteins**

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<tr>
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</tr>
<tr>
<td>Cadherin</td>
<td>↓ (total cellular)</td>
<td>Not determined</td>
<td>No effect</td>
</tr>
<tr>
<td>β-catenin</td>
<td>↓ (membrane)</td>
<td>↓ (membrane)</td>
<td>No effect</td>
</tr>
<tr>
<td>α-catenin</td>
<td>Not determined</td>
<td>↓ (total cellular)</td>
<td>No effect</td>
</tr>
<tr>
<td>EGF-R</td>
<td>↓↓ (membrane)</td>
<td>↓ (membrane)</td>
<td>No effect</td>
</tr>
<tr>
<td>Actin</td>
<td>Subcortical aggregate formation</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>Vinculin</td>
<td>No effect</td>
<td>Not determined</td>
<td>No determined</td>
</tr>
</tbody>
</table>
Table 7-1. Continued.

<table>
<thead>
<tr>
<th>RL95-2 Cells</th>
<th>HEC-1A Cells</th>
<th>HEC-1B Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BaP Effects on Protein Association</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-catenin/β-catenin</td>
<td>Not determined</td>
<td>↓ complex</td>
</tr>
<tr>
<td>β-catenin</td>
<td>Not determined</td>
<td>No change</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>Not determined</td>
<td></td>
</tr>
</tbody>
</table>

| **BaP Effects on Cell Cycle** |              |              |
| G0-G1 Phase | ↓ | ↓ |
| S Phase | ↑ | ↑ |
| G2-M Phase | ↑ | ↑ |
| Time course | Not determined | No change | No change |
| 6, 12, 24, 48 h | Not determined | Reversal of BaP effect between 12-24 hours; effect by 48 hours | Similar effects of BaP & control over time |

| **BaP Effects on Metabolic Enzyme Levels** |              |              |
| CYP1A1 mRNA | ↑ | ↑ |
| CYP1A1 Protein | ↑ | ↑ |
| CYP1B1 mRNA | ↑ | ↑ |
| PGHS-1 Protein | ↑↑ | ↑ |
| PGHS-2 Protein | Absent, not induced | Not determined | Not determined |
|              | Present, not induced | Present, not induced | Not determined | Not determined |


BIOGRAPHICAL SKETCH

Michelle Ann McGarry was born on June 8, 1975 in Rockville Centre, Long Island, New York, to Donald Patrick McGarry and Joan Arlene McGarry. She spent her childhood in Boca Raton, Florida where she attended Saint Joan of Arc elementary school and graduated Valedictorian of Pope John Paul II high school in 1993. Upon graduation, Michelle traveled to the Midwest for her college years to attend the University of Notre Dame, where she studied Biological Sciences and completed undergraduate research in organic chemistry under the direction of Dr. Xavier Creary.

Upon graduating from college with the Notre Dame Scholar distinction in May 1997, Michelle entered the Interdisciplinary Program in Biomedical Sciences at the University of Florida College of Medicine. She joined the laboratory of Dr. Kathleen Shiverick in the department of Pharmacology and Therapeutics to explore her interests in human cancer research. She simultaneously gained knowledge of the effects of chemically induced toxicity on human health and carcinogenesis through the Interdisciplinary Toxicology Graduate Training Program. After graduation, Michelle will attend medical school at the University of Florida, where she will apply her research knowledge to caring for patients as a physician-scientist.
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Kathleen T. Shiverick
Professor of Pharmacology and Therapeutics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Stephen M. Roberts
Professor of Pharmacology and Therapeutics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Dietmar W. Siemann
Professor of Pharmacology and Therapeutics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Michael R. Bubb
Associate Professor of Biochemistry and Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Naser Chegini
Professor of Anatomy and Cell Biology
This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 2001

[Signatures]

Dean, College of Medicine

Dean, Graduate School