THE ARYL HYDROCARBON RECEPTOR SIGNAL TRANSDUCTION PATHWAY: MECHANISM AND CONSEQUENCE OF ACTION IN HUMAN MAMMARY EPITHELIAL CELLS

by

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ABSTRACT

Polycyclic aryl hydrocarbons such as benzo[*a*]pyrene (BP) are produced during combustion or pyrolysis of organic matter and are thus ubiquitous in the human environment. BP has been shown to induce mammary carcinomas in laboratory animals and can accumulate in lipid stores such as the mammary fat pad. In the process of converting BP to a more water-soluble and excretable form, highly mutagenic intermediates are formed. BP can enhance expression of the metabolizing enzymes involved in this process by binding to a ligand-activated transcription factor known as the aryl hydrocarbon receptor (AhR). In its native state, cytosolic AhR is found in association with a dimer of 90 kDa heat shock proteins (HSP90) and an immunophilin-like protein. Ligand binding causes loss of accessory proteins and translocation to the nucleus where it heterodimerizes with the Arnt protein. The AhR/Arnt complex modulates gene expression by binding to cognate DNA sequences in the promoter region of Ah-responsive genes.

Two approaches were utilized to determine the effects of BP on human mammary epithelial cells (HMEC). Firstly, spontaneously immortalized nontransformed HMEC were subjected to multiple rounds of BP exposure and *in vitro* transformed clones were isolated based on a loss of contact inhibition and altered morphology. Unlike parental cells, the BP-transformed clones were found to possess chromosomal alterations that frequently occur in human breast cancer, including an isochromosome 8q. Secondly, the mechanism of resistance was investigated in a HMEC tumor cell line selected for growth in BP. AhR mRNA expression was significantly reduced in the BP-resistant cells, which led to decreased AhR protein and DNA-binding activity. Additionally, HSP90 was investigated for its ability to regulate AhR activity *in vivo* in HMEC tumor cell lines.

RÉSUMÉ

Les hydrocarbures polycycliques aromatiques, tel que le benzo[*a*]pyrene (BP) sont produits pendant la combustion des matières organiques et sont par conséquent abondant dans notre environnement. Il a été demontré que BP peut causer des carcinomes mammaires dans des animaux de laboratoire et peut s'accumuler dans les dépôts de matières grasses tels que le tissu adipeux mammaire. Au cours du procédé métabolique qui sert a rendre BP plus hydrophilique et donc plus facile à éliminer, des intermédiaires extrêmement mutagéniques sont formés. BP augmente l'expression des enzymes impliquées dans ce métabolisme. Cette induction est causée par l'activation d'un facteur de transcription ligand induit, connu sous le nom de récepteur d'aryles d'hydrocarbures (Ahr). Dans son état naturel, l'Ahr cytosolique s'associe a un dimère de protéines de stress de 90 kDa (HSP 90) et une protéine ressemblant à l'immunophiline. L'interaction d'un ligand avec ce récepteur entraine la perte des protéines accessoires et le déplacement du complexe ainsi formé vers le noyau, où une autre association avec Arnt se produit. Le complexe Ahr/Arnt modifie l'expression de certains gènes en reconaissant des séquences d'ADN spécifiques dans la région du promoteur des gènes répondant aux aryles d'hydrocarbures.

Deux approches ont été suivies pour étudier les effets de BP sur des cellules humaines mammaires épitheliales. Premièrement, des cellules HMEC non-transformées et spontanément immortalisées ont été assujeties à plusieurs cycles d'exposition au BP. Des clones transformés *in vitro* ont ete isolés selon le phenotype de perte d'inhibition de contact et une morphologie visiblement modifiée. Contrairement aux cellules parentales, les clones isolés apres le traitement au BP ont des alterations chromosomales qu'on retrouve fréquemment dans les cancers mammaires humains, dont un isochrome 8q. Deuxièmement, le méchanisme de résistance au BP a été étudié dans une lignée de HMEC tumorale selectionée au BP. L'expression d'ARNm de Ahr était réduite de façon significative dans les cellules resistantes au BP, et par conséquent, l'expression de la protéine et sa liaison à l'ADN. De plus, la capacite de HSP 90 de réguler l'activité de Ahr *in vivo* dans les cellules tumorales HMEC a été étudiée.

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NOMENCLATURE

Adr^R	Adriamycin-resistant
AhR	Aryl hydrocarbon receptor
AH	Aryl hydrocarbon
AHH	Aryl hydrocarbon hydroxylase
AIP	AhR-interacting protein
Arnt	Ah receptor nuclear translocator
ATP	Adenosine triphosphate
bHLH	Basic helix-loop-helix
BP	Benzo[*a*]pyrene
BPDE	(±)-*anti*-Benzo[*a*]pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide
BP^R	Benzo[*a*]pyrene-resistant
BSA	Bovine serum albumin
CAT	Chloramphenicol acetyltransferase
cDNA	Complementary deoxyribonucleic acid
CYP	Cytochrome P450
DBD	DNA-binding domain
DDE	1,1-Dichloro-2,2-bis(4-chlorophenyl)ethylene
DDT	1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGTA	Egtazic acid
EMSA	Electrophoretic mobility shift assay
ER	Estrogen receptor
ERE	Estrogen responsive element
EROD	Ethoxyresorufin *O*-Deethylase
FGF	Fibroblast growth factor
FKBP	FK506-binding protein
GA	Geldanamycin
GR	Glucocorticoid receptor
GST	Glutathione *S*-transferase
HMEC	Human mammary epithelial cell
HSP	Heat shock protein
IC_{50}	50% Inhibitory concentration

kb	Kilobases
kDa	Kilodalton
K_M	Michaelis constant
LBD	Ligand-binding domain
LOH	Loss of heterozygosity
MCDF	6-Methyl-1,3,8-trichlorodibenzofuran
mEH	Microsomal epoxide hydrolase
MEM	Minimal essential medium
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
NLS	Nuclear localization signal
PAH	Polycyclic aryl hydrocarbon
PAS	Per-Arnt-Sim homology
PBS	Phosphate-buffered saline
PCB	Polychlorinated biphenyls
PCR	Polymerase chain reaction
PKC	Protein kinase C
PR	Progesterone receptor
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RPM	Revolutions per minute
RT-PCR	Reverse transcription-polymerase chain reaction
SCID	Severe combined immune deficient
SDS	Sodium dodecyl sulfate
SHBG	Sex hormone-binding globulin
SSC	Standard saline citrate
SSCP	Single-stranded conformation polymorphism
TAF	Transcriptional activation function
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
UGT	Uridine 5'-diphosphate-glucuronosyltransferase
V_{max}	Maximal velocity (reaction rate)
WT	Wild-type
XRE	Xenobiotic responsive element

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PREFACE

The format of this dissertation is manuscript-based. In accordance with the Guidelines for Thesis Preparation, the thesis contains the following sections: an abstract in English and French, a table of contents, a literature review, three manuscripts, a final conclusion, and a comprehensive reference list. A nomenclature is included at the beginning of Chapter I that defines acronyms used throughout the thesis. Chapter V serves to summarize the major conclusions, make claims to originality, and offer suggestions for further research.

The submitted manuscripts are as follows:

Chapter II	Caruso JA, Emond J and Batist G Evaluation of genetic alterations in human mammary epithelial cell lines transformed *in vitro* with benzo[*a*]pyrene. Accepted pending revisions, *Mutation Research*
Chapter III	Caruso JA and Batist G Divergent mechanisms for loss of Ah-responsiveness in benzo[*a*]pyrene- and adriamycin-resistant MCF-7 cells In press, *Biochemical Pharmacology*
Chapter IV	Caruso JA, Laird DW and Batist G Role of HSP90 in mediating cross-talk between the estrogen receptor and Ah receptor signal transduction pathways. In press, *Biochemical Pharmacology*

Contributions of Authors

In Chapter II, the candidate was responsible for all experiments and the preparation of the manuscripts, with the exception of the cytogenetic analyses which were performed in the laboratory of Dr. Jaqueline Émond at the Montreal General Hospital.

In Chapter III, the candidate was responsible for all experiments and the preparation of the manuscripts.

In Chapter IV, the candidate was responsible for all experiments and the preparation of the manuscripts. Dr. Dale W. Laird provided expertise in confocal microscopy imaging.

Dedicated to my son,

Angelo Joseph Caruso

CHAPTER I

GENERAL INTRODUCTION

1. BREAST CANCER

Excluding skin cancer, breast cancer is the leading type of cancer in Canadian women [1]. Currently, one in nine women can expect to develop breast cancer in her lifetime and one in 23 will die from the disease. Breast cancer is very much a disease of Western countries: rates are nearly six times higher in Canada, the United States, or Northern Europe than in Asia or black Africa. Even more troubling, from 1981-1998 incidence rates for breast cancer in Canada have significantly increased ($p=0.01$) at an average annual rate of 1.5% [2].

1.1 Estrogen

The majority of known risk factors for breast cancer relate to the level of exposure to endogenous and exogenous estrogens. Early age at menarche and late age at menopause are associated with increased risk [3]. Although the rate of breast cancer incidence increases with age, it declines after age 50, the approximate age of natural menopause when ovarian function ceases and secretion of estrogen and progesterone drops. Breast cancer is markedly reduced by oophorectomy at a young age. Obesity, which increases risk in postmenopausal women, is thought to operate through increased estrogen levels: in the postmenopausal period, the major source of estrogen is extraglandular (largely adipose tissue) conversion of the adrenal androgen androstenedione to estrone [4, 5]. A slight increase in risk also occurs with the use of oral contraceptives and hormone replacement therapy [6, 7].

The role of estrogens in breast cancer has only recently been studied directly. In a large cohort study of postmenopausal New York women, Toniolo *et al.* [8] found that those who subsequently developed breast cancer show higher serum levels of estrone, total estradiol, and free estradiol, and a lower percent of

estradiol bound to sex hormone-binding globulin (SHBG) than women who remained free from cancer. Similarly, another report has shown that women in the highest quartile for non-SHBG bound estradiol had a 5.2-fold elevated risk compared to those women in the lowest quartile [9]. In a study designed to examine estrogen receptor alpha (ERα) expression in benign breast epithelium, women who were ER-positive had a 2.5-fold increase risk, after controlling for known breast cancer risk factors, compared to those who were ER-negative [10].

The biologic mechanisms involved are poorly understood, but the theory most often cited is that estrogens initiate carcinogenesis in nontransformed cells by providing a mitogenic stimulus (i.e. by enhancing the opportunity for genetic mishaps) [11]. Another possibility is that estrogen metabolism leads directly or indirectly to the production of carcinogenic products [12]. Although DNA adducts are not associated with estrogen exposure [13], it has been proposed that during the process of estrogen metabolism free radicals are created that can directly damage the phosphodiester bonds of DNA or alter the nucleotide base sequence [14, 15]. These free radicals are thought to arise by cytochrome P450 (CYP)- and CYP reductase-catalyzed redox cycling between catechol estrogen metabolites and their corresponding quinones. In this process semiquinone intermediates may react with molecular oxygen to form superoxide radicals, which are then converted to hydrogen peroxide and further to hydroxyl radicals [16, 17].

Humans are exposed to environmental estrogens in the form of synthetic compounds ("xenoestrogens") and naturally occurring plant estrogens ("phytoestrogens"). Structurally diverse organochlorine environmental contaminants such as polychlorinated biphenyls (PCBs), hydroxylated PCBs, kepone, methoxychlor, DDT, DDE, and related compounds exhibit weak estrogenic activity [18], whereas other compounds, specifically the dioxins and

some PCB congeners, possess antiestrogenic activity. Unlike naturally occurring estrogens which possess a half-life of minutes or hours, organochlorines are resistant to metabolism. Many of these highly lipophilic compounds persist in the body and accumulate in the food chain, and have been linked to reproductive failure in animals living in the wild or the laboratory [19]. Some studies have found associations between serum levels of organochlorine compounds and breast cancer [20-23], but most large studies have not found evidence of an association [24, 25]. In contrast, epidemiological evidence supports the hypothesis that phytoestrogens appear to have some protective effects in humans, including inhibition of breast cancer formation and growth [26].

1.2 Heredity

The proportion of all breast cancer cases that is attributed to hereditary factors is in the range of 5-10% [27, 28]. It has been estimated that 36% of the breast cancer cases diagnosed in women under 30 years of age are the result of an inherited genetic defect, making early age at onset the strongest indicator of hereditary involvement [29]. Genes currently thought to contribute to hereditary breast cancer include *BRCA1*, *BRCA2*, *p53*, *PTEN/MMAC1*, *ATM*, and *HRAS1*. Of these genes, mutations of the *BRCA1* gene have the most impact on breast cancer development [30]. About 1/500 to 1/1000 women carry mutant alleles for this gene, which are implicated in about 4% of breast cancers in all age groups, and close to 25% of those diagnosed before age 40 [31]. Women who carry a deleterious *BRCA1* allele have an 85% chance of developing breast cancer by the age of 80 and more than half of the cases will have occurred before age 50 [32]. *BRCA1* and *BRCA2* are very similar structurally and behave as classic tumor suppressor genes, in that loss of both alleles is required for tumorigenesis. These proteins appear to interact physically with Rad51, a protein involved in the

repair of DNA damage and in meiotic and mitotic recombination. BRCA1 and BRCA2 proteins appear to be involved in the response to DNA damage at several different levels including cell cycle checkpoint activation, induction of apoptosis, and DNA repair [33]. Interestingly, although approximately 90% of hereditary breast cancer involve *BRCA1* and *BRCA2*, neither of these genes have been implicated in sporadic breast cancer.

Familial breast cancer in Li-Fraumeni, Cowden and ataxia telangiectasia syndromes led to the identification of *p53*, *PTEN/MMAC1* and *ATM* genes, respectively, as genes involved in hereditary breast cancer. A genetic locus near the *HRAS1* proto-oncogene, 11p15, has been found to be highly polymorphic. Krontiris *et al.* [34] reported a 2-fold increase in breast cancer risk within the general population with certain 11p15 alleles. While the increased risk is not that large, these alleles are present in about 6% of the population and may, therefore, contribute to a significant fraction of breast cancer cases.

1.3 Environmental factors

Rockhill *et al.* [35] estimated that approximately 25% of breast cancer cases would be prevented in the United States if all white women were to undergo menarche at age 14 years or later, had no genetic or cultural/lifestyle predisposition to disease as reflected in family history of breast cancer, had no benign breast conditions detected by a biopsy, and had their first full-term pregnancy before age 20 years. This conjecture is relevant for two reasons: (i) it highlights the fact that established risk factors are largely unmodifiable, and (ii) it demonstrates that the majority of risk factors remain unknown. Since up to 80% of all cancers are thought to result from environmental causes [36], it is probable that carcinogens play a significant role in breast cancer etiology. Even among the established risk factors, carcinogens may be responsible, at least in part, for

initiating tumors which are proliferation-driven (i.e. hormone-responsive), and for mutation of the normal allele in cases of hereditary breast cancer.

Epidemiological evidence supports the hypothesis that environmental carcinogens contribute to human breast tumorigenesis. For example, the incidence of breast cancer among Japanese and Chinese women living in three United States geographic areas is more than twice that found in their native countries [37, 38]. Native Japanese women who migrate to the United States as young adults have a small increase in their breast cancer rates while living in the United States, whereas Japanese women born in the United States have rates approaching their white counterparts [39, 40]. Since established risk factors cannot account for the increase in breast cancer incidence among migrant women [38], these observations suggest that the risk of breast cancer is related to environmental exposures that are acquired early in life.

Despite epidemiogical studies that implicate environmental factors in the etiology of breast cancer, carcinogens of the breast have not as yet been clearly defined. Two factors that are known to increase the risk of breast cancer are exposure to ionizing radiation and alcohol consumption. Ionizing radiation has been linked to breast cancer from investigations on women subjected to medical procedures for tuberculosis [41] and Hodgkin's disease [42]. Airline cabin attendants are also reported to be at increased risk, which has been attributed to exposure to ionizing radiation during flights [43]. Mammographic equipment has been modified to reduce ionizing radiation exposure, although an additional 3.9 women per 100,000 who have annual two-view mammography from age 50 are expected to acquire breast cancer [44]. Moderate alcohol consumption slightly increases breast cancer risk, with a relative risk of 1.3 for women who consume three or more drinks per day [45]. However, the decrease in risk for cardiovascular disease that is associated with alcoholic beverages precludes a

recommendation against their use [46]. The mechanism by which alcohol is thought to increase cancer incidence is via induction of monooxygenase enzymes which have the potential to activate procarcinogens.

As mentioned above, timing of exposure to carcinogens appears to be an important determinant for mammary tumorigenesis. Studies have shown that radiation exposure in females under 20 years of age imparts a high risk of breast cancer, while exposure among those over 40 years of age show modest increased risk [47, 48]. Similarly, there are studies showing that cigarette smoke may be associated with greater risk among women who began smoking at an early age (reviewed in [49]).

As many as 160 chemicals were found to cause mammary tumors in the United States National Toxicology Program's carcinogenesis bioassay [50]. Two classic mammary tumor initiators in rodents, dimethylbenzanthracene and dimethylnitrosourea, act as mutagens by well established mechanisms [51], yet neither of these compounds occur in the human environment. Morris and Seifter [52] have suggested that aryl hydrocarbons (AHs) have a significant role in the genesis of breast cancer. They based their hypothesis on the following points: (i) humans ingest AHs on a daily basis, (ii) the breast is anatomically embedded in a major fat depot which stores and concentrates lipophilic Ahs, (iii) human mammary epithelial cells have the capacity to metabolize AHs to carcinogenic intermediates, and (iv) AHs have been identified as mammary carcinogens in animal studies [52]. AH compounds, including benzene, benzo[*a*]pyrene (BP), and dibenz[*ah*]anthracene, are formed during combustion or pyrolysis of organic matter. Thus, AHs are abundant in sources such as gasoline engine exhaust, cigarette smoke, urban air, soil, and in foods that are charcoal-broiled or smoked.

In addition to AHs, heterocyclic amines, aromatic amines, and nitro polycyclic amines are three additional classes of environmental agents that are

formed by combustion processes and have tested positive for mammary tumorigenesis in bioassays [53]. Sensitive analytical techniques are now available to monitor protein adducts, DNA adducts, and urinary metabolites of these compounds. However, failure to find associations in humans that reflect the experimental findings may be attributed to the inherent difficulty in characterizing exposure at the time of tumor initiation, which can occur 20 to 40 years before diagnosis.

1.4 Pathology

The human breast comprises structural elements (connective tissue, fat, blood vessels, lymphatic tissue) and functional elements (the mammary gland, composed of lobules and ducts lined by epithelial cells) (reviewed in [54-56]). The tissue organization of the breast is complex, even in the epithelial component where approximately 99% of tumors originate. There are two major epithelial phenotypes in the mammary gland, namely the luminal or secretory cell and the basal or myoepithelial cell. These cell types can be distinguished by immunological markers including expression of keratins 7, 8 and 18, ER, and polymorphic epithelial mucin in luminal cells; and keratins 5 and 14, vimentin, EGF receptor and laminin in basal cells. About 90% of breast tumors express keratins 8 and 18, and polymorphic epithelial mucin, indicating they are luminal in origin, and about 10% show some evidence of basal markers. The latter tumors tend to be more aggressive. Approximately half of all human mammary tumors express ER.

Breast cancer derives from the epithelial lining of the terminal mammary ducts or the glandular-like lobuli [57]. Accordingly, hyperplastic lesions are classified as "ductal" or "lobular".

For colon cancer, a paradigm has been described whereby tumorigenesis has been disseminated histologically into discrete steps [58]. In contrast, an unambiguous stepwise progression for mammary tumorigenesis has not been elucidated. This is largely attributable to the high number of morphologically complex and extremely common proliferative lesions of the breast, few of which are unequivocal precursors of breast cancer. Increasingly, epidemiologists have shown that certain subsets of these proliferative lesions are associated with the later development of breast cancer. Based on this work, breast cancer evolution has been proposed to progress histologically from normal cells, to hyperplasia, to dysplasia, to non-invasive "*in situ*" carcinoma, to primary invasive carcinoma, and finally metastatic carcinoma [59-62]. Each lesion in this model is envisioned as a non-obligatory precursor of the next, in the sense that some cells may pursue a stable natural history while others may progress to the next stage. Regression can occur at any stage, but becomes less likely with progression.

Breast cancer research has been hampered by the difficulty in immortalizing and transforming normal human mammary epithelial cells [63-65]. A likely explanation is that cells usually senesce before they can acquire the multiple changes required for immortalization and transformation. As with attempts to culture breast cells *in vitro*, attempts to grow breast cancers in the nude mouse have met with very limited success [66, 67], and those primary tumors that grow are not usually representative of the common invasive cancers.

1.5 Molecular genetics

Breast cancer accounts for less than 1% of all tumors examined for cytogenetic abnormalities [68]. From the relatively few studies that have described karyotypes of primary breast carcinomas, approximately one third of carcinomas are polyclonal [69, 70], whereas in other cases no cytogenetic

abnormalities are visible [69]. Structural arrangements often associated with monoclonal tumors include additions of chromosome 1q and 8q, losses of 1p, 1q, 3p, 6q, 11p, 11q, 16q, and 17p, and numerical aberrations +7, +18 and +20 [68, 69, 71]. Every human chromosome has been shown to exhibit allelic loss in at least a small fraction of breast tumors. The most frequently reported losses, in descending order, are seen on chromosome 17p, 16q, 17q, 18q, 7q, 8p, 1p, 13q, 3p, 6q and 11p [72-74].

Specific oncogenes and tumor suppressor genes are recurrently altered in human breast carcinomas; these are discussed in the following sections.

1.5.1 *c-Myc*

The *c-myc* proto-oncogene has been implicated in the control of a wide range of normal cellular processes including cell growth [75], differentiation [76], and apoptosis [77]. This gene encodes a *trans*-acting nuclear protein that can induce (e.g. cyclins A, E, and D1, dihydrofolate reductase, *p53*) and repress (e.g. *c-myc*, *c-erbB-2*, MHC class I) the expression of target genes [78]. Estradiol-induced cell proliferation has been associated with the transactivation of *c-myc* [79]. However, there is constitutive and relatively high expression of the *c-myc* gene in ER-negative cells [80]. Thus, overexpression of *c-myc* may be one mechanism by which cells progress to a hormone-independent phenotype. Amplification of *c-myc* has been reported in approximately 15% of over 5000 breast tumors [78], and in about 3-5% of tumors there is a rearranged allele [81]. Genetic alterations at this locus has been associated with aggressive, advanced tumors [82], high proliferative capacity [83], poor prognosis [84], and advanced age [85].

1.5.2 c-ErbB-2

The *c-erbB-2* proto-oncogene product is a member of the receptor tyrosine kinase superfamily that also includes the EGF receptor. Unlike the EGF receptor, however, an endogenous ligand for c-ErbB-2 has not been identified. Amplification and/or overexpression of *c-erbB-2* is found in approximately 20% of primary breast tumors, although the percentage of ductal carcinomas *in situ* with *c-erbB-2* overexpression tends to be much higher (40-50%) [81]. No evidence has yet been found for structural mutations of *c-erbB-2* in human tumors. Tumors positive for c-ErbB-2 overexpression tend to be ER- and progesterone receptor (PR)-negative, associated with high S-phase fraction, and correlated with a greater number of involved lymph nodes [86]. Patients with *c-erbB-2*-overexpressing tumors do not benefit as much from adjuvent therapy [87] and require more aggressive regimes [88].

1.5.3 *Int-2/Bcl-1/Hst-1*

Amplification of markers at chromosome position 11q13 occur in about 13% of 2422 primary breast cancers tested [89]. The *int-2*, *bcl-1*, and *hst-1* oncogenes colocalize in this region and are amplified either singly or in various combinations in breast tumors [90]. The *int-2* and *hst-1* genes encode two fibroblast growth factors (FGF) that are homologous to basic FGF, a protein associated with multiple cellular functions including proliferation, angiogenesis, wound repair and embryonal morphogenesis [91]. The role of Int-2 and Hst-1 in tumor progression is emphasized by the observation that the *bek* and *flg* genes (which code for acidic and basic FGF receptors, respectively) are also frequently amplified in breast cancer [92].

The *bcl-1* gene product is cyclin D1, a protein involved with cell cycle regulation. Overexpression of cyclin D1 may result in accelerated proliferation by

overcoming regulatory constraints normally imposed from its association with phosphorylated retinoblastoma protein. As a prognostic factor, the strongest association of 11q13 amplification is with ER-positive status (the opposite of *c-erbB-2*) [93].

1.5.4 *H-ras*

The family of Ras proteins are responsible for regulating the flow of information that is triggered from activated cell surface receptors. Ras ultimately controls the activity of nuclear transcription factors which function to control the expression of key genes that regulate cell growth and differentiation. The four *ras* genes, *H-ras*, *K-ras4A*, *K-ras4B*, and *N-ras*, can be constitutively activated, and in the process converted to oncogenes, by mutation of codons 12, 13, or 61. In contrast to the *p53* gene, *ras* mutation rates vary quite widely between tumors of different origin. Mutations of *ras* genes have been reported to occur in about 90% of pancreatic tumors [94, 95], 50% of colon and thyroid tumors [96, 97], 30% of lung tumors [98, 99], and only about 5% of breast tumors [100].

1.5.5 *p53*

Mutation of the *p53* gene has been reported in more than 50% of all cancers [101], including breast cancer [102]. Among its known functions, p53 acts as a transcription factor to regulate cell proliferation [103] and apoptosis [104]. Point mutations in the *p53* gene fall predominantly within highly conserved regions [105]. These mutations produce missense proteins with altered transcriptional regulation activities, and often result in high concentrations of p53 protein detectable by immunohistochemistry [106]. Mutant p53 can act in a dominant-negative fashion by binding to and inactivating wild-type p53, while null mutations are presumably recessive at the cellular level [107]. Five studies

have directly correlated *p53* mutations with breast cancer prognosis and most of these studies found relative risks for recurrence or death between 2.2 and 3.3 (reviewed in [108]).

1.5.6 *RB*

Phosphorylation of the retinoblastoma protein on serine and threonine residues oscillates during the cell cycle. The unphosphorylated form of RB predominates in the G_1 phase, and phosphorylated forms appear as cells enter the S phase and persist during G_2 and M phases [109, 110]. Cell cycle regulation by RB is mediated, at least in part, through its interaction with the E2F transcription factor. Phosphorylation of RB initiates release of E2F from RB, allowing E2F to transactivate several growth-related genes including *c-myc*, *c-myb*, DNA polymerase α, ribonucleotide reductase, and thymidylate synthase (reviewed in [111]). Loss of heterozygosity (LOH) for the *RB* gene occurs in approximately 36% of breast tumors examined [112-116]. It appears that alteration of RB is associated with the latter stages of tumor progression: smaller breast tumors (<2 cm) rarely have a loss of RB expression, whereas LOH in tumors greater than 2 cm is frequent [112, 115]. Moreover, Varley *et al.*, [117] have shown that a proportion of tumor cells express RB in all cases where loss of RB expression was found, indicating that loss of RB is likely a progressive event in breast cancer rather than an initiating stimulus.

Other genes that have been identified as potential prognostic markers for breast cancer in some studies, but which have either undetermined or minimal genetic rearrangement, include *nm23* [118], *c-myb* [119], *c-fos* [120], *DCC* [121] and the gene encoding the IGF-1 receptor [122].

2. BENZO[A]PYRENE

BP has been at the forefront of carcinogenesis research for well over a century. Possibly the earliest reference to BP-induced carcinogenicity was the report by Percival Pott in 1775 that several young men who had been exposed to coal soot through their occupation as chimney sweeps developed cancer of the scrotum [123]. In the nineteenth century, high incidence of skin cancer among workers producing paraffin, shale oil and coal tar led to the pursuit of a carcinogenic agent [124]. In 1915 Yamagiwa and Ichikawa [125] were the first to experimentally induce skin tumors by repeatedly painting the ears of rabbits with coal tar, and by 1933 BP had been purified and identified as the active component of coal tar [126].

Although Boyland proposed in 1950 that epoxides were the reactive intermediates of PAHs [127], he and others continued to subscribe to the theory that carcinogenesis by PAHs was mediated by their physical intercalation into DNA. This idea was put to rest in 1968 when it was demonstrated that a PAH could be metabolized to an epoxide [128], and covalent binding of BP to DNA or protein was dependent upon a microsome-dependent metabolism step [129, 130]. In 1973, Bruce Ames and coworkers developed several histidine-requiring (His^-) strains of *Salmonella typhimurium* which underwent back-mutation to a His^+ phenotype when exposed to a chemical mutagen [131, 132]. They realized that bacteria do not duplicate mammalian metabolism in activating carcinogens. To overcome this limitation, they added rat liver homogenates to the culture plates. By this method, Ames was able to show that carcinogenic PAHs, including BP, are only mutagenic in the presence of mammalian metabolizing enzymes [131].

Sims and colleagues [133] in 1974 identified the metabolite, benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE), that bound to DNA (Fig.

1). They accomplished this by synthesizing BPDE and showing that the chromatographic profiles of DNA digests obtained from cells treated with BP matched those of digests of DNA reacted with BPDE. Within the following three years, the absolute stereochemistry of each active intermediate of BP had been determined and their biological activity assayed in animal and bacteria studies [134-138]. These studies showed that activation of BP to its ultimate carcinogenic form occurs in three stereospecific enzymatic steps: (i) oxygenation at the 7,8-double bond of BP by cytochrome P450 enzymes to a 7,8-epoxide, (ii) hydration of the 7,8-epoxide by epoxide hydrolase to a (-)-*trans*-7,8-diol, and (iii) oxygenation at the 9,10-double bond of the (-)-*trans*-7,8-diol by CYPs to yield (+)-*anti*-BPDE and (-)-*syn*-BPDE. The majority of adducts formed in tissues or cells treated with BP are derived from (+)-*anti*-BPDE, the predominant isomer formed metabolically. During this time, it was also shown that (+)-BPDE reacted principally with DNA at the 2-amino group of deoxyguanosine to a 20-fold greater extent than the (-)-enantiomer, and that small and approximately equal amounts of both enantiomers were bound to the exocyclic amino group of deoxyadenosine. However, there was no difference between (+)- and (-)-BPDE in binding to deoxyguanosine when single-stranded DNA was used [139]. These results suggest a highly stereoselective orientation of the (+)-enantiomer of BPDE to chiral centres in double-stranded DNA prior to the covalent binding to deoxyguanosine.

In addition to BPDE, numerous metabolites have been isolated after incubating cells or tissue preparations with BP. The primary metabolites include three epoxide, five phenol, three quinone, one hydroxymethyl and three dihydrodiol derivatives (reviewed in [140]).

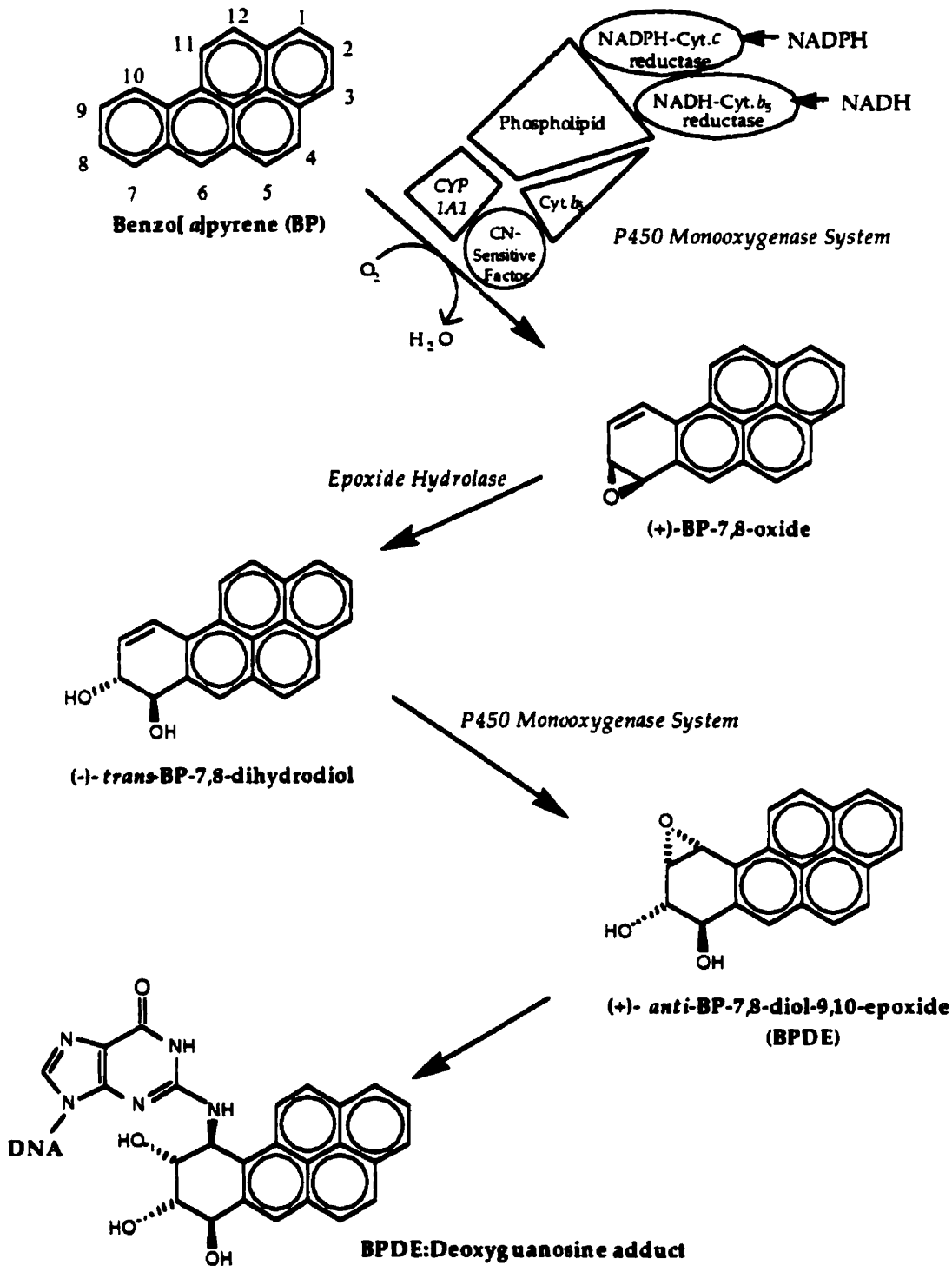

Figure 1. Metabolic pathway of benzo[*a*]pyrene activation and DNA binding.

2.1 Mammary carcinogenesis

As early as 1936, Maisin and Coolen induced mammary tumorigenesis by repeatedly painting the skin of mice with BP [141]. Moreover, Huggins and Yang [142] reported in 1962 that a single feeding of BP led to mammary cancer in eight of nine female Sprague-Dawley rats. More recently, mammary tumors were observed in rats that were administered BP via intramammary injection [143] and by gavage [144, 145]. Compared to other PAHs that are known to induce mammary tumorigenesis in animals [51], BP ranks among the highest for carcinogenic potency [145, 146].

2.2 Human exposure

BP is a polycyclic aryl hydrocarbon that is found in fossil fuels and occurs in products of incomplete combustion and pyrolysis of organic matter. Human exposure to BP is via three routes: inhalation, diet, and through intact skin. Cooked foods are the major source of AH ingestion. For example, a 200 g charcoal-broiled steak contains about 10 μg of BP, smoking 20 cigarettes will lead to the inhalation of 0.4-0.8 μg of BP, daily exposure to ambient air amounts to 9-40 ng BP, while intake from drinking water may be 1 ng per day [147]. Cooking practices dramatically alter the concentration of BP in foods: beef patties that are charcoal broiled have approximately 7 μg/kg of BP, cooking over a soft wood grill increases BP to 42 μg/kg, whereas hard wood grilled patties have 1 μg/kg BP [148]. BP has been identified in various foods including those that are processed or refined (up to 33.5 μg/kg); oils, butter, margarine and fat (up to 62 μg/kg); fruit, vegetables and cereal (up to 48.1 μg/kg); and roasted coffee (up to 16.5 μg/kg) [149].

Emissions from coal burning utilities, home heating units, and automobiles increase the amount of BP in the atmosphere. Thus, concentrations

are higher in the winter than in the summer, and in urban centres compared to rural areas [150]. Although BP occurs in the volatile phase of exhaust fumes, its concentration is much higher in the particulate phase, especially in diesel exhaust [151]. BP has been reported to occur in cigarette, cigar and marijuana smoke, and appears to be more abundant in sidestream smoke rather than mainstream smoke [149, 152].

2.3 Metabolism

To expedite excretion, lipophilic xenobiotic compounds such as BP are converted to more polar and water-soluble derivatives. This is accomplished via "phase I" enzymes which oxygenate the compounds (commonly referred to as 'activation') and "phase II" detoxifying enzymes which conjugate a substrate to electrophilic metabolites.

2.3.1 *Cytochromes P450*

Cytochrome P450 enzymes were named for their carbon monoxide-difference spectrum which contained a major band at an unusually long wavelength (about 450 nm) (reviewed in [153, 154]). Although they are referred to as cytochromes, in many instances they also act as oxygenases in addition to their role as electron carriers. Most of the reactions begin with the transfer of electrons from NADPH, through a reductase, and then to a CYP, leading to a reductive activation of one oxygen atom into the substrate. The reactions that have been demonstrated include hydroxylation, epoxidation, peroxygenation, deamination, desulfuration, and dehalogenation, as well as reduction. The CYP superfamily is composed of ten families in mammals. Some of the CYPs, such as those involved in steroid transformations, have a very narrow range of substrate specificities, whereas other CYPs possess unusually broad and overlapping

substrate specificities. Members of the first three CYP families are principally involved in xenobiotic metabolism.

Several CYPs are known to metabolize BP, and these enzymes show varying affinity for BP and its metabolites. Shou *et al.* [155] examined the metabolism of BP by seven different cDNA-expressed human CYPs. These studies determined that the relative potency of the CYP isoforms to metabolize BP, in descending order, to be 1A1, 1A2, 2C9, 2B6, 3A4, 2E1, and 2C8. Activity of mouse 1A1 is forty times higher than for the human homologue, indicating the potential pitfalls of extrapolating drug metabolism studies across species [155]. 1A2 is a liver-specific enzyme which has not been detected in breast tissue, whereas 1A1 is an inducible CYP found primarily in extrahepatic tissues, including the breast [156-158]. Recently, a second dioxin-inducible CYP1 subfamily member has been identified, 1B1 [159], that is more frequently expressed at the mRNA level in normal human breast tissue than 1A1 [157]. Shimada and his colleagues have investigated the ability of human 1A1 and 1B1 enzymes to activate BP and four of its metabolites [160]. These studies indicated that BP is actively catalyzed by 1A1, but is not a good substrate for 1B1. However, the activity of 1B1 for the (+)-7,8-diol metabolite is 2-fold greater compared to 1A1, whereas 1A1 has a slightly greater affinity for the (-)-7,8-diol enantiomer, a precursor of BPDE. In addition to members of the CYP1 family, immunoblot and RT-PCR techniques have revealed the presence of 2A, 2C, 2D, 3A and 4A subfamilies, as well as the individual forms 2E1, 3A4, 3A5 and 2D6 in normal breast tissue [157, 161].

2.3.2 *Sulfotransferases*

Although BPDE is considered the primary carcinogenic metabolite, other reactive metabolites may also contribute to the carcinogenicity of BP. The

laboratory of James Miller [162, 163] has shown that 6-hydroxymethyl-BP, a product of *S*-adenosyl-*L*-methionine-dependent methylation activity [164], can be activated *in vivo* to an electrophilic and mutagenic sulfuric acid ester metabolite by rat and mouse liver sulfotransferase activity. However, the extent of participation of these enzymes in BP carcinogenic processes is not known. In one study, sulfotransferase activity was detected in about half of the human breast tumor and peritumoral tissues examined [165].

2.3.3 *Epoxide Hydrolases*

The epoxide hydrolases are a group of enzymes which catalyze the conversion of epoxides, frequently produced through the action of CYP-mediated metabolism, to less toxic dihydrodiols. These enzymes potentiate secondary oxidation reactions, leading to highly toxic dihydrodiol-epoxides such as BPDE. Although microsomal epoxide hydrolase (mEH) is the main form of this enzyme, at least three different isoforms are expressed in humans. mEH protein [158] and activity [165] have been detected in breast tumor samples and in adjacent normal breast tissue.

2.3.4 *Glutathione S-transferases*

The glutathione *S*-transferases (GSTs) are multifunctional proteins that have the capacity to catalyze the conjugation of electrophilic toxins with glutathione resulting in more polar and readily excreted metabolites; to sequester toxins through high affinity binding; and to remove toxic peroxides through intrinsic organic peroxidase activity (reviewed in [166]). Nine members of the GST supergene family have been characterized in humans that are classified in four soluble families (α, μ, π, θ) and one membrane-bound form (microsomal).

Since BPDE is a poor substrate for the epoxide hydrolases, the most important mechanism for its detoxification is conjugation with glutathione. Robertson *et al.* [167] examined the catalytic efficiencies of three types of human GST to conjugate glutathione to BPDE. These studied showed that 59%, 60% and 90% of (+)-*anti*-BPDE were conjugated with glutathione in the presence of purified GST α, μ, and π, respectively. Although GST π has a higher catalytic activity toward (+)-*anti*-BPDE, the μ form shows a higher efficiency for conjugating epoxides such as (-)-*anti*-BPDE and BP-4,5-oxide [167, 168].

GSTs are expressed in all human tissues, however, the relative distribution of the various isozymes is tissue- and cell-specific. The α form is found in livers of every adult, whereas GST μ is more variable in expression among individuals, and most extrahepatic organs express class π. The breast is not an exception to this general rule: studies have shown little to no expression of α in breast tumor or peritumoral tissues, expression of μ in roughly 60% of the samples, and consistent expression of π in nearly all samples tested [165, 169].

2.3.5 *UDP-Glucuronosyltransferases*

The uridine 5'-diphosphate-glucuronosyltransferases (UGTs) are a family of enzymes in the endoplasmic reticulum of cells that covalently attach glucuronic acid to a vast array of lipophilic compounds, thereby conferring greater polarity and water-solubility on them and enhancing their excretion in urine or bile. Nine human cDNAs that encode UGTs have been cloned, and are classified into two families, UGT1 and UGT2 [170]. In contrast to the GSTs, BP-epoxides are not substrates for UGTs [171]. Hydroxylated derivatives of BP, however, have been shown to be substrates for UGT types 1*6, 2B1, 2B2 and 2B7 [172, 173], and studies in UGT-deficient rat fibroblasts suggest that UGTs may have a role in BP-induced carcinogenesis [174]. Presently, expression of

individual UGT isoforms have not been characterized in human breast tissue, although UGT activity has been detected in normal breast tissue in at least one study [165].

2.4 Molecular epidemiology

Polymorphisms have been identified in key enzymes associated with BP activation. Some of these allelic differences confer a change in specificity or activity towards BP. In most epidemiological studies, however, either the low number of subjects and/or the low frequency of the deleterious allele have rendered any conclusions to be only suggestive.

2.4.1 *Cytochromes P450*

There are four polymorphisms within the *CYP1A1* gene that have been investigated in relation to breast cancer incidence: *m1*, an *MspI* restriction fragment length polymorphism (RFLP) in the 3'-noncoding region [175]; *m2*, an adenine to guanine transition (codon 462: isoleucine to valine) in the heme-binding domain of exon 7 [176]; *m3*, a *MspI* RFLP found in African-Americans [177]; and *m4*, another amino acid substitution (codon 461: threonine to asparagine) in exon 7 [178]. None of these genotypes, either individually or combined, have been associated with increased breast cancer risk [179-184]. Two studies have found an association in a subset of women who smoke and have the *m1* [184] or the *m2* polymorphisms [180, 184]. Data from Ishibe and coworkers' study of 466 breast cancer cases and 466 matched controls enrolled in the Nurses' Health Study was suggestive that women who initiated smoking before 18 years of age and had the *m2* genotype were at increased risk [184]. This is in agreement with a model proposed by Russo who utilized animal studies to show that there is narrow time frame during mammary development in which the breast is most

susceptible to carcinogenesis [57]. However, the proportion of breast cancers attributable to cigarette smoking at a young age among women with the variant forms of *CYP1A1* was low [184]. Perrson *et al.*[185] showed that are no differences in K_M or V_{max} for CYP1A1-dependent 3-hydroxylation of BP and *O*-dealkylation of ethoxyresorufin between the different *m2* alleles, although the polymorphism could be affecting mRNA or protein stability or may be linked to another genetic locus.

Smoking is also a confounding factor in studies examining the effect of *CYP2E1* polymorphisms on breast cancer risk. Similar to studies above, only women who were classified as smokers and harbored a specific polymorphism (a *DraI* RFLP in intron 6) were found be at increased risk [186]. While there are no clear *in vitro* data showing that the polymorphic alleles affect enzymatic function, the *CYP2E1* intron 6 polymorphism has been associated with altered CYP2E1 protein levels in human liver samples [187] and increased 7-methyl-2-deoxyguanosine adduct levels in human lung [188].

2.4.2 *Glutathione S-transferases*

GSTM1 and GSTP1 detoxify carcinogenic PAHs such as BP, while smaller reactive hydrocarbons, such as ethylene oxide and diepoxybutane, are substrates for GSTT1. GSTM1 enzyme activity is absent in about 45-50% of Caucasian populations [189, 190]. The absence of GSTM1 activity is caused by inheritance of two null alleles. To determine if BP induces less DNA adducts in people with a GSTM1 null genotype, Liu *et al.* [191] incubated human liver cytosols from GSTM1-positive or -negative individuals with calf thymus DNA and BP. BPDE-deoxyguanosine adducts were decreased equally from either low or high conjugators. Based on these experiments, it was proposed that other GST isoforms were compensating for the lack of GSTM1 in null individuals [191]. In

over a thousand breast cancer patients examined, no increased risk has been found for those with a null genotype when compared to a matched control group [180, 182, 183, 189, 192, 193]. One study has suggested that a slight increase in risk may exist for postmenopausal women with the null genotype [193].

Two polymorphic forms of human GSTP1, differing in their primary structure by a single amino acid at position 104, possess different affinities for (+)-*anti*-BPDE: the V_{max} of glutathione-BPDE conjugation was approximately 3.4-fold higher for valine (V104) than for isoleucine (I104) [194]. Although the results are still preliminary, Helzlsouer *et al.* [193] found that postmenopausal women with a V104/V104 genotype may be at increased risk for breast cancer.

2.4.3 *Epoxide hydrolases*

Two point mutations have been described in the human *mEH* gene that affect enzyme activity. The first (codon 113: tyrosine to histidine) reduces enzyme activity by at least 50%, and the second (codon 139: histidine to arginine) produces an enzyme with activity that is increased by at least 25% [195]. Presently, the role of *mEH* polymorphisms in relation to breast cancer risk is not known.

3. Signal Transduction via the Ah Receptor

Halogenated aromatics such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are formed as by-products of numerous industrial processes and have been identified as contaminants in almost every component of the global ecosystem [196, 197]. Unlike PAHs, TCDD is not metabolized and bioaccumulates in the food chain. Epidemiologic studies of accidentally exposed human populations, as well as various animal and cell culture models, indicate

that halogenated aromatics are responsible for species-specific toxic and biochemical responses such as dermal toxicity (including chloracne), hepatotoxicity and porphoryria, immunotoxicity, teratogenicity, carcinogenesis, tissue-specific hypo- and hyperplastic responses, wasting, modulation of endocrine homeostasis, lethality, and induction and inhibition of diverse enzyme activities [198-203]. TCDD is dissimilar to most carcinogens in that it is nongenotoxic: it does not covalently bind DNA, RNA, or protein nor is it mutagenic in the Ames assay [204, 205]. Proposed mechanisms for the carcinogenic effects of TCDD include increased CYP-mediated metabolic activation of endogenous or exogenous compounds, DNA single-strand breaks resulting from lipid peroxidation, and alterations in cell proliferation through transcriptional regulation of cytokines and growth factors [206].

3.1 Receptor activation

Early studies supported the hypothesis that TCDD-induced responses were mediated through a receptor. Firstly, the induction of aryl hydrocarbon hydroxylase (AHH) activity and several other microsomal monooxygenase activities varied in inbred strains of mice [207, 208]. In genetic crosses of Ah-responsive C57BL/6 and Ah-nonresponsive DBA/2 mice, Ah-responsiveness was found to be inherited as a simple autosomal trait. "Nonresponsive" mice are actually less responsive, requiring 10- to 100-fold higher doses of TCDD to attain the same level of enzyme induction as responsive strains [209]. Secondly, the effects of TCDD occurred in a dose-responsive, stereospecific, and tissue-specific manner [209]. Poland and colleagues first identified the Ah receptor (AhR) biochemically by demonstrating that a hepatic protein in C57BL/6 mice had very high affinity for radiolabeled TCDD, whereas liver extracts prepared from DBA/2 mice had much less affinity [209].

It was determined from velocity sedimentation experiments that cytosolic liganded AhR formed a specifically bound peak at about 9-10S [210, 211]. This form of the receptor binds weakly to DNA. At higher temperature or salt concentration the AhR is "transformed" into a 5-6S form which can bind to DNA with high affinity [210, 211]. Two laboratories identified the 90 kDa heat shock protein (HSP90) as a component of the AhR cytosolic complex [212, 213]. One of these groups, Gustafsson and coworkers [212], recognized that characteristics of the AhR closely resembled that of steroid hormone receptors, which were known to be bound to a HSP90 dimer [214]. In their experiments, they showed that molybdate stabilized the AhR in a 9-10S form in a similar fashion as it does for the glucocorticoid receptor, and that an antibody raised against the glucocorticoid-associated HSP90 protein also interacts with the 9-10S AhR complex [212].

Recently, a second protein has been identified in the nontransformed AhR complex that has been termed AhR-interacting protein (AIP) [215, 216]. AIP is a novel cytoplasmic protein that is closely related to FKBP52, an immunophilin that is a component of the multiprotein complex containing unliganded steroid receptor as well as HSP90, HSP70, and p23 [217, 218]. Members of the FKBP family are thought to facilitate protein folding, steroid receptor signaling, heat shock responses, and drug-induced immunosuppression [218, 219]. One significant difference between FKBP52 and AIP is that while FKBP52 has been found to associate directly with HSP90, AIP does not appear to stably interact with HSP90 in the absence of the AhR [216].

Upon stimulation with ligand, AhR translocates to the nucleus where it heterodimerizes with the Ah receptor nuclear translocator (Arnt) protein (Fig. 2). At some point during this process, HSP90 and AIP become dissociated, but it is not certain whether ligand or Arnt initiates their release from the AhR. The

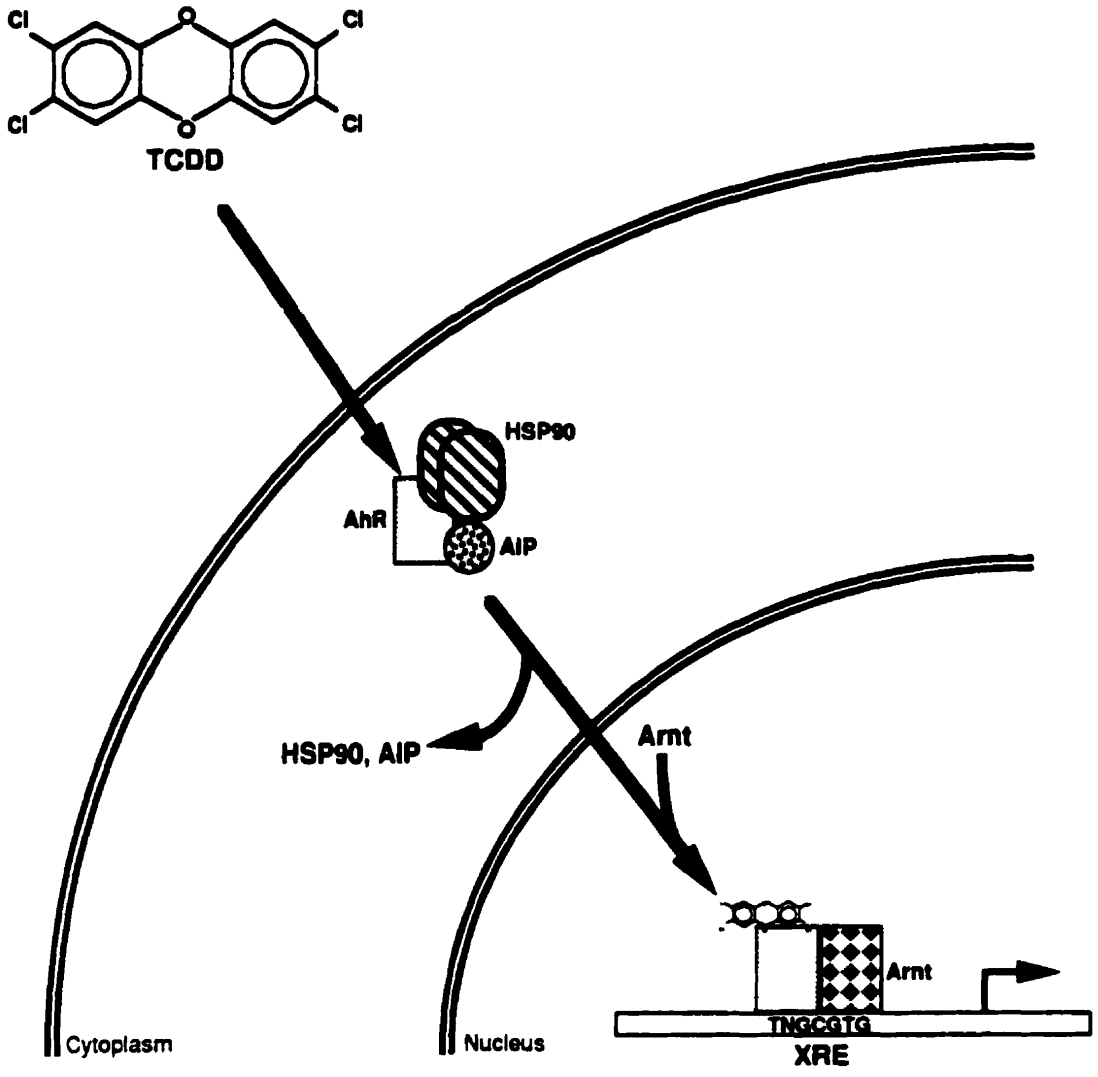

Figure 2. Schematic representation of the Ah receptor (AhR) signal transduction pathway. In its native state, AhR exists in the cytoplasm in a complex with a 90 kDa heat shock protein (HSP90) dimer and the AhR-interacting protein (AIP). Tetrachlorodibenzo-*p*-dioxin (TCDD), the prototypical AhR agonist, can enter the cell by passive diffusion. Binding of TCDD to the AhR initiates a process whereby HSP90 and AIP dissociate and the AhR translocates to the nucleus and heterodimerizes with the Arnt protein. The AhR/Arnt complex transactivates the expression of Ah-responsive genes by binding to *cis*-acting xenobiotic responsive elements (XREs).

cDNA for Arnt was cloned by complementation of a mutant hepatoma cell line that phenotypically appeared to be defective in the translocation of liganded AhR from the cytoplasm to the nucleus [220]. However, the Arnt nomenclature turned out to be misleading: Arnt-defective cells appear to be deficient in translocation because liganded AhR cannot bind DNA in the absence of Arnt. In fact, Arnt resides in the nucleus in the presence or absence of inducer [221].

3.2 Genetic and protein structures of the AhR and Arnt

The *AhR* gene is over 37 kb long and is divided into 11 exons [222, 223]. The human *AhR* (*hAhR*) gene has been mapped to chromosome 7p21 [224], and the *hArnt* gene, which has not yet been fully characterized, is localized on chromosome 1q21 [225]. Primary structures of the murine AhR (*mAhR*) [226, 227] and *hArnt* [220] were first reported in 1992 and 1993, respectively. The 6.3 kb *mAhR* cDNA was found to encode a polypeptide of 805 amino acids. The predicted amino acid sequence of AhR showed a significant similarity to that of Arnt, and carries characteristic domain structures such as basic helix-loop-helix (bHLH) motifs at the amino-terminal, Per-Arnt-Sim (PAS) homology domains, and glutamine-rich domains at the carboxyl-terminal (Fig. 3). The PAS superfamily of regulatory proteins include Per and Sim, two *Drosophila* proteins involved in circadian rhythm and central nervous system development, respectively, and mammalian hypoxia-induced transcription factors HIF-1α and HLF.

3.2.1 PAS region

The PAS region of the AhR, composed of two imperfect inverted repeats of 51 amino acids (PAS-A and PAS-B) separated by 110 amino acids, is functionally associated with ligand- and HSP90-binding processes as well as

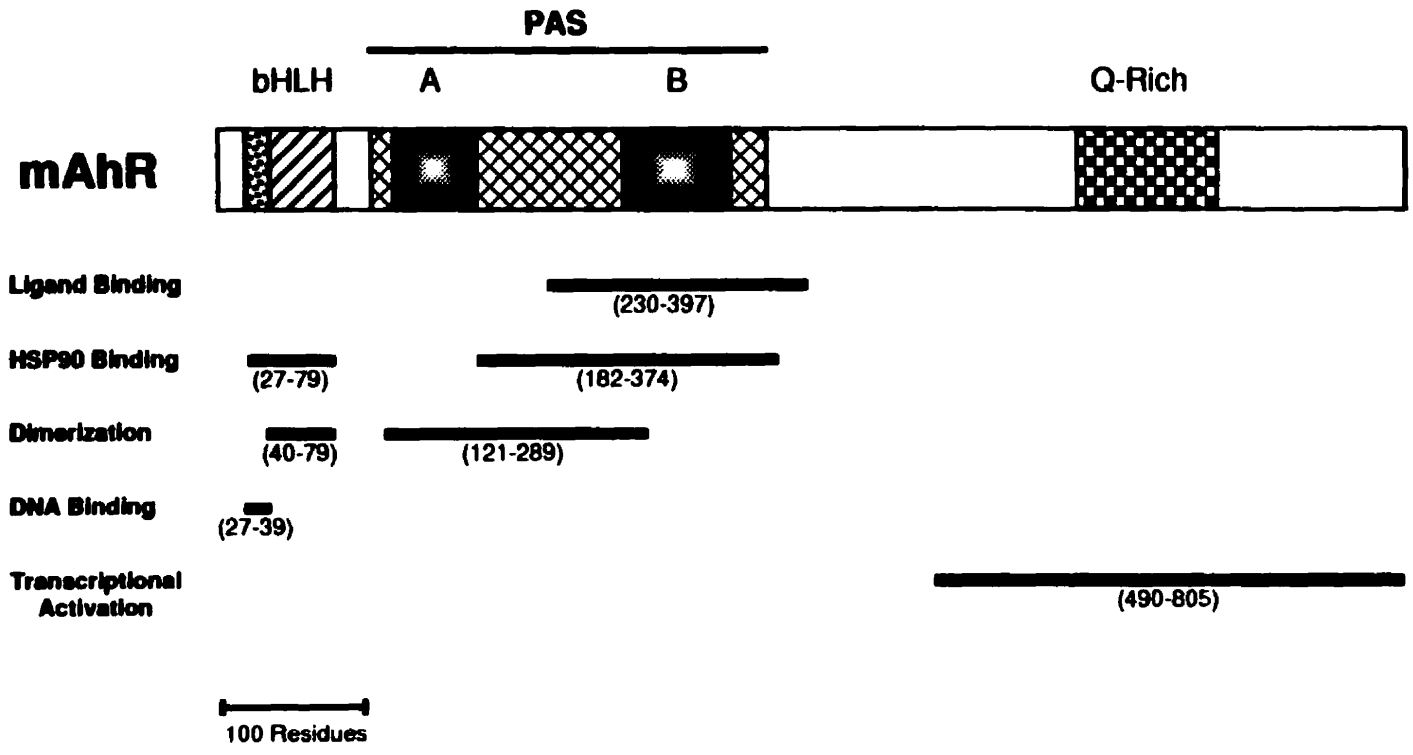

Figure 3. Functional domains of the mouse Ah receptor. Diagram is based on a figure by Fukanaga *et al.* [228].

dimerization. Although Arnt also contains a PAS motif, it does not bind either to ligand or HSP90. For the AhR, deletion of PAS-A was found to reduce ligand binding by 30% compared to full-length AhR, whereas deletion of PAS-B led to a complete inhibition [228]. By similar techniques it was determined that both PAS regions are absolutely required for HSP90 binding [228]. Whitelaw *et al.* [229] replaced the PAS and bHLH domains of AhR and Arnt with a heterologous zinc finger DNA-binding domain. Both of the resultant chimeric proteins were potent and constitutive transcription factors. However, inclusion of the PAS HSP90-binding region of AhR was found to mediate an irreversible *cis*-repressive function on distant transactivation domains. The ability of HSP90 to confer repression from an intact PAS domain was conditional upon the absence of ligand [229].

Based on RT-PCR analysis of the AhR PAS region in living representatives of early vertebrates (jawless, cartilaginous and bony fish), Hahn *et al.* [230] have estimated that the AhR existed early in vertebrate evolution, at least 450-510 million years ago. The conservation of AhR-like sequences in all vertebrate groups suggests that it performs a vital function.

3.2.2 bHLH region

The bHLH motif has been demonstrated to harbor subdomains that have a role in DNA binding (basic region) and dimerization (HLH region) [231]. A feature of many bHLH proteins is the presence of a secondary dimerization surface adjacent to the HLH domain. For example, c-Myc and c-Max proteins contain a leucine zipper, whereas the PAS regions fulfill this role in the AhR and Arnt [228, 232]. The activated AhR/Arnt complex recognizes a conserved DNA sequence known as the xenobiotic responsive element (XRE) that is composed of the asymmetric sequence TNGCGTG, where N could be any nucleotide [233-

236]. This is in contrast to the classical bHLH transcription factors which bind the palindromic E-box sequence CACGTG. Covalent cross-linking analysis and immunoprecipitation with antibodies specific for the AhR or Arnt demonstrated that the AhR binds to the 5' half-site TNGC, and Arnt occupies the 3' GTG E-box-like sequence [233]. Both proteins directly contact the thymine in their respective half-site. Deletion studies on the first and second α-helices indicate that both of these motifs are required for dimerization [231]. Furthermore, deletion of the HLH domain was shown to abolish DNA-binding (most likely as the result of the inability to form dimers) and cause a 50% decrease in HSP90 binding (indicating that both HLH and PAS domains have a role in HSP90-binding) [228].

3.2.3 *Transcriptional activation domains*

Four distinct types of transcriptional activation domains have been identified in transcription factors by deletion analysis and classified according to their primary sequence: acidic, proline-, glutamine-, and serine/threonine-rich [237, 238]. For the AhR and Arnt, transactivation domains were predicted to occur in the carboxyl-terminal domains based on the presence of high concentrations of glutamine residues. Indeed, several studies have shown that the amino-termini of the AhR and Arnt appear to be devoid of transactivational activity, whereas the carboxyl-termini of both AhR and Arnt are capable of transactivation [229, 239-242]. The transactivation characteristics of the AhR and Arnt are dissimilar. For the AhR, transactivation capability is only revealed with uncoupling of HSP90 binding [229]. Rowlands *et al.* [240] subdivided the carboxyl-terminal of AhR into acidic (residues 545-600), glutamine-rich (residues 600-713) and proline-, serine-, threonine-rich (residues 714-848) domains. These regions were found to independently enhance transcription, and function synergistically [240]. In contrast, the transactivation function of Arnt is

constitutive and independent of the glutamine-rich region [243]. Furthermore, there is evidence that the relative strengths of the AhR and Arnt transactivation domains are promoter- and cell type-specific [229, 243].

3.3 The AhR gene battery

In humans, genes transactivated by the transformed AhR complex can be subdivided into genes coding for drug-metabolizing enzymes (*CYP1A1*, *CYP1A2*, *CYP1B1*, *NQO1*, *NQO2*, *ALDH-3c*, *UGT1*6* and *PGHS-2*) and growth/differentiation genes (*PAI-2*, *TGFα* and *IL-1β*) [198, 244]. Another gene regulated by the AhR is rat *GST Ya* [245]. Although it remains to be determined whether the human homologues of this gene, *GSTA1* and *GSTA2*, are up-regulated by AhR agonists, XREs have not been located within 1300 nucleotides upstream of their transcriptional start sites. In one study TCDD was found to induce GST activity in human liver samples, but it was not determined whether the α class GSTs were involved [246].

Members of the AhR gene battery are differentially regulated. For example, CYP1A1 is not normally expressed in the absence of AhR activation, whereas most extrahepatic tissues express low levels of CYP1B1 constitutively [247]. In the presence of TCDD, CYP1B1 levels are greatly induced, however, the level of CYP1A1 induction appears to be regulated by other factors, most notably expression of ER [248]. This may have ramifications for estradiol-mediated carcinogenesis in ER-negative cells due to the efficiency of CYP1B1 in catalyzing the 4-hydroxylation of estradiol (leading to a known carcinogenic metabolite) compared to CYP1A1, which more efficiently metabolizes estradiol to 2-hydroxyestradiol (non-carcinogenic) [249, 250].

Inducibility of a diverse set of phase I and II metabolizing enzymes implies that the AhR signaling pathway has evolved, at least in part, to protect

organisms from exposure to aromatic structures. These compounds have the capacity to accumulate in tissues and act as nonspecific inhibitors of enzymatic reactions and to intercalate into nuclear and mitochondrial DNA [251].

Among the diverse range of effects known to be caused by TCDD, thymic atrophy, chloracne, and immunosuppression are most likely the result of altered regulation of genes related to cell growth and differentiation. In human keratinocytes, plasminogen activator inhibitor 2 (*PAI-2*) and interleukin-1β (*IL-1β*) have been shown to be activated by TCDD [252]. Moreover, the cytokine genes *IL-2*, *IL-6*, *TGF-β* and *TNF-α* are also up-regulated by TCDD [253, 254]. The regulation of the latter genes by AhR has not been fully characterized, although in some instances putative XREs have been located [255], and for TGF-α it appears that TCDD can exert post-transcriptional control over its expression [256]. Control of these secretory proteins indicates that actions of TCDD on one cell type can influence the behaviour distal cell populations via endocrine or paracrine pathways.

3.4 Agonists and antagonists of the AhR

Diverse structural classes of compounds are known to bind the AhR, and these include TCDD and related polyhalogenated aromatics, PAHs, indole-3-carbinol and related hetero-PAHs, phenanthrolines, phenanthridinones, benzocoumarins and various substituted flavinoids [257-262]. Computer modeling based on three-dimensional quantitative structure-activity relationships has been used to predict the binding affinities of AhR ligands [199, 259]. Waller and McKinney used this approach to deduce that AhR ligands must be at least 12.0 Å in length and must not exceed 14.0 Å [263]. Furthermore, the preference of the AhR for planar or near planar molecules dictates that the dimensions of ligands along the depth axis does not exceed 5.0 Å [263].

Systematic structure-activity relationships have been reported for the halogenated aromatics [200, 264], which revealed a rank order correlation between their cytosolic Ah receptor binding affinities and their potencies as AhR agonists for *CYP1A1* induction and other AhR-mediated biochemical and toxic responses.

Since the known ligands of the AhR are mainly environmental contaminants of industrial origin, they are most probably not the natural ligands for this receptor. AhR is able to modulate differentiation processes, including development of the liver, kidney, and palate [265, 266]. Thus, it is possible that the endogenous AhR ligand may represent an unknown morphogen. However, it cannot be excluded that natural ligands of the AhR are xenobiotic. For instance, PAHs from forest fires have been present in the environment throughout the evolution of mammals.

Presently, the only known endogenous ligand of the AhR is bilirubin. In order to be excreted, this heme by-product is conjugated by a bilirubin-specific UGT enzyme, or, to a lesser extent, is oxygenated by CYP1A1/CYP1A2 [267]. Studies have convincingly shown that bilirubin is an AhR agonist since it: (i) induces *CYP1A1* mRNA in wild-type Hepa1c1c7 cells in a time- and dose-dependent manner but not in mutants deficient in either AhR or Arnt expression, (ii) induces EROD and XRE-luciferase activity, (iii) leads to formation of AhR/XRE complexes, and (iv) transforms the AhR to a nuclear complex in cell lines derived from various species [268, 269]. Therefore, like BP this data suggests that bilirubin, like BP, can induce the expression of enzymes involved in its own metabolism. However, since the oxidative metabolism of bilirubin is only a secondary excretion pathway (bilirubin toxicity has not been reported in AhR knockout mice), bilirubin is most likely not the primary AhR agonist.

Alternatively, the natural ligand for the AhR may occur in the diet. It has been demonstrated that indolo[3,2-*b*]carbazole, a heteroaromatic polycycle capable of being produced *in vitro* under acidic conditions from indoles commonly found in cruciferous vegetables (i.e. 3,3'-diindolylmethane and indole-3-carbinol), binds to the AhR with high affinity [270]. The naturally-occurring indoles have been reported to induce AHH activity and to protect against PAH-induced neoplasia [271]. While it is possible that the protective effect of indolo[3,2-*b*]carbazole is a function of increased metabolism of PAHs via AhR activation, it more likely that biological activity of this type may result through competitive antagonism at the AhR ligand-binding site.

Partial support of the latter hypothesis comes from studies in which α-naphthoflavone (7,8-benzoflavone) was determined to be antagonistic to the biological effects of TCDD by directly binding to the AhR. Bioflavinoids and related synthetic analogs exhibit a broad spectrum of biological activity including xenoestrogenic, antimutagenic and anticarcinogenic activities, as well as inhibition of protein kinases, porcine-5-lipoxygenase, ornithine decarboxylase, glutathione reductase, CYP enzymes, and HIV proteinase ([272] and references therein). In various cancer cell lines, α-naphthoflavone was found to inhibit TCDD-induced *CYP1A1* gene expression at concentrations $\leq 10^{-6}$ M and this was paralleled by decreased formation of the nuclear AhR complex [273]. However, α-naphthoflavone is an AhR agonist at a concentration of 10^{-5} M [274]. A related compound, β-naphthoflavone (5,6-benzoflavone), also binds to the AhR and is often used as a prototypical inducer of AhR-mediated *CYP1A1* and *CYP1A2* gene expression.

In addition to α-naphthoflavone and other flavone derivatives [272], compounds that bind to the AhR and have antagonist activity include 1-amino-2,7,8-trichlorodibenzo-*p*-dioxin [275], 6-methyl-1,3,8-trichlorodibenzofuran

(MCDF) [276], and several polychlorinated biphenyl congeners [277]. It is thought that binding of these molecules to the AhR results in a conformational change in the receptor that decreases its affinity for DNA.

3.5 Regulation of AhR activity

Several lines of evidence suggest that AhR signaling is regulated by the phosphorylation status of AhR and Arnt. Treatment with either alkaline phosphatase [278], acid phosphatase [279, 280] or 2-aminopurine [281], an inhibitor of protein kinase activity, inhibits the XRE binding activity of the AhR, but not ligand binding nor formation of the AhR/Arnt heterodimer. Puga and coworkers [281] investigated the ability of nine specific protein kinase inhibitors to inhibit TCDD responses, but only staurosporine, an inhibitor of protein kinase C (PKC), was found to duplicate the results found with 2-aminopurine. The involvement of PKC was verified independently by other groups by using different PKC inhibitors or by pretreatment with phorbol esters, which are strong activators of PKC but lead to a depletion of PKC activity after prolonged treatment [282, 283]. However, Schafer *et al.* [284] have shown that the AhR present in guinea pig hepatic cytosol can be transformed to a DNA binding state by TCDD treatment in the absence of any detectable PKC activity. Therefore, the involvement of PKC in directly phosphorylating the AhR is inconclusive.

Dephosphorylation experiments with the individual receptor subunits prior to assembly of the transformed heterodimer indicated that phosphorylation seemed to be important for the DNA binding activity of the AhR, whereas Arnt appeared to require phosphorylation to interact with the receptor [283]. Gasiewicz and colleagues have mapped the phosphorylation sites of the mAhR to two regions: between residues 368 and 605 (a region which overlaps with the PAS domain); and between residues 636 and 759 at the glutamine-rich carboxyl-

terminal [280]. These regions do not encompass the DNA-binding domain, however, Gasiewicz hypothesizes that the DNA-binding activity of the AhR may be regulated via the phosphorylation status of a DNA-binding repressor domain [280, 285].

3.6 AhR knockout mice

There has been some debate in the past as to whether a portion of the toxic effects elicited by TCDD occur independently of the AhR [286]. These questions have been answered with the creation of mice that are defective in AhR expression. The first knockout mouse to be reported was created by deleting exon 1 of the *AhR* gene in C57BL/6 mice [265]. Almost half of the AhR-null mice died shortly after birth whereas the survivors reached maturity and were fertile. A probable cause for the high rate of mortality is opportunistic infection, since necropsy revealed the presence of lymphocyte infiltration in various organs, most notably the gut, lung and urinary tract [287]. This data indicated that the $AhR^{\Delta 1/\Delta 1}$ mice were immunocompromised, and indeed more detailed analysis showed decreased accumulation of lymphocytes in the spleen and lymph nodes, but not in the thymus. The most surprising changes, however, were found in the liver, which were reduced in size by 50% and showed signs of bile duct fibrosis, eosinophilia of periportal hepatocytes, and glycogen depletion [265]. The deficiencies in immune and hepatic function in this model are suggestive of a physiological role for AhR. In addition, studies on $AhR^{\Delta 1/\Delta 1}$ mice indicate that most of the toxic effects associated with TCDD exposure are dependent upon a functional AhR [288].

A second *AhR* knockout mouse was created by deleting exon 2, which encodes the bHLH domain. Similar to the previous model, Schmidt *et al.* [289] found that the $AhR^{\Delta 2/\Delta 2}$ mouse was unresponsive to TCDD-induced

monooxygenase expression, and possessed a slower rate of growth within the first four weeks of age, a decrease in fertility, and a significant reduction (25%) in liver size. Among the differences were the observations that all $AhR^{\Delta 2/\Delta 2}$ mice were viable, microscopic alterations of the spleen, and an extensive microvesicular fatty metamorphosis of the liver that was suggestive of a metabolic deficit in hepatocyte function [289]. Interestingly, the $AhR^{\Delta 2/\Delta 2}$ mice did not display a decrease in the number of splenocyte and lymphocyte cells, which may explain the lack of neonatal lethality in these mice compared to the $AhR^{\Delta 1/\Delta 1}$ mice.

It is not known at the present time why there are differences between the $AhR^{\Delta 1/\Delta 1}$ and $AhR^{\Delta 2/\Delta 2}$ mice. Presumably, they are complete knockouts since *CYP1A1* expression cannot be induced by TCDD in either model. The effects of genetic background on the phenotype of the knockout mice is probably minimal since both models were derived from C57BL/6 embryonic stem cells. Bradfield [290] has suggested that discrepancies may be due to measurement of different endpoints in different laboratories at different ages; cloning strategies which may have affected the expression of neighbouring genes; and altered housing environments which may have differentially exposed the mice to toxic compounds that cannot be excreted due to the absence of AhR. Finally, the $AhR^{\Delta 1/\Delta 1}$ mice were created by deleting the translational start site, whereas the $AhR^{\Delta 2/\Delta 2}$ mice express *AhR* mRNAs lacking exon 2. Thus, another possibility is that differences between the two models may be the result of functions of the AhR that are independent of its gene transactivation activity.

The hallmarks of low-dose TCDD exposure in experimental animals are induction of monooxygenase enzymes, notably in the liver; immunosuppression; atrophy of the thymus; and, at higher doses, atrophy of the lymphoid organs [291]. Therefore, it was somewhat surprising that AhR-null mice do not possess

morphologic alterations of the thymus. To determine which cell type of the thymus was sensitive to TCDD and whether thymus toxicity was dependent upon the AhR, Staples *et al.* produced chimeric mice with TCDD-responsive ($AhR^{+/+}$) stromal components and TCDD-unresponsive ($AhR^{\Delta 1/\Delta 1}$) hemopoietic components, and the reverse [292]. Results indicated that thymic alterations induced by TCDD are strictly dependent on AhR activation in hemopoietic cells.

The laboratory of Fujii-Kuriyama was the third group to construct an AhR knockout mouse [266]. AhR-null mice were viable, fertile and otherwise apparently normal at birth, but displayed a slightly slower growth rate than wild-type mice for the first few weeks of life. When pregnant mice were administered TCDD by gavage, none of the $AhR^{\Delta 3/\Delta 3}$ mutant fetuses were sensitive to the teratogenic effects of TCDD, although almost all wild-type fetuses suffered from cleft palate and hydronephrosis. These results clearly demonstrated the involvement of the AhR in the malformation of the palate and kidney in mouse embryos caused by TCDD.

Finally, there is evidence that development of the mammary gland is also partially dependent upon the AhR. Hushka *et al.* [293] compared $AhR^{\Delta 2/\Delta 2}$ and $AhR^{+/+}$ littermates and found a 50% reduction in terminal end buds and an increase in the blunt-ended terminal ducts in the mammary glands of AhR-null animals. This observation was further supported by data showing that exposure to 2,3,7,8-tetrachlorodibenzofuran, a potent AhR agonist, led to a greater than 2-fold decrease in lobule number and size [293]. In summary, AhR knockout studies indicate that virtually all the toxic effects associated with TCDD exposure can be linked to AhR expression, and that AhR has a significant role in liver and possibly mammary gland development.

3.7 Tissue-specific expression

In the rat and mouse, *AhR* mRNA expression was found in all tissues examined. By far the greatest expression was observed in the lung, with much less expression in the thymus, kidney, liver, brain, and heart [294, 295]. *Arnt* mRNA expression was usually expressed in a coordinate fashion, although the highest levels were found in the placenta [294]. This trend is conserved in humans: *AhR* and *Arnt* mRNA are expressed in lung > kidney > heart > spleen > liver, but not the pancreas [296].

4. Cross-Talk Between the AhR and ER Signal Transduction Pathways

4.1 Interference

Tobacco smoke, which contains several different PAH compounds that bind to the AhR, has antiestrogenic effects that manifest in a variety of biological parameters such as early onset of menopause, increased risk of postmenopausal osteoporosis and, among women taking oral estrogens, lower circulating estrogen levels [297, 298]. In addition, smoking is inversely correlated with endometrial cancer risk [299] and possibly breast cancer [300]. TCDD is responsible for species-specific toxic syndromes including wasting, immunosuppression, teratogenesis, hyperkeratosis, and chloracne, and is a hepatocarcinogen in bioassays [198]. In other tissues TCDD can have protective effects: long term feeding of low levels of TCDD resulted in a significant decrease in spontaneous mammary and uterine tumors in female rats [301]. Since these tumors are hormone-dependent, this led to the possibility that ligands for the AhR may be antiestrogenic.

The initial experiments which directly implicated TCDD as an antiestrogenic agent showed that estrogen-induced uterine growth could be

blocked in mice and rats by cotreating the animals with TCDD [302-304]. Subsequently, numerous studies have shown that AhR agonists induce a broad range of antiestrogenic activities in animal models including suppression of estrogen-induced peroxidase activity, EGF receptor binding and mRNA levels, *c-fos* proto-oncogene mRNA levels, and cytosolic and nuclear PR binding (reviewed in [305]); and in human breast cancer cell lines TCDD and related compounds inhibit estrogen-induced cell proliferation [306, 307], postconfluent foci formation [308], glucose metabolism [309], secretion of procathepsin D, cathepsin D, and pS2 proteins [306, 310], tissue plasminogen activator activity [311], and PR mRNA and protein expression and binding [312]. It is known that both the AhR and ER are directly involved in the antiestrogenic activity of TCDD from studies showing the absence of an effect in cells which lack either receptor [311, 313], and from structure-activity relationships which show that agonists with a high affinity for the AhR possess greater antiestrogenic activity compared to those congeners with less affinity [307, 314, 315]. The interference between the AhR and ER signal transduction pathways occurs despite the lack of affinity between TCDD and the ER, or between estrogens and the AhR [303]. Furthermore, DNA binding experiments indicate that the ER does not bind to the XRE, nor does the AhR have any affinity for the estrogen responsive element (ERE) [316].

One of the earliest findings was that TCDD decreases ER protein levels by about 50-75% in *in vivo* [303, 317] and *in vitro* [307, 313, 315] models, thereby suggesting a possible mechanism of action for the antiestrogenic nature of TCDD. An alternate mechanism was put forth by Gierthy, who proposed that the antiestrogenic effect of TCDD was the result of enhanced metabolism of estrogen [311, 318-322]. This hypothesis was based on work showing that estrogen metabolism was only minimal in untreated MCF-7 cells, whereas treatment with

TCDD led to a greater than 10-fold increase in the rate of NADPH-dependent microsomal hydroxylation of 17β-estradiol at the C-2, -4, -6α, and -15α positions, resulting in depletion of the hormone from the culture medium. Additionally, exposure to compounds that inhibit CYP1A enzymes was reported to inhibit TCDD-induced estrogen metabolism in MCF-7 cells [320].

However, several studies from the laboratory of Stephen Safe produced results that were contrary to this hypothesis and suggested that TCDD induces an antiestrogenic effect by decreasing ER protein levels. First, MCDF was shown to be active as an antiestrogen at dose levels which caused only minimal induction of AHH activity [314]. Second, exposure of cells to TCDD at concentrations which do not induce monooxygenase enzymes led to decreased ER levels [315]. Third, TCDD-mediated decrease in ER was reported to occur within 1.5 h [313], whereas only minimum induction of AHH activity occurs within this time frame [323]. Fourth, TCDD caused a decrease of uterine and hepatic ER in CD-1 mice which was not accompanied by changes in serum estrogen levels [317].

White *et al.* [324] proposed that the activated AhR complex decreases ER protein levels by acting as a repressor of *ER* gene transcription. They based their hypothesis on the presence of a XRE-like sequence in the 5' flanking region of the human ER gene. They showed that TCDD-activated nuclear extracts from mouse and human cell lines specifically bound to the putative XRE, and that this binding could be eliminated via competition with a XRE-containing oligonucleotide complementary to the mouse *CYP1A1* gene [324]. Subsequently, Safe showed that TCDD does not significantly affect steady state *ER* mRNA levels or the rate of *ER* gene transcription in MCF-7 cells [325], data which suggests that the TCDD-mediated decrease in ER protein expression occurs at the translational or post-translational level.

The hypothesis that TCDD exerts antiestrogenic effects primarily by decreasing cellular ER levels has also been challenged. Recently, two reports demonstrate that TCDD is strongly antiestrogenic in cells in which the levels of ER cannot be down-regulated due to constitutive expression from an expression vector [316, 326]. Therefore, these observations suggest that TCDD acts as an antiestrogen independently of changes in ER expression.

Thus far, the antiestrogenic mechanism of action is understood for only one estrogen-responsive gene. Treatment of MCF-7 cells with 17β-estradiol results in transactivation of cathepsin D gene expression and increased intra- and extracellular levels of procathepsin D and cathepsin D. An imperfect ERE half-site and a Sp1 binding site were identified in the cathepsin D promoter, and subsequent studies with wild-type and mutant ER/Sp1 oligonucleotides in electrophoretic mobility shift and transient transfection assays showed that formation of an ER-Sp1 complex was required for the estrogen-induced response [327]. Krishnan *et al.* [328] found that an imperfect XRE was located in close vicinity to the ERE/Sp1 site. In addition, they showed that estrogen-induced CAT activity was inhibited by TCDD when the wild-type ERE/Sp1/XRE promoter sequence was inserted behind the CAT gene and transfected into MCF-7 cells, whereas no inhibition was observed when the XRE was mutated. Therefore, in this instance the activated AhR complex was found to act as a negative regulator of transcription.

Kharat and Saatcioglu [316] have proposed that the antiestrogenic effects of TCDD are mediated by direct transcriptional interference with the liganded ER. They based their conclusions on studies showing that cotreatment of MCF-7 cells with TCDD and 17β-estradiol for one hour abrogated the binding of nuclear extracts to an oligonucleotide containing an internal ERE, and reciprocally, inhibited the binding of the activated AhR complex to a XRE oligonucleotide.

These observations suggest the mechanism of action does not involve reduction of ER levels, estrogen metabolism, or transactivation of an inhibitor. Soon thereafter, however, Safe reported that these results could not be repeated in his laboratory using the same cell lines [329].

Apart from the AhR and ER signaling pathways, there are numerous examples of receptor interference. For example, AhR has been shown to engage in cross-talk with the glucocorticoid receptor [330, 331] and the retinoic acid receptors [332-335], and cross-talk occurs amongst steroid hormone receptors [336, 337], and between steroid hormone receptors and thyroid/retinoid receptor family members [338-340]. Thus, explication of the interference between the AhR and ER may uncover a mechanism by which other receptor signal transduction pathways overlap with one another.

4.2 Cooperation

Expression of the AhR is not a sufficient indicator for Ah-responsiveness in human breast cancer cell lines. Vickers *et al.* [341] have suggested that Ah-responsiveness is dependent upon the ER content of the cell. For example, MCF-7 cells have high ER expression and AHH activity, T47-D and ZR-75B cells are moderate for both parameters, whereas MDA-MB-231 and HS578T cells have undetectable levels of ER and AHH activity. Moreover, MCF-7 cells selected for resistance to adriamycin (Adr^R) were found to have a loss of ER expression and Ah-responsiveness [342]. The correlation between ER expression and Ah-responsiveness is not universal, however, since AhR signaling was subsequently found to be functional in ER-negative MCF-10A and MDA-MB-468 cell lines [343, 344].

Treatment of ER-negative MDA-MB-231 cells with TCDD was shown to result in increased formation of the transformed AhR complex, which bound to a

XRE oligonucleotide in electrophoretic mobility shift assays [345-347]. Paradoxically, TCDD does not induce *CYP1A1* gene expression in this cell line, indicating a failure at the level of AhR-mediated transcriptional activation. Thomsen *et al.* [347] showed that the Ah-nonresponsive phenotype of MDA-MB-231 cells could be reversed by introduction of ER. In transient cotransfection studies involving a TCDD-responsive reporter vector, hER expression led to a 10-fold increase in CAT activity, whereas transient expression of PR and Jun nuclear proteins did not restore Ah-responsiveness in this cell line.

Investigation into cross-talk between the AhR and ER signal transduction pathways has been an interesting and often times controversial area of research. Presently, the precise mechanisms involved have yet to be resolved.

5. The 90 kDa Heat Shock Protein Family

HSP90 homologues have been cloned from diverse organisms including mammals, chickens, fruit flies, yeast and bacteria (reviewed in [348-350]). Member proteins from even the most distantly related eukaryotic species possess 50% amino acid identity, and all have greater than 40% identity with the *Escherichia coli* HSP90 protein. Proteins of the HSP90 family are abundant at normal temperatures, accounting for 1-2% of cytosolic protein, and are further induced by heat. Very little HSP90 is detectable in the nucleus. However, heat shock induces a reversible time-dependent nuclear translocation, reaching a plateau 20 hours after nuclear HSP90 levels have doubled [351]. In higher eukaryotes, HSP90 exists *in vivo* as a dimer of α and/or β subunits that are produced in equal amounts. Moreover, higher eukaryotes have a distinct form of HSP90 (GRP94/GP96) localized in the endoplasmic reticulum, where it is

involved in the assembly of immunoglobulins and other proteins destined for secretion or surface presentation.

It is widely thought that HSP90 proteins are essential for the viability of eukaryotic cells. This is based on studies showing that several critical proteins related to cell morphology and cell cycle regulation interact with HSP90; HSP90 is expressed in every eukaryotic cell type examined thus far; and yeast are unable to survive when both of their HSP90 homologues are knocked out [352]. Under physiological conditions, HSP90 has been found associated with a wide range of proteins including tyrosine kinases ($pp60^{src}$, Sevenless, ErbB-2, EGF, Yes, Fps, Fes, Fgr, Wee1 and Abl), serine/threonine kinases (Raf-1, members of the mitogen-activated protein (MAP) family, eIF-2α, eEF-2, casein kinase II, yeast PKC and Cdk4), helix-loop-helix transcription factors (AhR, MyoD1 and E47), tumor suppressor genes (RB and p53), cytoskeletal proteins (actin and tubulin), steroid receptors (glucocorticoid, estrogen, progesterone, mineralocorticoid) and other signaling proteins (G-proteins and calmodulin) [348-350, 353-355].

5.1 Chaperone functions

During protein synthesis, intermediates are prone to aggregation. To overcome this problem, molecular chaperones bind to and stabilize nascent polypeptides, thereby preventing the occurrence of inappropriate inter- and intramolecular reactions. All cells possess a large complement of molecular chaperones to carry out these functions, but the precise role of most chaperones are not known. Nathan *et al.* [356] examined the ability of HSP90 to prevent aggregation of newly synthesized proteins in a *Saccharomyces cerevisiae* strain harboring a temperature-sensitive HSP90 mutant. No increase in aggregation was observed among proteins synthesized in HSP90-deficient cells. Although a general role for HSP90 has not been firmly established, the chaperone functions

of HSP90 are most likely restricted to the maturation and maintenance of associated proteins. This is based on studies showing that proteins that are complexed with HSP90 not only have difficulty achieving a stable conformation, but also require continual association with HSP90 to maintain an active conformation (e.g. [357-361]).

HSP90 is functionally associated in multiprotein complexes. Initially, client proteins are brought to HSP90 in a complex involving the ATPase chaperone HSP70 and cochaperones Ydj1 [362] and Hip [363], which interact with HSP90 via p60 [364]. These accessory proteins are subsequently replaced in the HSP90-client complex by an immunophilin (e.g. FKBP52 or Cyp40), when the client is a steroid receptor [218], or p50 when the client is a protein kinase [365]. p60, p50 and the various immunophilins compete for binding to HSP90 and are presumed to interact with a common site [366]. The final step of conformational maturation requires the acidic p23 protein, whose binding to HSP90 complexes appears to be ATP-dependent [367]. In contrast to the related chaperone HSP70, HSP90 does not exhibit detectable ATPase activity. However, crystallization [353] and biochemical studies [368] indicate that HSP90 does bind ATP, albeit weakly.

5.2 Intracellular trafficking

HSP90 is thought to have a role in the intracellular trafficking of proteins. One mechanism by which HSP90 facilitates the cytoplasmic localization of steroid receptors is by masking *cis*-acting nuclear localization signals (NLSs). For example, binding of ligand to the cytoplasmic glucocorticoid receptor (GR) causes release of HSP90, accessibility of its NLS, and transport to the nucleus [369]. Conversely, the NLS of the PR is fully accessible, and this receptor is exclusively localized in the nucleus.

However, the NLS is not the sole determinant of subcellular localization for steroid receptors. Kang [370] targeted chicken HSP90 to the nucleus by fusing it to the nucleoplasmin NLS. When expressed individually in COS-7 cells, expression of HSP90-NLS was localized to the nucleus, and GR or PR mutants lacking a NLS were distributed in the cytoplasm. However, when the receptor mutants were coexpressed with HSP90-NLS, the receptors localized entirely to the nucleus [370]. These studies indicate that HSP90 is capable of sequestering associated proteins.

In another approach, Yang & DeFranco [371] introduced molybdate into cells via a liposome-mediated delivery system. Molybdate, which is known to stabilize receptor-HSP90 complexes *in vivo*, inhibited hormone-dependent nuclear import of the GR. In cells chronically exposed to hormone, molybdate trapped both GR and PR in the cytoplasm, suggesting that receptors can export from nuclei but cannot be reimported into the nuclei when in stable association with HSP90 [371]. These observations suggest that association of receptors with HSP90 must be dynamic in order for nuclear trafficking to proceed.

The naturally occurring antibiotic geldanamycin (GA) was found to show potent activity in the National Cancer Institute's (United States) *in vitro* screen for antitumor agents, achieving 50% growth inhibition at concentrations as low as 13 nM against the most responsive cell lines [372]. GA's target has been identified as HSP90 [373]. Crystallization studies indicate that the GA-binding domain occurs within residues 9-232 of HSP90, a region which is highly conserved across species [354]. GA treatment has been shown to inhibit steroid-dependent GR translocation from the cytoplasm to the nucleus [374]. Furthermore, GA disruption of the Raf-1-HSP90 complex interferes with trafficking of the newly synthesized Raf-1 from cytosol to the plasma membrane [375].

5.3 Geldanamycin studies

Benzoquinone ansomycin compounds such as GA and herbimycin were found to revert transformation by tyrosine kinase oncogenes such as *src*, *yes*, *fps*, *abl* and *c-erbB-2* [376, 377]. One of the most pronounced effects elicited by GA is the rapid turnover of HSP90-associated proteins [373, 378-381]. In HeLa and Hepa 1c1c7 cells, AhR was depleted to 75% of normal levels within one hour exposure to GA [355]. Thus, a common consequence of persistent association with HSP90 appears to be stabilization of the bound protein.

Hormone binding abilities of receptors for glucocorticoid, progesterone, androgen, and estrogen were found to be inhibited upon exposing cells to GA, suggesting that HSP90 plays the same role for hormone binding throughout the entire class of steroid hormone receptors [378]. Inhibition was only observed when GA was applied to cell cultures under growth conditions or was present during *in vitro* synthesis; presynthesized receptors in cell extracts were not affected. Upon withdrawal of GA, glucocorticoid binding ability was regained, which was partially independent of *de novo* synthesis [378]. These data support the view that HSP90 actively participates in steroid-induced signal transduction.

5.4 Genetic studies

Yeast express two genes, *HSP82* and *HSC82*, that encode homologues of the mammalian HSP90 proteins. Mutants with defects in both genes are inviable, whereas either of the two genes alone is sufficient for growth at normal temperatures [352]. Picard *et al.* [382] constructed a strain lacking both chromosomal genes and harboring a plasmid baring *HSP82* expressed under control of a galactose-inducible promoter. These cells die in glucose, but live in galactose media, in which HSP82 accumulates to a level around that of HSP82 and HSC82 combined in the parental cells. A clone was isolated, GRS4, by its

ability to grow in glucose. GRS4 cells achieve this by producing about 5% of the wild-type level of HSP90 in glucose, and normal levels in galactose. In experiments in which a receptor and reporter gene were cotransfected into GRS4 cells, results showed that at low levels of HSP90 receptors seem to be mostly free of HSP82, yet failed to enhance transcription. Upon hormone addition, the receptors were found to be activated but with markedly reduced efficiency [382]. When these experiments were repeated using an AhR signaling system, reduced levels of HSP90 caused a complete blockage of agonist-induced response [383, 384]. These data indicate that HSP90 does not inhibit receptor function solely by steric interference (i.e. by masking the DNA-binding domain).

5.5 Steric interference

The genetic studies in yeast emphasize the role of HSP90 in maintaining receptors in a ligand-binding conformation, but they do not necessary exclude steric interference as a means by which HSP90 modulates receptor activity. As mentioned earlier, HSP90 can mask the NLS of proteins, thereby directing their intracellular localization. Moreover, HSP90 can inhibit the intrinsic DNA-binding activity of receptors by physically blocking their DNA-binding domain. For example, the hormone/HSP90-binding domain of steroid receptors was found to confer hormonal control onto constitutively activated transcription factors E1A [385] and Myc [386].

Several studies have shown that ligand is not a prerequisite for DNA-binding activity. Deletion of the entire ligand-binding domains of either ER [387] or GR [388] was found to produce truncated receptor mutants that are constitutive transcriptional activators and do not form 8-9S complexes. Sabbah *et al.* [389] performed gel shift experiments with purified ER and HSP90. Their results indicated that at low HSP90 levels ER was capable of binding with its

cognate ERE, whereas high concentrations of HSP90 specifically inhibited the ERE-binding activity of ER. Furthermore, this effect could be reversed by increasing the concentration of DNA in the reaction [389], indicating that HSP90 is capable of dissociating ER from the ERE by a dynamic and specific process.

In a similar fashion, studies from the laboratory of Lorenz Poellinger have shown that the AhR is capable of binding to its cognate responsive element in the absence of either ligand or HSP90. In one study, untransformed cytosolic 9S AhR complexes and transformed nuclear 6S AhR complexes were isolated using anion exchange chromatography [390]. Gel shift studies indicated that the smaller HSP90-free form of AhR could bind to a XRE, whereas the larger form did not [390]. This was corroborated by work showing that salt-disrupted AhR, which was not associated with HSP90, bound to DNA constitutively [361]. Furthermore, TCDD was not capable of binding to the HSP90-free form of AhR. Based on these results, Poellinger proposed that HSP90 plays dual roles in the modulation of AhR activity: (i) it represses the intrinsic DNA-binding activity of the receptor, and (ii) it appears to determine the ability of the receptor to assume and/or maintain a ligand binding conformation [361].

Although HSP90 has a role in modulating the activities of AhR, ER, and GR, there are specific points of differences between them. First, there is evidence that the AhR [361] and GR [358] must be bound to HSP90 in order to bind ligand, however, this requirement is debatable for ER [378, 391-394]. Second, the ligand-binding domain of GR constitutes the only structural requirement for high affinity HSP90 binding [395], whereas the HSP90-binding domains of AhR [396] and ER [387] are composed of separate regions which overlap with their respective ligand- and DNA-binding domains.

5.6 Heat shock

In all organisms induction of the heat shock response leads to a rapid and intense up-regulation of a subset of proteins. For different organisms the response is induced at different temperatures that reflect the conditions of the host environment. For example, heat shock is induced at 60°C in thermophilic bacteria growing at 50°C; at 5-10°C in Arctic fishes growing at 0°C; whereas in soybeans or fruit flies it can be induced on hot sunny days [349].

At elevated temperatures, the higher order three-dimensional structure of proteins break down. Induction of heat shock proteins has been shown to both inhibit denaturation and facilitate refolding into the native structures. It does not appear that HSP90 has a role in the overall protection and renaturation of cellular proteins during heat shock, but it may function to maintain a small group. In yeast, HSP90 deficiencies had no effect on the thermal inactivation profiles of either firefly or bacterial luciferase proteins [356]. These defects did, however, reduce the rate at which heat-inactivated luciferase was reactivated [356]. In agreement with these studies, geldanamycin has been shown to inhibit the refolding of luciferase both *in vivo* and *in vitro*, and as a result luciferase is retained in the HSP90 complex in an unfolded, degradation-sensitive state [381, 397]. It was demonstrated that HSP90 cooperates in this process with HSP70, HSP40, p60, Hip, and p23 [381, 397]. Thus, the HSP90 complexes formed with thermally denatured polypeptides resemble those of hormone receptors and protein kinases.

In summary, HSP90 is involved in the maturation step of a small set of proteins, and the continued dynamic association with HSP90 leads to protection against degradation and maintenance of a proper functional conformation. In some instances, HSP90 may act by masking domains which are activated upon disruption of the protein-HSP90 complex. It is thought that HSP90 levels increase

at higher temperatures to more effectively chaperone these proteins and to aid otherwise stable proteins which encounter problems in folding and stability.

CHAPTER II

EVALUATION OF GENETIC ALTERATIONS IN HUMAN MAMMARY EPITHELIAL CELL LINES TRANSFORMED *IN VITRO* WITH BENZO[*A*]PYRENE

PREFACE TO CHAPTER II

Species-specific differences in xenobiotic metabolism and tumor morphology create uncertainty when attempting to extrapolate transformation experiments from animal models to humans.

In our experiments we have utilized a novel spontaneously immortalized human mammary epithelial cell line, MCF-10A, that possesses a near-normal karyotype. Although immortalization is one aspect of malignant transformation, this cell line has been used for transformation studies by us and others because it offers the following advantages: (i) it is thoroughly characterized with respect to morphology, cytogenetics, signaling pathways, and cell type-specific protein expression, (ii) immortalization allows for the accumulation of sufficient cellular material to perform several reproducible assays, and (iii) it is derived from human tissue and therefore more relevant to breast cancer research.

The objective of the experiments described in this chapter was to characterize the genetic alterations associated with benzo[*a*]pyrene-induced transformation of human mammary epithelial cells. The results obtained concur with the hypothesis that alterations of chromosome 8 may be an early event in breast carcinogenesis; and show that benzo[*a*]pyrene can elicit genetic alterations *in vitro* that are found in primary human breast carcinomas.

ABSTRACT

While some epidemiological risk factors for breast cancer have been identified, the factors responsible for the initiation and transformation of mammary epithelial cells are not clear. We have exposed the spontaneously immortalized human mammary epithelial cell line MCF-10A to benzo[*a*]pyrene and selected transformed clones based on a loss of contact inhibition and altered morphology. Cytogenetic studies showed that each of the transformed cell lines E3.2, E3.3 and E3.7 possess an isochromosome 8q aberration. The *c-myc* proto-oncogene, which is positioned at 8q24, was analyzed for changes in expression. Both *c-myc* mRNA and protein levels were found to be increased in all the transformed clones relative to the parental cells. E3.3 cells have only one copy of chromosomal arm 5q, which is the location of a putative tumor suppressor gene that regulates *c-myc* expression. Furthermore, E3.3 cells were found to have the highest c-Myc protein levels. No mutations were detected in *c-myc*, *p53* and *H-ras* genes by sequencing or single-stranded conformation polymorphism analysis. The transformed clones were not able to grow as tumors *in vivo* when injected into nude or SCID mice. Data from this model shows that benzo[*a*]pyrene, a ubiquitous environmental carcinogen, can induce selectable morphologic changes in a near-normal human mammary epithelial cell line and that these transformed cells possess chromosomal aberrations frequently found in clinical investigations.

INTRODUCTION

Traditionally accepted risk factors for breast cancer represent less than half of all cases [398], and improved screening practices cannot completely account for the rise in incidence rates over the past two decades [399]. Although numerous chemicals, including polycyclic hydrocarbon and aromatic amine compounds, are known to induce mammary carcinomas in rodents [53], it has not been possible to determine whether chemical carcinogens are etiologic agents for human breast cancer. The failure to find associations that reflect the experimental findings may be attributed in part to our inability to adequately characterize exposure at the time of tumor initiation, which occurs 20 to 40 years before diagnosis. Environmental exposures can be prevented, thus, identification of carcinogens is a critical objective for breast cancer research.

Benzo[*a*]pyrene (BP) is a polycyclic aromatic hydrocarbon that forms during combustion or pyrolysis of organic matter and is thus found in broiled and smoked foods, cigarette smoke, and urban air [52, 400]. Lipophilic compounds such as BP can be stored and concentrated in the mammary fat pad [401] and secreted by the alveolar duct system of nursing mothers [402]. Cytochrome P450 and peroxidase enzymes metabolize BP to approximately 20 oxidized by-products. Although several metabolites are able to induce mutations, transform cells, and bind to cellular macromolecules, only (±)-*anti*-benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) is considered an ultimate carcinogen [140]. BPDE covalently binds to DNA forming adducts, preferentially at guanine residues and to a much lesser degree at adenine residues. In addition, BPDE causes single- and double-strand DNA breaks [403]. Although the exact mechanisms are not clear, this chromosomal damage probably results from radical oxygen species [404] and inefficient repair of DNA adducts by the

nucleotide excision repair pathway [405].

There is preliminary evidence which suggests that BP is a potential human mammary carcinogen. Li and co-workers [406] have shown that BP-like DNA adducts in the breast tissue of cancer patients (n=87) were about 4-fold higher than in noncancer controls (n=37). One specific BP-like adduct in particular was detected at significant levels in normal adjacent breast tissues of 41% of the breast cancer patients, but was absent in all non-cancer controls [406].

The objective of this work was to determine whether human mammary epithelial cells treated *in vitro* with BP manifest genotypic changes associated with breast cancer. For this purpose MCF-10A cells were utilized. This untransformed cell line was established as a spontaneous outgrowth of mastectomy tissue obtained from a woman with fibrocystic breast disease and retains a diploid karyotype with minimal rearrangement [407, 408]. Cells were subjected to multiple rounds of BP exposure and transformed clones were selected based on a loss of contact inhibition and altered morphology. Transformants were characterized by cytogenetic analysis, and genes implicated in breast cancer (*c-myc*, *p53*, *H-ras*) were examined for mutation and/or changes in expression.

MATERIALS AND METHODS

1. Reagents

Antibodies were utilized that recognize different epitopes near the C-terminal of c-Myc: Ab-1 monoclonal anti-c-Myc antibodies (Cedarlane, Hornby, Ontario, Canada) recognize amino acids 411-420; and amino acids 429-440 were used to generate 1537-15 polyclonal anti-c-Myc antibodies [409]. Monoclonal anti-β-actin antibodies were purchased from Boehringer Mannheim (Laval, Quebec,

Canada). The *c-myc* probe was synthesized by polymerase chain reaction (PCR) using human genomic DNA as a template (see Table 1 for primers) followed by gel purification of the 1084 base pair oligonucleotide.

2. Cell culture

The MCF-10A cell line was obtained from the American Type Culture Collection at passage number 44. MCF-10A cells, including BP-exposed cells and the transformed clones, were maintained in a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagles medium supplemented with 100 ng/ml cholera toxin, 10 μg/ml insulin, 0.5 μg/ml hydrocortisol, 20 ng/ml epidermal growth factor and 5% v/v horse serum in an atmosphere of 37°C/5% CO_2. For carcinogen treatments, BP (Sigma, St. Louis, MO) was dissolved in dimethyl sulfoxide and added to the medium at a concentration of 0.5% v/v.

3. Carcinogen treatment and anchorage-independent growth

MCF-10A cells were exposed to 1 μM BP for 48 h and then cultured in normal medium for 2 weeks. Cells were routinely passaged before confluency was achieved. This regimen was repeated for a total of 4 consecutive treatments. Exposed cells were designated MCF-10A.E1 to MCF-10A.E4. Mock-treated MCF-10A cells were exposed to dimethyl sulfoxide alone. The BP-exposed cells were assayed for anchorage-independent growth by plating each group of cells in two 6-well plates. The semi-solid medium was prepared by mixing 1.4% agar, 2X complete MCF-10A medium, horse serum and cells, for a final concentration of 0.35% agar, 1X medium, 25% serum and 400 cells/well. Cell aggregates that were evident after suspending in agar were excluded in the analysis of colony formation. Plates were incubated at 37°C/5% CO_2 and the medium replenished twice weekly. After 5 weeks, anchorage-independent growth (spherical

TABLE 1. Amplification primers for *c-myc*, *p53* and *H-ras*

Gene	Exon(s)	Assay[a]	Primer Sequence (5'->3')[b]
c-myc	2	SSCP	U-AGACTGCCTCCCGCTTTGTGTG
			L-AGAAGGGTGTGACCGCAACG
	2	NB,SB	U-CCAAGCCGCTGGTTCACTAAG
			L-GGCCCGTTAAATAAGCTGCC
p53	5, 6	SQ	U-CACTTGTGCCCTGACTTTCAACT
			L-ACCCATTTACTTTGCACATCTC
	7, 8	SQ	U-CCTGTGTTATCTCCTAGGTTGGC
			L-CCACCGCTTCTTGTCCTGCTTGCT
H-ras	1, 2	SQ	U-AGCGATGACGGAATATAAGC
			L-AGACTTGGTGTTGTTGATGG

[a]SSCP, single-stranded conformation polymorphism; NB, Northern blot; SB, Southern blot; SQ, sequencing

[b]U, upper; L, lower

formation of >20 cells) was scored using a light microscope.

4. Isolation of BP-transformed clones

MCF-10A.E3 cells were seeded in a 100 mm tissue culture dish. When cells became confluent 10 foci were carefully removed with the use of a microscope under sterile conditions and transferred to a 24-well plate. Extra precaution was taken to exclude surrounding cells. Three of these clones were expanded into the transformed cell lines designated E3.2, E3.3 and E3.7.

5. Cytogenetic analysis

MCF-10A wild-type cells and BP-transformed clones were harvested by trypsinization after an 18 h *in situ* exposure to 0.02 μg/ml Colcemid. The cells were exposed to a hypotonic solution (0.4% KCl) for 20 min and then to a modified Carnoy's fixative (methanol:acetic acid=3:1). Two drops of cell suspension placed on frozen slides were immediately exposed to steam to improve chromosome spreading. G-banding was achieved by dipping the slides in a 0.01% trypsin solution and staining for 1.5 min in a 0.06% Leishman solution. The chromosome study was done on well-spread metaphases with a resolution of 400 bands.

6. SSCP analysis

This assay was performed according to the nonisotopic method of Yap and McGee [410]. Briefly, a 291 base pair oligonucleotide encompassing a portion of exon 2 of the *c-myc* gene was amplified by PCR. The 50 μl reaction volume contained 100 nM primers (Table 1), 200 μM dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1 μg of genomic DNA and 2.5 Units of Taq polymerase (Pharmacia, Baie d'Urfe, Quebec, Canada). Cycling consisted of a 3 min

denaturation step at 94°C followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 70°C for 30 s; and ending with a 70°C extension step for 5 min. For each sample, 20 µl of PCR product was denatured by addition of 2 µl of 0.5 M NaOH, 10 mM EDTA followed by incubation at 42°C for 5 min. Samples were resolved through 10% polyacrylamide gels containing ethidium bromide and TBE buffer [90 mM Tris, 90 mM boric acid, 3 mM EDTA]. Gels were exposed to ultraviolet light and photographed.

7. Sequencing

Tumor suppressor gene *p53* exons 5 and 6; *p53* exons 7 and 8 ; and *H-ras* exons 1 and 2 were PCR-amplified (see Table 1 for primers) and purified from a 1.2% agarose gel using Wizard PCR Preps (Promega, Madison, WI). These oligonucleotides were sequenced from both ends using the dsDNA Cycle Sequencing System (Canada Life Technologies, Burlington, Ontario, Canada) with the same primers. Reaction products were resolved through 6% polyacrylamide gels containing 7 M urea at 50°C. Gels were transferred to Whatmann 3MM paper, dried, and autoradiographed.

8. Northern blot analysis

Total RNA was isolated from cells in log growth phase using the RNAzol B (Tel-Test, Friendswood, TX) method by the manufacturer's guidelines. RNA (25 µg/lane) was loaded onto 1.1% agarose/formaldehyde/ethidium bromide gels. The samples were run at 100 V for 3 h and a photograph of the gels were taken. The RNA was transferred to Zeta-Probe membranes (Bio-Rad, Mississauga, Ontario, Canada) by capillary transfer overnight and hybridized with ultraviolet light. The *c-myc* probe was radiolabeled with [α-^{32}P]dCTP by random primer extension (Oligo Labeling Kit; Pharmacia). Hybridization was performed in 40%

formamide, 4X standard saline citrate, 4X Denhardt's reagent, 0.2 mg/ml salmon sperm DNA, 1.2% sodium dodecyl sulfate and 10% dextran sulphate at 42°C overnight.

9. Immunoblot analysis

Cells in exponential phase growth on 100 mm plates were washed with PBS, detached with a cell scraper, and sedimented. The cells were resuspended in 0.5 ml lysis buffer [10 mM Tris-HCl (pH 8.0), 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 0.01 mg/ml leupeptin, 0.01 mg/ml pepstatin, 0.01 mg/ml aprotinin] per $2x10^7$ cells and incubated on ice for 10 min. Samples were spun down at high speed and the protein content of the supernatant was quantitated by the Bradford assay (Bio-Rad). Protein samples (25 μg/lane) were resolved through 12% polyacrylamide-sodium dodecyl sulfate gels and electrophoretically transferred to nitrocellulose (BioBlot-NC; Costar, Cambridge, MA) by standard techniques. The blots were stained with Ponceau S to verify the efficiency of transfer and the gel loading. If areas of the gel did not transfer properly or if differences in gel loading were apparent, the blot was not used. Membranes were blocked overnight in BLOTTO buffer [50 mM Tris (pH 7.5), 0.2% Tween-20, 150 mM NaCl, 5% dry milk powder] at 4°C and then washed 4 times in TTBS+ buffer [50 mM Tris, 0.5% Tween-20, 300 mM NaCl (pH 7.5)] for a total of 40 min. Immunohistochemical staining was carried out with a 3 μg/ml dilution of anti-c-Myc (Ab-1) antibodies, 2 μg/ml of anti-c-Myc (1537-15) antibodies, or 1:1000 of anti-β-actin antibodies in BLOTTO buffer for 2 h at room temperature. Membranes were washed as previously and incubated with 1:10,000 horseradish peroxidase-conjugated secondary antibody in BLOTTO buffer for 1 h at room temperature. Following another wash cycle, specific proteins were detected by the enhanced chemiluminescence (ECL)

system from Amersham (Oakville, Ontario) as described by the manufacturer.

10. Southern blot analysis

For each sample, 10 μg of genomic DNA was digested with *EcoRI* restriction enzyme, precipitated, and resuspended in water. Samples were loaded onto 0.8% agarose gels and electrophoretic separation of DNA was performed overnight at 1 V/cm. Gels were denatured for 45 min in 1.5 M NaCl, 0.5 N NaOH and neutralized for 30 min in 1 M Tris (pH 7.4), 1.5 M NaCl. DNA samples were transferred to Zeta-Probe membranes by capillary transfer and hybridized with ultraviolet light. Membranes were probed with an oligonucleotide complimentary to *c-myc* as described for the Northern blot assay.

11. Tumorigenic assay

Female mice at 4 weeks of age were obtained from Jackson Laboratories (Bar Harbor, MN). For each cell line tested, two BALB/c nude (nu-/nu-) mice and two SCID (C3HSNM.C.Scid/J) mice were injected subcutaneously into the dorsal flank with 10 million cells suspended in either 200 μl of PBS or 200 μl Matrigel (10 mg/ml; Collaborative Research, Bedford, MA). The mice were palpated at least twice a week and sacrificed after 5 months.

12. Densitometry

Radiographic and chemiluminescent exposures were scanned into a Power Macintosh computer with a UMAX VistaS-12 scanner and Adobe Photoshop 3.0.5 software. Images were then quantified with the use of National Institutes of Health Image 1.61b7 software. Quantification was performed as follows. A rectangular tool was produced that surrounded the largest band of interest. The size of this tool was held constant for all measurements within a

specific set of samples. The mean value of the intensity within the tool was then determined for: (i) the band of interest, (ii) the area directly above the band, and (iii) the area directly below the band. The intensities of the area above and below the band of interest were then averaged and subtracted from the band of interest.

RESULTS

Treatment of MCF-10A cells with BP resulted in morphologic changes associated with cell transformation. Anchorage-independent growth increased in a non-stochastic manner with escalating exposures of BP (Table 2). In tissue culture, MCF10A.E1 and MCF-10A.E2 cells maintained the appearance and properties of the parental cell line. After the third BP exposure, however, foci of cells were detected on the tissue culture plate (Fig. 4B). Under the microscope, these foci were found to be initiated soon after passaging and appeared as a disorientated group of rounded cells within the regular pattern of surrounding cells. By the time confluency was achieved, large foci were visualized as cells grew one atop the other in a semi-spherical pattern. Three of the foci were expanded into the transformed cell lines designated E3.2, E3.3 and E3.7. When the transformed clones were grown as a monolayer, the cells possessed a homogenous spindle-shaped morphology (Fig. 4C), in contrast to MCF-10A cells (Fig. 4A) where individual cells varied in size and shape.

During the establishment of the MCF-10A cell line from primary breast epithelial cells, Soule *et al.* [407] noted that the cells acquired a balanced translocation t(3;9)(p13;p22) by the 846th day in culture (passage 13). This rearrangement occurred at about the same time as the cells had 'spontaneously immortalized.' Subsequently, by the 1009th day (passage 34) 9p had undergone

TABLE 2. Anchorage-independent growth of benzo[*a*]pyrene (BP)-exposed MCF-10A cells

Cell Type	BP Exposure(s)[a]	Colonies/well[b]
MCF-10A	0	0
MCF-10A.E1	1	0
MCF-10A.E2	2	0.33
MCF-10A.E3	3	2.72
MCF-10A.E4	4	5.57

[a]For each exposure, cells were treated with 1 μM BP for 48 h and then cultured in drug-free medium for 2 weeks.

[b]Colony counts were taken 5 weeks after seeding 400 cells/well in 6-well plates containing nutrient agar as describe in "Materials and Methods." Results are the arithmetic mean of 12 wells per cell type.

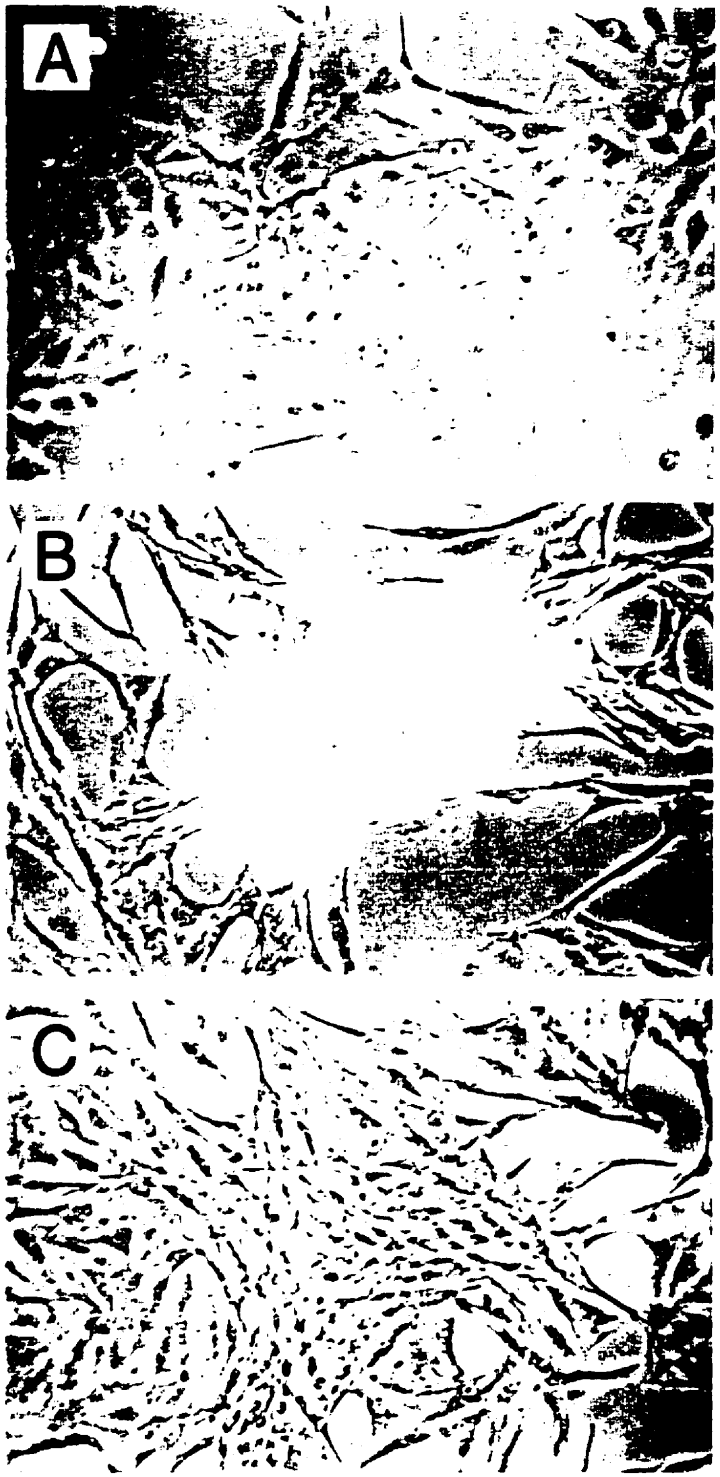

Figure 4. Morphology of MCF-10A wild-type and benzo[*a*]pyrene-exposed sublines under anchorage-dependent conditions. (A) MCF-10A cells. (B) MCF-10A cells exposed to 1 μM benzo[a]pyrene for three 48 h intervals (MCF10A.E3) showing loss of contact inhibition: growing cells overlap forming foci. (C) *In vitro* transformed cells. Individual MCF-10A.E3 foci were isolated and expanded. The transformed cells display a spindle-shaped morphology and frequently form a criss-cross orientation at high density.

further modification which included duplication and addition of 9q(q21-q33) and 5q(q31-qter) to the original 9p22 breakpoint and loss of the translocated 3p segment [407]. These structural aberrations of chromosomes 3 and 9 were present in our cytogenetic analysis of MCF-10A cells at passage 50 (Table 3). Abnormalities reported by Soule *et al.* that occurred in late passages, including a der(6) by day 1229 and trisomy of chromosomes 8 and 16 in one subline, were not found in our studies, indicating that the MCF-10A cells we had obtained were derived from an earlier period in the evolution of this cell line.

Cytogenetic analysis of the transformed clones revealed chromosomal abnormalities not present in the parental cell line (Table 3 and Fig. 5). Each of the transformed clones possessed an isochromosome 8q that replaced one of their complements of chromosome 8. For the transformed cell lines, this aberration was present in 100% of the cells analyzed (n=41), whereas none of the wild-type MCF-10A cells displayed an isochromosome 8q. The second chromosomal abnormality identified was an isochromosome 5p. This aberration was more variable: in E3.2 cells isochromosome 5p was present in addition to its disomic complement of chromosome 5 in each of the cells examined; whereas E3.3 cells possessed one normal chromosome 5 and one isochromosome 5p in 5/13 cells examined and a normal disomic complement of chromosome 5 in the remaining cells.

Genes frequently altered in breast cancer were analyzed for genetic defects. The majority of human cancers have a mutated *p53* tumor suppressor gene, a finding that has been associated with the latter stages of tumor development [411-416]. Activating point mutation of codons 12, 13 and 61 of the *H-ras* proto-oncogene have been reported in chemically-induced mammary carcinomas [417], but rarely in primary human breast tumors [418]. We sequenced exons 5, 6, 7 and 8 of *p53* and exons 1 and 2 of *H-ras*, however,

TABLE 3. Cytogenetic analysis of MCF-10A parental cells and benzo[*a*]pyrene-transformed clones.

Cell line	Cells analyzed / karyotyped	Chromosome range [a]	Common abnormality	Recurrent abnormality	Modal karyotype
MCF-10A	8/8	44-46	der(3) [b],der(9) [c]		46,XX,der(3),der(9)
E3.2	6/2	46-47	der(3),+i(5p),i(8q),der(9)		47,XX,der(3),+i(5p),i(8q),der(9)
E3.3	13/3	38-46	der(3),i(8q),der(9)	i(5p) [5/13]	46,XX,der(3),i(5p),i(8q),der(9)
E3.7	16/4	44-46	der(3),i(8q),der(9)		46,XX,der(3),i(8q),der(9)

[a] Variation of the chromosome number from the modal number is due to random loss of chromosomes.

[b] der(3) is equivalent to der(3)t(3;9)(p13;p22).

[c] der(9) is a complex rearrangement that can be described as an ulterior replacement of the initially translocated seqment 3p13-pter on 9p22 by a duplicated segment 9q21-q33 followed terminally by a duplicated segment 5q31-qter (see also [13]).

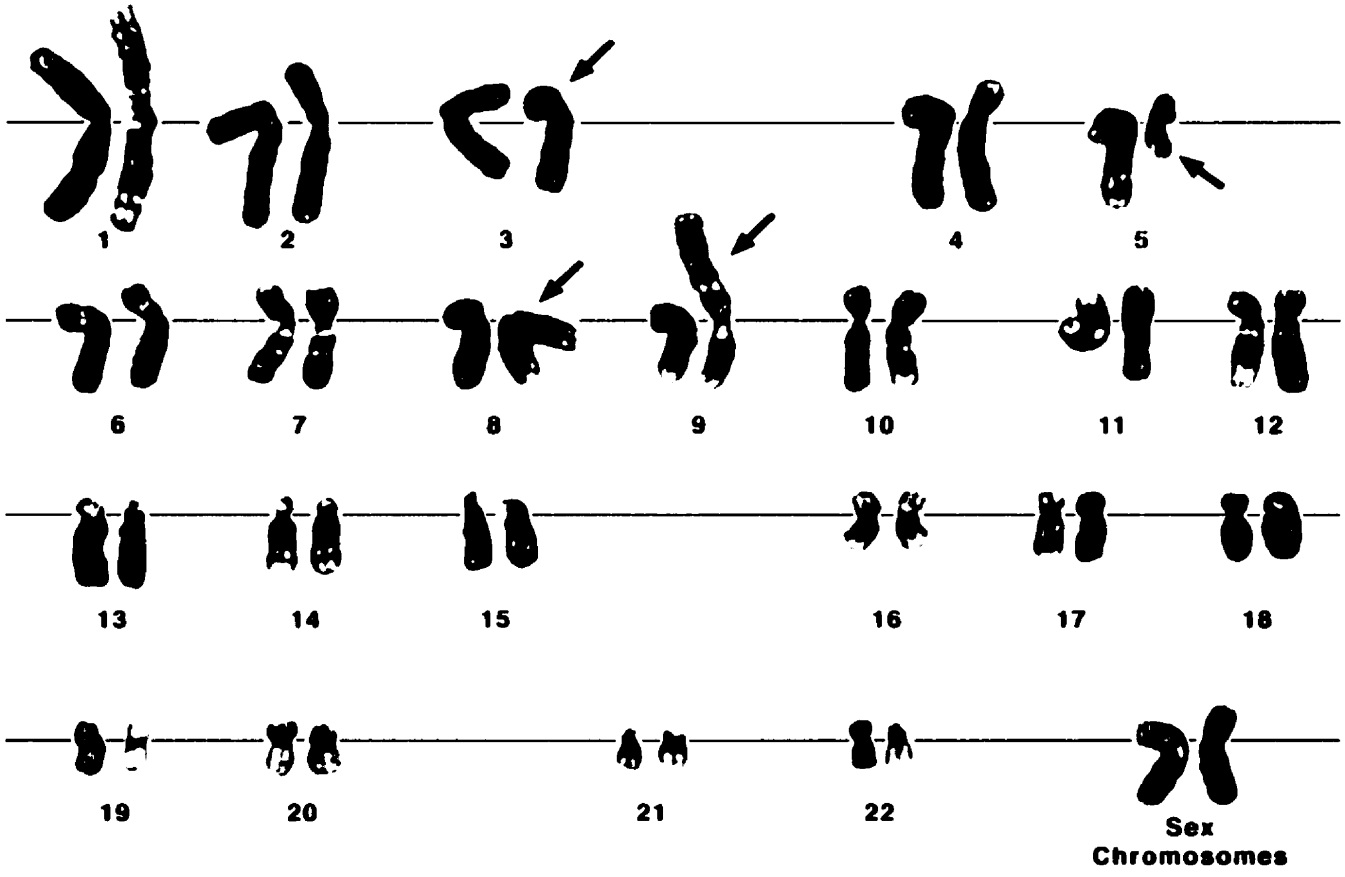

Figure 5. Karyotype of the benzo[*a*]pyrene-transformed MCF-10A cell line E3.3 [46,XX,der(3)t(3;9)(p13;p22),i(5p),i(8q),der(9)]. *Arrows* indicate chromosomal aberrations.

mutations were not found in the BP-transformed clones. To determine whether BP induced mutations in *c-myc*, single-stranded conformation polymorphism (SSCP) analysis was performed (Fig. 6). Data from this experiment indicated that exon 2 was not altered relative to wild-type MCF-10A cells. In human mammary tumors point mutations are not normally observed in *c-myc*, but mutations have been reported to occur in exon 2 in lymphomas [419, 420]. Finally, *c-myc* was not amplified in the transformed clones relative to the parental cells as determined by Southern blot analysis (data not shown).

Levels of *c-myc* mRNA are elevated about 2-fold in each of the transformed cell lines (Fig. 7). This increase concurs with the change in chromosome 8q copy number found in the respective karyotypes. As shown in Fig. 8, three protein bands that vary in size from 53-61 kDa are recognized by anti-c-Myc (Ab-1) antibodies in MCF-10A wild-type cells and the BP-transformed clones, and E3.3 cells have an additional *c-myc* product at 65 kDa. To confirm the specificity of the anti-c-Myc antibodies, this data was verified with antibodies raised against a different epitope (1537-15 anti-c-Myc IgG, not shown). Analysis of the band intensities indicated that c-Myc protein expression is up-regulated 2-fold in E3.2 and E3.7 cell lines relative to parental MCF-10A cells (Fig. 8), whereas E3.3 cells have a 10-fold increase in expression.

Despite a predicted molecular mass of 49 kDa, c-Myc is most often detected as a 65 kDa phosphoprotein, although numerous cytoplasmic and nuclear proteins have been reported to be recognized by antibodies against c-Myc peptides that range in size from 32 to 85 kDa ([421] and references therein). The diversity in molecular mass may arise for several reasons. For example, multiple promoters produce different primary transcripts; alternate splicing patterns can generate mRNAs of varying configurations; and various post-translational modifications alter the molecular mass of the final product

Figure 6. Single-stranded conformation polymorphism analysis of the *c-myc* protooncogene in MCF-10A wild-type (WT) and benzo[*a*]pyrene-transformed cell lines. A portion of exon 2 was PCR-amplified, denatured, and resolved through a polyacrylamide gel under renaturing conditions as described in "Materials and Methods." There is no difference in the mobility of *c-myc* oligonucleotides between WT cells and the transformed clones, indicating the absence of benzo[*a*]pyrene-induced genetic mutation in this region. UD, undenatured; ss, single-stranded oligonucleotide; ds, double-stranded oligonucleotide.

Figure 7. (A) Analysis of *c-myc* mRNA content in MCF-10A wild-type (WT) and benzo[*a*]pyrene-transformed cell lines. (B) To assess the integrity of RNA and gel-loading, a photograph of the agarose/ethidium bromide gel was taken prior to nitrocellulose transfer. 18S and 28S indicate the location of ribosomal RNA bands. Northern blot analysis was performed as described in "Materials and Methods."

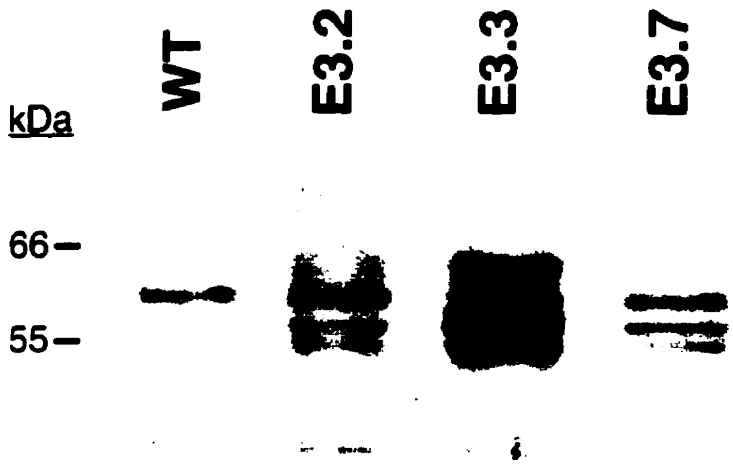

Figure 8. c-Myc expression in MCF-10A wild-type (WT) and benzo[*a*]pyrene-transformed cell lines. The presence of c-Myc in total cellular extract was detected by immunoblot analysis using antibodies that recognize an epitope near the C-terminal of the protein (Ab-1 monoclonal anti-c-Myc IgG). Membranes were stripped and reprobed for β-actin expression to standardize for loading differences.

(reviewed in [76]). Since we did not find an alteration in the size of the *c-myc* mRNA by Northern blot analysis (Fig. 7), the expression of a 65 kDa c-Myc protein in E3.3 cells is most likely the result of a post-translational event, although the exact nature of this modification remains to be determined.

The tumorigenic potential of MCF-10A, E3.2, E3.3 and E3.7 cells were tested in immunocompromised mice. Cells were suspended in PBS and injected subcutaneously into the dorsal flank of athymic nude mice. No tumors were detected in any of the animals during a 5 month observation period. This was repeated in SCID mice, which lack functional T cells and B cells, with the same result. It has been shown that the use of Matrigel, a solubilized tissue basement membrane preparation, enhances the growth of human small-cell lung cancer cell lines as tumors in nude mice [422]. However, suspension of cells in Matrigel prior to injection did not lead to tumor formation with any of the cell lines tested. This data indicates that the transformed clones are not tumorigenic by this method, although the intrinsic lack of efficiency in the murine tumorigenicity assays [423] precludes a definitive phenotyping.

Discussion

An important property of normal cells is the ability to cease proliferating once a high cell density is achieved. In our model, foci form in confluent BP-exposed MCF-10A cells that are not observed in untreated cells. Several of these foci were carefully isolated and expanded, and three clones were further assayed for genotypic and phenotypic characteristics of transformation. Cytogenetic analysis showed that the transformants have unique chromosomal aberrations. The alteration common to all three transformed cell lines is an isochromosome 8q. This is a significant finding since one third of human breast tumors are

reported to have cytogenetic abnormalities of chromosome 8 [71]. Deletions of 8p are most frequent, but trisomy and monosomy of chromosome 8, isochromosome 8q, homogeneously staining regions and translocations are also observed [71, 424-427].

The short arm of chromosome 8, in which one copy is lost in the transformed clones, is the site for a putative tumor suppressor gene(s) [428-431]. Loss of heterozygosity (LOH) of discrete regions of chromosome 8p has been observed in cultured human breast carcinoma cells [432] and in approximately half of clinical breast cancers [430, 433, 434]. In addition to losses of loci on chromosome 8p, the BP-transformed clones have additions of chromosome 8q. A gain of chromosome 8q was described as the second most frequent abnormality in a series of breast cancers studied by direct preparation [435]. Furthermore, trisomy of chromosomes 7, 8 and 18 were present in cytogenetic investigations of malignant breast tumors, but trisomy of chromosome 8 was the only recurrent karyotypic abnormality, and in some cases the sole visible chromosomal aberration [426, 436].

The *c-myc* proto-oncogene, positioned at 8q24, is amplified in 10-30% of human mammary tumors [437, 438]. Amplification of *c-myc* has been correlated with high proliferative capacity [83] and poor prognosis [439, 440] in breast cancer patients, and has been postulated to result primarily through polysomy of chromosome 8 and/or genomic endoreduplication (i.e. DNA aneuploidy), rather than gene duplication [441]. We screened *c-myc* for genetic mutation and changes in expression. Southern blot analysis did not show amplification of *c-myc*, and no mutations were detected by SSCP analysis. The BP-transformed clones were found to have increased *c-myc* mRNA and protein levels. Most studies of *c-myc* in tumor samples have focused on gene amplification. The relatively few studies that have reported *c-myc* mRNA and protein expression *in vivo* consistently show

a much higher frequency of overexpression than can be explained by amplification [442-446].

In one study, c-Myc protein levels in the majority of human colon adenocarcinoma cell lines tested were found to be 8- to 37-fold higher than the levels of c-Myc in normal colorectal cell lines [447]. The constitutively elevated c-Myc protein expression in the carcinoma cell lines was not typically accompanied by gross rearrangement or amplification of the gene. There is data to suggest that a putative tumor-suppressor gene on chromosome 5 regulates expression of c-Myc [448]. Deregulation of c-Myc expression has been shown to be linked with LOH for polymorphic markers on chromosome 5q [448]. Transfer of normal human chromosome 5 into two human colon carcinoma cell lines with 20- to 30-fold overexpression of c-Myc resulted in suppression of c-Myc levels and abrogation of tumorigenicity in nude mice [449]. Loss of the transferred chromosome resulted in re-expression of the tumorigenic phenotype and in constitutive elevated expression of c-Myc. Two of the BP-transformed clones described here have an isochromosome 5p. E3.2 cells have an i(5p) chromosome in addition to its disomic complement of chromosome 5, whereas E3.3 cells have one normal chromosome 5 and one i(5p) in about one-third of cells examined. It is interesting to note that E3.3 cells have a loss of one set of loci on 5q and a 10-fold increase in c-Myc expression, however, further studies are required to determine if gene(s) on chromosome 5q are responsible for deregulation of c-Myc expression in this cell line.

There are a number of indicators which suggest that chromosome 8 aberrations are associated with the early stages of human mammary tumorigenesis. As outlined above, one or more loci on chromosome 8 are altered in the majority of breast cancers. Secondly, Yaremko and colleagues [434] found that LOH on chromosome 8p was present in 11 of 20 breast carcinoma resections.

They suggest that 8p LOH is an early event since it occurs with equal frequency in small (<2 cm) or large (>2 cm) breast tumors, and in tumors without regional metastases. In addition, the *c-myc* proto-oncogene is thought to have a role in the initial stages of breast cancer development from studies showing an association of c-Myc overexpression with high-grade tumors [82, 450], early recurrence, and poor prognosis [84, 451], but less so with nodal status or metastasis [85, 452, 453]. The data presented here support the hypothesis that loci on chromosome 8 are involved in the transformation of human mammary epithelial cells. Ongoing studies in this laboratory are designed to specifically address this question.

BP is known to induce tumors in the mammary gland of rats [143], however, species variation in xenobiotic metabolism and differences in tumor genotypes and morphology do not allow us to characterize BP as a human breast carcinogen. Therefore, *in vitro* models of human mammary carcinogenesis may help to bridge the gap between experimental and clinical findings. Our results show that BP is capable of inducing a transformed phenotype in human mammary epithelial cells. Characterization of clones selected for morphological characteristics of transformation did not uncover mutations in *p53* and *H-ras* genes, however, the BP-transformed cell lines were found to possess an alteration of chromosome 8 and increased *c-myc* expression, two parameters frequently associated with clinical disease.

CHAPTER III

DIVERGENT MECHANISMS FOR LOSS OF AH-RESPONSIVENESS IN BENZO[*A*]PYRENE- AND ADRIAMYCIN-RESISTANT MCF-7 CELLS

PREFACE TO CHAPTER III

One of the reasons for the development of cancers and their relentless malignant progression, even in the presence of highly toxic anticancer therapies, is an enhanced ability to bypass mechanisms responsible for cell death. Drug resistance, either intrinsic or acquired, can be mediated by a variety of mechanisms. These include increased DNA repair, drug efflux, and drug detoxification, to cite a few of the more prevalent examples. It follows that uncovering the underlying enhanced survival capability of tumor cells is fundamental to gaining a better understanding of tumor progression and response to therapy.

This chapter describes the basis for loss of Ah-responsiveness in MCF-7 breast cancer cells selected for resistance to benzo[*a*]pyrene and adriamycin. These studies show that AhR activity can be down-regulated by different mechanisms in xenobiotic-resistant human mammary epithelial cells.

ABSTRACT

The intracellular aryl hydrocarbon receptor (AhR) mediates signal transduction by environmental pollutants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and benzo[*a*]pyrene by functioning as a ligand-activated transcription factor. We have investigated AhR signaling in sublines of the human breast cancer cell line MCF-7 selected for resistance to adriamycin (Adr^R) and benzo[*a*]pyrene (BP^R). Previously we reported that Adr^R cells have a loss of estrogen receptor (ER) expression and are Ah-nonresponsive. Here we show that AhR mRNA and protein are expressed at normal levels in Adr^R cells and the activated AhR complex is functionally capable of binding a xenobiotic responsive element. In MCF-7 cells AhR was depleted to 15% of normal levels after 4 h TCDD treatment, however, 45% of AhR remained in Adr^R cells during this time-course. In BP^R cells AhR mRNA levels were found to be decreased relative to wild-type cells, which led to decreased AhR protein levels and DNA-binding activity. Cellular ER content has been shown to correlate with Ah-responsiveness in human breast cancer cell lines. BP^R cells were found to be ER-positive, although chronic (BP^R cells) and acute (24 h) exposure to benzo[*a*]pyrene led to significantly lower ER protein levels in MCF-7 cells. We conclude that loss of Ah-responsiveness occurs by different mechanisms in xenobiotic-resistant MCF-7 sublines: AhR mRNA is down-regulated in BP^R cells, whereas Adr^R cells are deficient in AhR signaling by a mechanism unrelated to AhR expression and activity.

INTRODUCTION

The AhR signaling pathway mediates the cytotoxic and genotoxic effects of several environmental contaminants (reviewed in [454, 455]). Dioxins and PAHs are able to induce the transcription of genes by binding to the AhR. In the absence of ligand, cytoplasmic AhR is complexed to a 90 kDa heat shock protein dimer and an immunophilin-like protein [215, 216]. Binding of ligand to AhR initiates a process leading to dissociation of heat shock proteins and heterodimerization with Arnt in the nucleus. This complex functions to transactivate a discrete set of genes possessing upstream xenobiotic responsive elements. Members of the *AhR* gene battery include *CYP1A1, CYP1A2, CYP1B1,* NADPH:quinone oxireductase, aldehyde dehydrogenase 3c, UDP-glucuronosyltransferase, and glutathione S-transferase Ya. Studies in various animal models indicate that dioxins are responsible for species-specific toxic syndromes including wasting, immunosuppression, teratogenesis, hyperkeratosis, and chloracne, as well as cancer [198]. PAHs such as BP can be metabolized by cytochrome P450 enzymes to reactive intermediates and are thus more carcinogenic than dioxins. It has been suggested that exposure to PAHs may lead to breast cancer since these lipophilic compounds can be stored and concentrated in the mammary fat pad [52, 401], and enzymes that activate PAHs are expressed in human mammary epithelial cells [157, 158, 165, 456].

Mammary tumors which are initially responsive to chemotherapeutic treatment quickly progress to a hormone-independent and cytotoxic drug-resistant phenotype. *In vitro* models of xenobiotic resistance have been extensively studied by us [457-463] and others to elucidate the mechanisms by which cells cope with environmental stress and to gain insight for strategies to counteract chemotherapy resistance. MCF-7 human breast cancer cells are ideally

suited for investigating drug resistance since many hormone, growth factor, and xenobiotic receptor signaling pathways have been extensively studied in this cell line. Previously we characterized MCF-7 cells selected for resistance to adriamycin. This subline was found to have increased glutathione *S*-transferase activity [457], overexpression of P-glycoprotein [464, 465], cross-resistance to BP [458] and radiation [460, 463], and loss of ER expression [458]. This study reports the isolation and characterization of a BP-resistant MCF-7 subline. Vickers *et al.* [341] have shown that a correlation exists between Ah-responsiveness and ER expression in human breast cancer cell lines. Our data shows that BP^R cells retain the ER-positive status of the parental cell line. Therefore, these cell lines provide a model to compare AhR signaling in MCF-7 cells or derivatives thereof with AhR^+ER^+ (WT), AhR^-ER^+ (BP^R), and AhR^-ER^- (Adr^R) phenotypes. To this end, we have analyzed AhR and Arnt for genetic mutation, changes in mRNA and protein expression, and DNA-binding activity. Results from this study show that *AhR* mRNA is down-regulated in the BP^R subline. In contrast, AhR expression and activity are normal in Adr^R cells but TCDD treatment does not deplete AhR protein to the extent observed in wild-type cells. This data suggests that AhR is protected from ligand-binding *in vivo* in Adr^R cells.

MATERIALS AND METHODS

1. Drugs and reagents

TCDD and BPDE were purchased from the Midwest Research Institute (Kansas City, MO); [^{3}H]TCDD from ChemSyn Laboratories (Lenexa, KS); and BP from Sigma Chemical Co. (St. Louis, MO). BP and BPDE were dissolved in dimethyl sulfoxide and TCDD in acetone. A 1200 base pair fragment from a *PstI* digestion of rat *CYP1A1* cDNA (plasmid courtesy of Dr. Alan Anderson) was

used as a probe for Northern blot analysis. Human β-actin oligonucleotide probe was purchased from Clontech (Palo Alto, CA). Polyclonal anti-AhR antibodies were obtained from Dr. Allan Okey and Affinity BioReagents (Golden, CO); polyclonal anti-Arnt antibody was a gift from Dr. Oliver Hankinson; monoclonal α-tubulin was purchased from Boehringer Mannheim Canada, Laval, Quebec; and monoclonal anti-ER (Ab-1, clone AER314) from NeoMarkers, Freemont, CA.

2. Cell lines and culture conditions

MCF-7 wild-type cells and resistant sublines were maintained in RPMI media supplemented with 10% fetal bovine serum, 50 International Units/ml penicillin, and 50 μg/ml streptomycin. The BP^R cell line was selected by exposing MCF-7 cells to increasing concentrations of BP. When cells were able to survive at a given level of drug, the concentration was increased in a stepwise manner until growth at 10 μM BP was achieved. Adr^R cells were described previously [458]. LS180 is a human colon adenocarcinoma cell line known to express high levels of AhR [466].

3. Northern blot analysis

Total RNA was isolated from cells in log growth phase using the RNAzol B (Tel-Test, Friendswood, TX) method by the manufacturer's guidelines. Twenty-five μg of RNA for each sample was loaded onto a 1.1% agarose/formaldehyde/ethidium bromide gel. The samples were run at 100 V for 3 h and a photograph of the gel was taken. The RNA was transferred to a Zeta-Probe membrane (Bio-Rad, Mississauga, Ontario, Canada) by capillary transfer overnight and cross-linked with ultraviolet light. Oligonucleotide probes were radiolabeled with [α-^{32}P]dCTP by random primer extension (Oligo Labeling Kit; Pharmacia, Baie d'Urfe, Quebec, Canada). Hybridization was

performed in 40% formamide, 4X SSC, 4X Denhardt's reagent, 0.2 mg/ml salmon sperm DNA, 1.2% SDS and 10% dextran sulphate at 42°C overnight. Final high-stringency washes were carried out in 0.1X SSC/0.1% SDS at 65°C. Membranes were reprobed for β-actin expression to standardize for loading differences.

4. Cytotoxicity assay

Cells were plated out in 200 μl of medium at a concentration of 1000 cells/flat-bottomed well in 96-well microtiter plates and incubated overnight at 37°C in an atmosphere of 5% carbon dioxide. 200 μl of medium containing drug dissolved in appropriate solvent were added to wells and incubated for a further 72 h. Medium was then removed from each well and replaced by 150 μl of medium containing 10 mM 1,4-piperazinediethanesulfonic acid (pH 7.4) and 50 μl MTT (Sigma) at 2 mg/ml in PBS. Plates were then wrapped in aluminum foil and incubated for 4 h at 37°C. Medium was removed and the formazan crystals that remained were dissolved in 200 μl dimethyl sulfoxide and 25 μl glycine buffer [0.1 M glycine, 0.1 M NaCl (pH 10.5)]. Quantitation of cell viability was performed by measuring the absorbance at a wavelength of 570 nm on a microtiter plate reader. The negative control (background) consisted of wells which were administered medium although no cells were seeded. For the positive control (100% viability) cells did not receive drug treatment.

5. Sequencing

The DNA-binding domains of AhR and Arnt [467] and the ligand-binding domain of AhR [468] were PCR-amplified (see Table 4 for primers) and purified from a 1.2% agarose gel using Wizard PCR Preps (Promega, Madison, WI). These oligonucleotides were sequenced from both ends using the GibcoBRL dsDNA Cycle Sequencing System (Canada Life Technologies, Burlington, Ontario,

TABLE 4. Sequence of oligonucleotides

Oligonucleotide†	Sequence*	Assay‡
U-AhR/DBD	GCAGTGGTCCCAGCCTACAC	Seq
L-AhR/DBD	GCAGGCTAGCCAAACGGTCC	
U-AhR/LBD	CATCTAAGCTTGCCGCCATGAATTTCCAAGGGAAG	Seq
L-AhR/LBD	AGTCCCTCGAGGTTAGGGATCCATTATGGCA	
U-Arnt/DBD	AGGTCGGATGATGAGCAGAGC	Seq
L-Arnt/DBD	ATGTGTTGCCAGTTCCCCGC	
U-β-Actin	CGTGATGGACTCCGGTGACGGGG	Q-PCR
L-β-Actin	GATGGAGTTGAAGGTAGTTTCGTG	
U-AhR	ATACTTCCACCTCAGTTGGC	Q-PCR
L-AhR	AAAGCAGGCGTGCATTAGAC	
U-Arnt	CGGAACAAGATGACAGCCTAC	Q-PCR
L-Arnt	ACAGAAAGCCATCTGCTGCC	
XRE	GATCTGGCTCTTCTCACGCAACTCCG	EMSA

†U, upper; L, lower; AhR, Ah receptor; DBD, DNA-binding domain; LBD, ligand-binding domain; XRE, xenobiotic responsive element

*All oligonucleotides are single-stranded , except for XRE which is double-stranded

‡Seq, sequencing; Q-PCR, semi-quantitative reverse transcribed-polymerase chain reaction; EMSA, electrophoretic mobility shift assay

Canada) with the same primers. Reaction products were resolved through 6% polyacrylamide gels containing 7 M urea at 50°C. Gels were transferred to Whatmann 3MM paper, dried, and autoradiographed.

6. Preparation of cell lysates

Cells in exponential phase growth were washed with PBS and detached from flasks by trypsinization [0.05% trypsin, 0.5 mM EDTA] and sedimented. All proceeding steps were carried out on ice. Cell pellets were washed again in PBS and then resuspended in lysis buffer containing 25 mM HEPES (pH 7.4), 20 mM sodium molybdate, 5 mM EGTA, 3 mM $MgCl_2$, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 0.5% NP-40 and 10% glycerol. Suspensions were sonicated for 10 sec and supplemented with phenylmethylsulfonyl fluoride (100 μM) and 150 units of DNase. The lysate was incubated for 4 min and then sonicated for an additional 10 sec. At this time, a sample was removed for protein determination by the Bradford assay (Bio-Rad) and the remainder of the sample was combined with an equal volume of 2X gel sample buffer [125 mM Tris (pH 6.8), 4% SDS, 25% glycerol, 4 mM EDTA, 20 mM dithiothreitol, 0.005% Bromophenol blue]. Samples were heated at 100°C for 5 min and stored at -20°C.

7. Gel electrophoresis and Western blotting

Protein samples were resolved through 7.5% polyacrylamide-sodium dodecyl sulfate gels and electrophoretically transferred to nitrocellulose by standard techniques. The blots were stained with Ponceau S to verify the efficiency of transfer and the gel loading. If areas of the gel did not transfer properly or if differences in gel loading were apparent, the blot was not used. Membranes were blocked overnight in BLOTTO buffer [50 mM Tris (pH 7.5), 0.2% Tween-20, 150 mM NaCl, 5% dry milk powder] at 4°C and then washed 4

times in TTBS+ buffer [50 mM Tris, 0.5% Tween-20, 300 mM NaCl (pH 7.5)] for a total of 40 min. Immunohistochemical staining was carried out with 1:1000 anti-AhR antibody or 90 ng/ml anti-Arnt antibody in BLOTTO buffer for 2 h at room temperature. Membranes were washed as previously and incubated with 1:10,000 horseradish peroxidase-conjugated secondary antibody in BLOTTO buffer for 1 h at room temperature. Following another wash cycle, the blots were developed with the enhanced chemiluminescence kit (Amersham Canada, Oakville, Ontario). In some cases the blot was stripped and reprobed with antibodies against α-tubulin to ensure gel loading was equivalent from sample to sample.

8. Semi-quantitative RT-PCR analysis

Samples of total RNA (see above) were subjected to DNase treatment and reprecipitated. Five μg of RNA was used to prime cDNA synthesis (Gibco/BRL Superscript preamplification system; Canada Life). The 50 μl reaction volume contained 100 nM primers, 200 μM dNTPs, 3 μCi [α-^{32}P]dCTP, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), cDNA and 2.5 Units of Taq polymerase (Pharmacia). After 4 min at 94°C, PCR comprised of 25 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final round of extension at 72°C for 10 min. Samples (10 μl of each PCR product) were subjected to 10% polyacrylamide gel electrophoresis in TBE buffer [90 mM Tris, 90 mM boric acid, 3 mM EDTA]. Gels were transferred to Whatmann 3MM paper, dried, and autoradiographed. The primer sequences for β-actin, AhR [296, 469] and Arnt [296] are found in Table 4. To ensure that PCR amplification was linear, a series of PCR reactions were initially performed over a range of MCF-7 cDNA dilutions. These data were plotted in a graph of optical density units versus cDNA volume and a suitable cDNA dilution was chosen for each set of primers

such that PCR amplification fell within the linear range. Endogenous β-actin mRNA expression was used as an internal control to account for differences in RNA quantity and integrity between samples.

9. Preparation of nuclear extracts

Nuclear extracts were prepared according to Whitlock and Galeazzi [470], with minor changes. All steps were performed on ice or at 4°C. Confluent cells were washed with PBS (without Ca^{2+} or Mg^{2+}), harvested by scraping, and centrifuged at 1500 RPM in a Sorvall RT6000B centrifuge. The cellular pellet was resuspended in 5 volumes of 10 mM HEPES (pH 7.5). After swelling 10 min, the cells were collected by centrifugation as before and resuspended in 5 volumes of 3 mM $MgCl_2$, 1 mM dithiothreitol, 25 mM HEPES (pH 7.5), and 0.01 mg/ml each of the protease inhibitors aprotinin, leupeptin, and pepstatin. Cells were again collected by centrifugation and resuspended in 2 volumes of the same buffer. Cells were broken with 15 strokes in a Dounce homogenizer using a tight pestle and immediately centrifuged at 15,000g. The crude nuclear pellet was resuspended in 2 volumes of 0.1 M KCl, 25 mM HEPES (pH 7.5), 1 mM dithiothreitol, and protease inhibitors. Nuclei were lysed by adding 2 M KCl to a final concentration of 0.4 M and mixing gently for 30 min. The lysate was adjusted to 20% glycerol and centrifuged at high speed in an Eppendorf 5402 microcentrifuge for 30 min. The transparent supernatant was aliquotted in small volumes, snap frozen on dry ice, and stored at -80°C.

10. Electrophoretic mobility shift assay

15 μg of nuclear extract protein was incubated with a [^{32}P] end-labeled oligonucleotide (Table 4) containing an internal XRE from the human *CYP1A1* gene [471] in the presence of 1 μg of poly(dI-dC)·poly(dI-dC). Binding reactions

were carried out in 25 mM HEPES (pH 7.5), 1 mM dithiothreitol, 0.15 M KCl, and 10% glycerol in a volume of 30 µl at 20°C. Samples were layered onto 5% polyacrylamide gels and electrophoresed in buffer consisting of 20 mM HEPES, 20 mM Tris, 1 mM EDTA (pH 8.0), at 4°C. Gels were fixed in 10% (v/v) methanol and 10% (v/v) acetic acid, transferred to Whatmann 3MM paper, dried, and autoradiographed.

11. Immunohistochemistry

Cells that had been plated the previous day on coverslips were fixed with 4% paraformaldehyde/PBS for 15 min at 37°C. Membranes were made permeable with a 0.3% Triton X-100/PBS solution for 5 min followed by blocking for 30 min with 5% BSA/PBS. The specimens were incubated for 2 h with AhR antibody (1:1000 dilution in 5% BSA/PBS) at 37°C. The cells were washed 3 times with PBS. The secondary antibody consisted of rhodamine-conjugated rat anti-rabbit IgG (1:100 dilution in 5% BSA/PBS) and was applied for 1 h at 37°C. Following another wash cycle, the coverslips were fixed to slides with polyvinyl alcohol. AhR cellular localization was determined using a Zeiss LSM410 confocal microscope.

12. TCDD uptake studies

Transport of TCDD in wild-type and adriamycin-resistant MCF-7 cells was determined according to the methods described by Schilsky *et al.* [472]. Briefly, MCF-7 wild-type and Adr^R cells were plated in T25 flasks in complete medium. When the cells were at 90-95% confluency, the medium was removed and replaced with 2 ml of 10 nM [^{3}H]TCDD in serum-free RPMI for various periods at 37°C. Drug uptake was stopped by the rapid addition of 10 ml of ice-cold PBS to the flasks. The cells were washed three times with 5 ml PBS, and

digested by the addition of 1 ml of 1 N NaOH. An aliquot of the lysate was taken for scintillation counting and another for protein assay. Results were determined as the mean of experiments performed in duplicate and expressed as pmoles TCDD per gram total cellular protein.

13. Steroid receptor assay

Cells were collected by scraping in PBS and centrifuged at 1500 RPM in a Sorvall RT6000B centrifuge at 4°C. Cells were washed again in PBS and recentrifuged. The pellet was resuspended in 10 volumes phosphate-glycerol buffer [1 mM NaH_2PO_4, 4 mM Na_2HPO_4, 10 mM sodium molybdate, 0.1% monothioglycerol, 10% glycerol (pH 7.5)] and homogenized with a Polytron homogenizer. Extracts were incubated with 7.6 nM [^{3}H]estradiol (New England Nuclear Corp., Boston, MA) alone or in the presence of 100-fold excess of unlabeled estradiol (Sigma). Specific binding was determined using a dextran-coated charcoal assay procedure described previously [473].

14. Densitometry

Radiographic and chemiluminescent exposures were scanned into a Power Macintosh computer with a UMAX VistaS-12 scanner and Adobe Photoshop 3.0.5 software. Images were then quantified with the use of National Institutes of Health Image 1.61 software. Quantification was performed as follows. A rectangular tool was produced that surrounded the largest band of interest. The size of this tool was held constant for all measurements within a specific set of samples. The mean value of the intensity within the tool was then determined for: (i) the band of interest, (ii) the area directly above the band, and (iii) the area directly below the band. The intensities of the area above and below the band of interest were then averaged and subtracted from the band of interest.

RESULTS

MCF-7 cells were cultured in escalating concentrations of benzo[*a*]pyrene over a 5 month period leading to the establishment of BP^R cells. This was the identical procedure used for selection of adriamycin-resistant MCF-7 cells [457]. Induction of CYP1A1, an essential enzyme in the metabolism of BP to BPDE, was analyzed to determine the mechanism of resistance. As shown in Fig. 9, BP^R cells possess a very limited capacity to transactivate *CYP1A1* gene expression in the presence of either BP or TCDD. Cytotoxicity studies based on the MTT assay show that the IC_{50} for BP is approximately 2 orders of magnitude greater for BP^R cells compared to WT cells (Fig. 10A). To rule out the possibility that resistance is associated with other mechanisms such as enhanced phase II detoxification, DNA repair, or efflux of BP out of the cell, MTT assays were carried out using the activated metabolite, BPDE (Fig. 10B). These experiments show that WT and BP^R cells are equally sensitive to BPDE, indicating that resistance is mediated through decreased phase I activation of BP.

We analyzed components of the Ah receptor signaling pathway for alterations in genetic structure, expression, and activity. AhR and Arnt are members of a family of transcription factors containing basic, helix-loop-helix, and PAS motifs. The functional domains of the AhR and Arnt have been investigated by deletion, site-specific mutation, and chimeric protein analyses [228, 232, 235, 474]. These studies suggest that residues within the basic region specify the DNA recognition site, whereas the ligand-binding domain of AhR overlaps with the PAS region. We sequenced the DNA-binding domains of AhR and Arnt and the ligand-binding domain of AhR. However, no mutations were detected in the benzo[*a*]pyrene- and adriamycin-resistant MCF-7 cell lines.

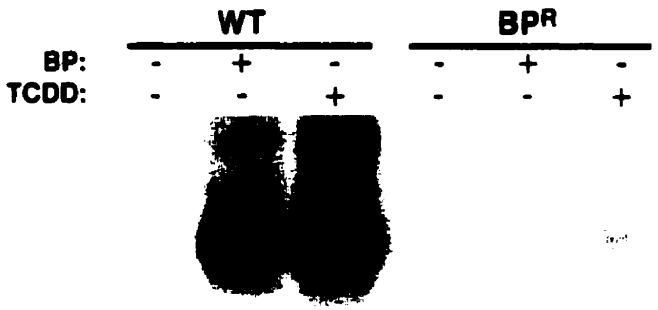

Figure 9. *CYP1A1* mRNA levels in MCF-7 wild-type (WT) and benzo[*a*]pyrene-resistant (BP^R) cell lines. Cells were treated with either solvent, 1 μM BP, or 100 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) for 24 h prior to RNA isolation. Northern blot analysis was performed with 25 μg of RNA in each lane. Membranes were stripped and rehybridized with a β-actin probe to standardize for loading differences. These data are representative of three separate experiments.

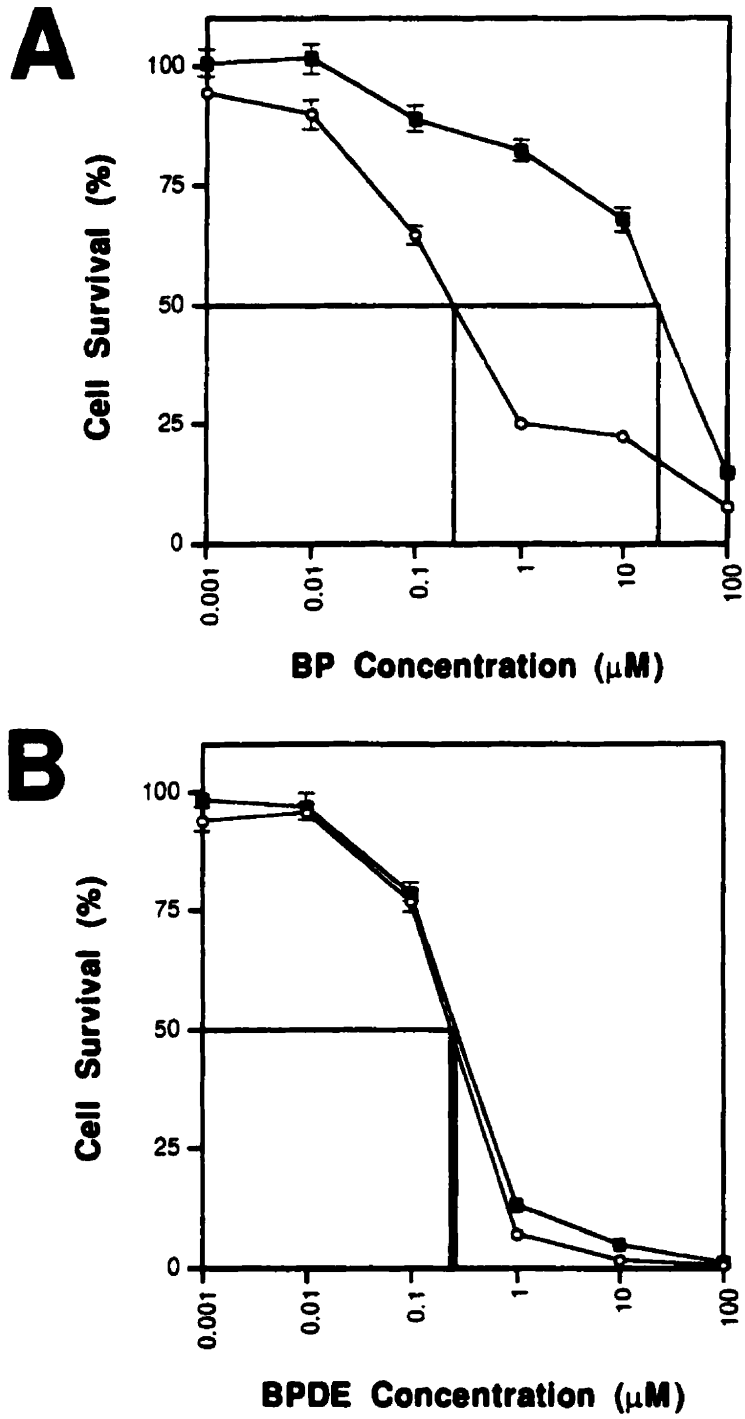

Figure 10. Cytotoxicity studies. MCF-7 wild-type (O) and benzo[*a*]pyrene-resistant (■) cells were treated with benzo[*a*]pyrene (BP) or the activated metabolite, BP-7,8-dihydrodiol-9,10-epoxide (BPDE), for 72 h in 96-well microtiter plates. Cell survival was determined by a colorimetric assay described in "Materials and Methods" which measures the ability of living cells to reduce a dye to a formazan by-product. These data are representative of three experiments. Results are the average of 12 wells per drug concentration per cell line and are expressed as ±SEM.

Immunoblot analyses were performed to determine whether the deficiency in *CYP1A1* induction could be attributed to decreased AhR and Arnt expression. As shown in Fig. 11, AhR protein levels in Adr^R cells are equivalent to WT cells, whereas BP^R cells have about a 4-fold decrease in expression. In addition, Adr^R cells have a 3-fold decrease in Arnt protein expression relative to WT and BP^R cells (Fig. 11). Semi-quantitative RT-PCR analysis was utilized to determine if the decrease in AhR expression found in BP^R cells was a result of lower *AhR* mRNA levels (Fig. 12). Indeed, *AhR* mRNA levels are decreased 3-fold in the BP^R subline, and Arnt levels are not altered. Therefore, benzo[*a*]pyrene-resistance is associated with down-regulation of *AhR* mRNA in this model.

Nuclear extracts were combined with a radiolabeled XRE to determine if the DNA-binding capability of activated AhR is decreased in the resistant sublines (Fig. 13). LS180 cells, which have previously been shown to overexpress AhR, were used as a control. The *arrow* in Fig. 13 indicates the AhR/Arnt complex, since this band is induced by TCDD (lane 3 versus lane 2), and anti-AhR or -Arnt antibodies completely inhibited the specific retardation of the XRE (lanes 5 and 6, respectively), whereas a non-specific control antibody does not (anti-ER, lane 7). The epitope recognized by the anti-AhR antibody occurs within the DNA-binding domain (amino acids 13-31), whereas the anti-Arnt antibody does not obstruct DNA-binding or dimerization which causes the AhR/Arnt/antibody complex to be supershifted in the loading well (lane 6). In these experiments nuclear extracts from BP^R cells showed little affinity for the XRE, which is concordant with AhR expression in this cell line. Although Adr^R cells have no detectable increase in AHH activity when exposed to TCDD [342, 458], and express a relatively low level of Arnt, nuclear proteins from this subline possessed a greater ability to bind a XRE compared to extracts prepared from WT

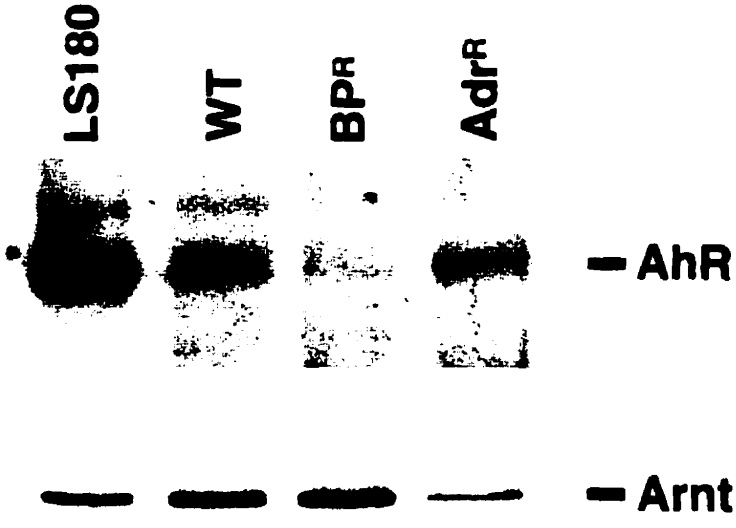

Figure 11. Analysis of Ah receptor (AhR) and Arnt protein levels in MCF-7 wild-type and xenobiotic-resistant cell lines. The presence of AhR and Arnt in total cellular extracts were detected by immunoblot analyses. LS180 is a human colon adenocarcinoma cell line known to overexpress AhR. Amounts loaded: AhR, 100 μg protein/lane; Arnt, 20 μg protein/lane. Relative protein levels (as a percentage of wild-type) for BP^R and Adr^R cells are: AhR protein, 28.0±7.6 and 140.7±41.6, respectively; Arnt protein, 120.4±8.7 and 33.0±0.3, respectively. Results are expressed as ± SEM for 3 determinations for each experiment.

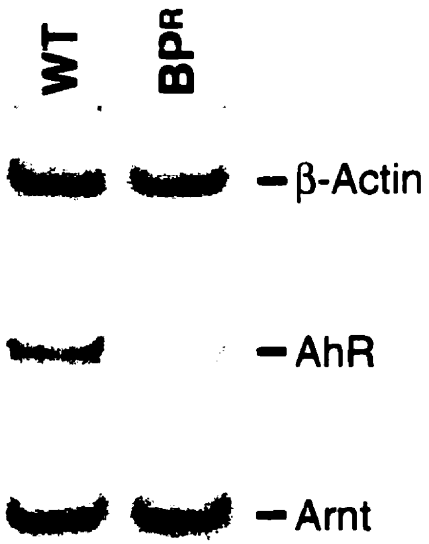

Figure 12. Representative semi-quantitative RT-PCR analysis of Ah receptor and Arnt expression in MCF-7 wild-type and benzo[*a*]pyrene-resistant cell lines. 5 μg of DNase-treated total RNA was reverse transcribed in a final volume of 20 μl. A volume of this cDNA (1/10 μl for AhR; 1/100 μl for Arnt; 1/10000 μl for β-actin) was chosen such that PCR amplification was linear for each gene of interest. Conditions for "hot PCR" and subsequent gel electrophoresis are described in "Materials and Methods." β-actin expression was used as an internal control. Relative mRNA levels (as a percentage of wild-type) for BP^R cells are: AhR mRNA, 30.1±4.4; Arnt mRNA, 73.1±10.4. These results are expressed as ± SEM for 2 experiments performed with different RNA preparations.

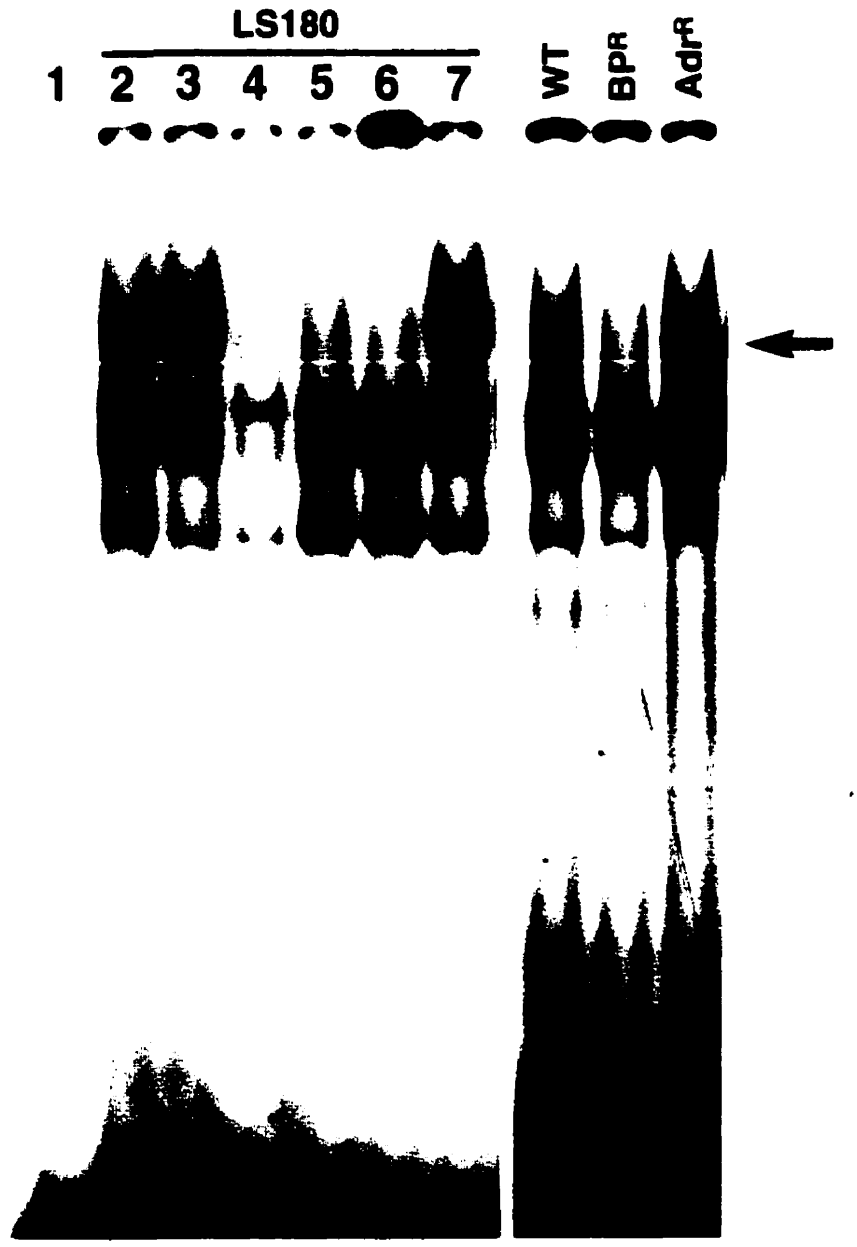

Figure 13. Electrophoretic mobility shift assays using an oligonucleotide containing an internal xenobiotic responsive element (XRE). *Arrow* indicates the Ah receptor/Arnt/XRE complex. Lane 1 was run with radiolabeled probe only. All remaining lanes were loaded with 15 μg nuclear protein that had been incubated with probe as described in "Materials and Methods." Nuclear extracts were obtained after cells were treated with 10 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in serum-free medium for 1 h, except for lane 2 in which cells were treated with solvent only. Lane 4 was competed with 100 M excess cold XRE. Lanes 5-7 had additions of anti-Ah receptor, -Arnt, and -estrogen receptor antibodies, respectively. This data is representative of at least three separate experiments.

cells. These data show that Arnt is not a limiting factor for AhR signaling in MCF-7 cells, and that loss of Ah-responsiveness in Adr^R cells is independent of AhR/Arnt expression and DNA-binding activity.

It is conceivable that MCF-7 cells selected for resistance to adriamycin are transformed to an Ah-nonresponsive phenotype through an alteration in the subcellular distribution of AhR. To address this question we investigated AhR localization by confocal microscopy. WT and Adr^R cells were plated out on coverslips. The following day cells were exposed to 1 nM TCDD for 0 h, 1 h or 2.5 h. As shown in Fig. 14, the pattern of AhR subcellular distribution is not parallel between the two cell lines. In WT cells (panel 1A) outlines of the nuclei are clearly demarcated and AhR is visualized throughout the cytoplasm, whereas in Adr^R cells (panel 2A) AhR appears in globular pockets towards the cell periphery. Treatment with ligand resulted in a rapid depletion of AhR in WT cells (panels 1A-C). In contrast, TCDD had less of an effect on AhR expression in Adr^R cells (panels 2A-C). Triplicate immunoblot experiments confirmed that a difference in the rate of AhR protein turnover exists between WT and Adr^R cells (data not shown). Quantitation of AhR expression by densitometry determined that 4 h TCDD exposure (10 nM) depleted AhR to 15% of basal levels in WT cells, compared to 45% in Adr^R cells. These results prompted us to investigate TCDD transport in these cell lines. Surprisingly, our results indicated that the rate of TCDD uptake and total intracellular level of TCDD in Adr^R cells was higher than in the parental MCF-7 cell line (Fig. 15).

Vickers *et al.* [341] noted that there was a correlation between Ah-responsiveness and ER expression in human breast cancer cell lines and suggested that there may be common factor(s) which regulate both AhR and ER signal transduction pathways, although these remain unknown. To determine the ER status of the BP^R sublines, protein levels were quantitated by competitive

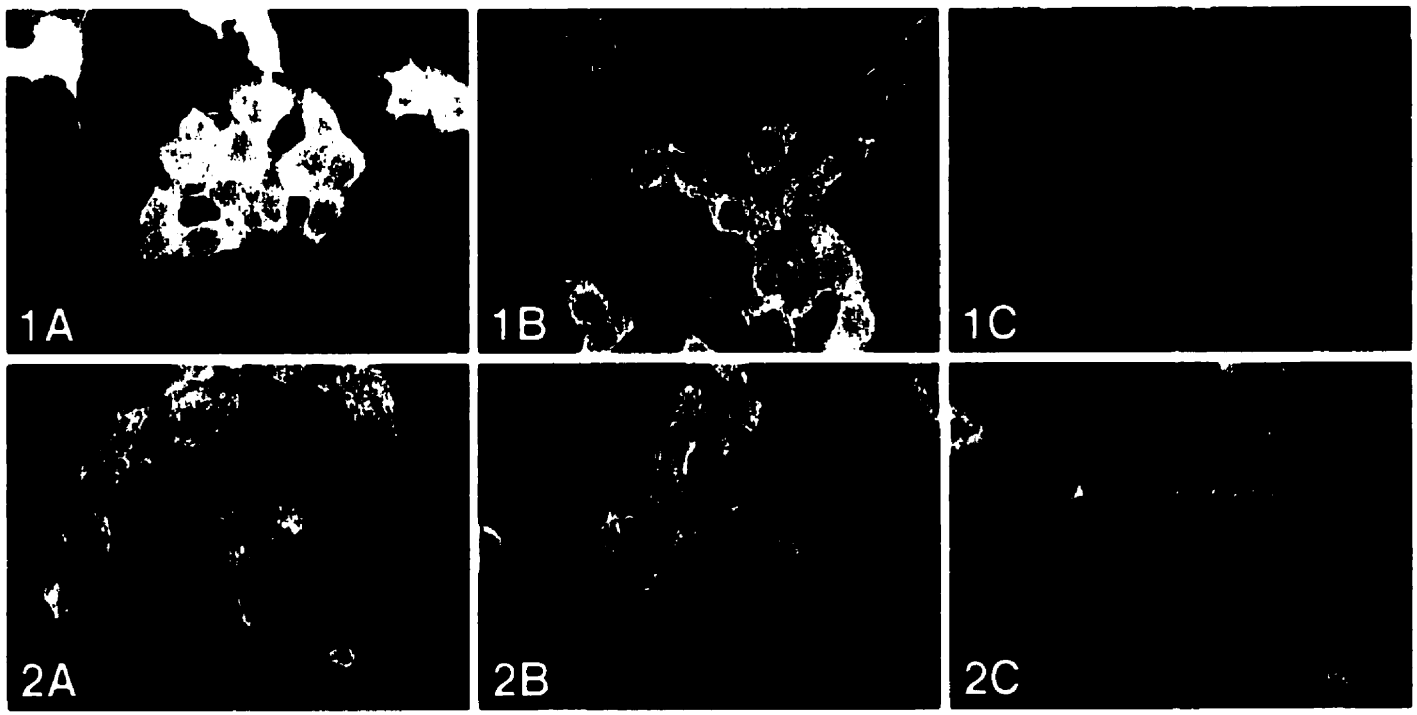

Figure 14. Effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposure on Ah receptor localization in (1) wild-type and (2) adriamycin-resistant MCF-7 cell lines. Cells plated the previous day on coverslips were incubated with 10 nM TCDD for (A) 0 h, (B) 1 h, or (C) 2.5 h followed by immunohistochemical labeling of Ah receptor as described in "Materials and Methods." Fluorescence was visualized by confocal microscopy. This experiment was repeated with similar results.

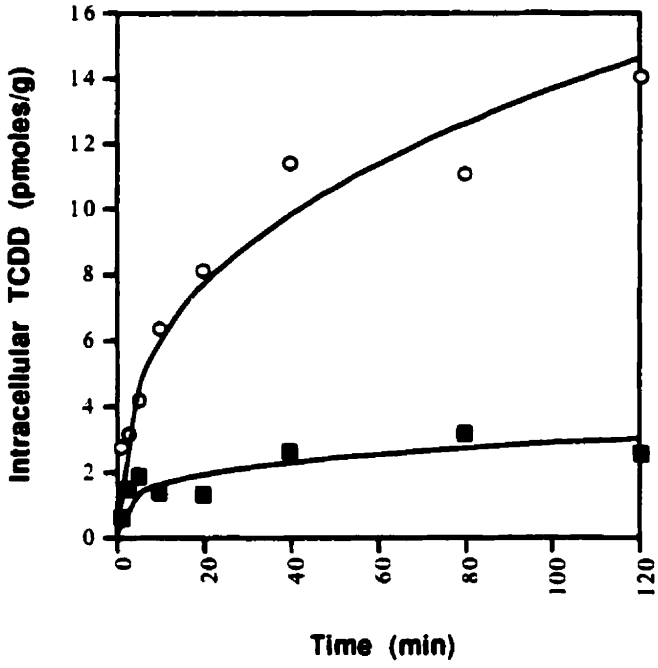

Figure 15. TCDD uptake in MCF-7 wild-type (o) and adriamycin-resistant (■) cell lines. Cells growing in T25 flasks were exposed to 10 nM [^{3}H]TCDD (specific activity 22.2 Ci/mmol) in serum-free RPMI medium. Following incubation at 37°C for the designated times, the cells were washed and the total intracellular counts were determined as described under "Materials and Methods." This data is representative of two experiments, each performed in duplicate.

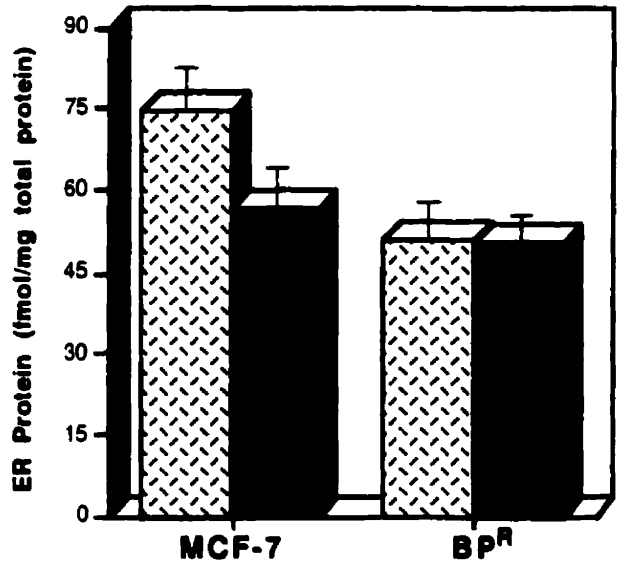

Figure 16. Determination of estrogen receptor (ER) levels in MCF-7 wild-type and benzo[*a*]pyrene-resistant cell lines. Cells were treated with solvent (*stippled* bars) or agonist (1 μM benzo[*a*]pyrene or 100 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; *solid* bars) for 24 h. Aliquots of total cellular extract were incubated for 4 h at 4°C with [^{3}H]estradiol either alone or in the presence of 100-fold excess of unlabeled estradiol as described in "Materials and Methods." Specific binding was determined using a dextran-coated charcoal assay procedure. Results shown are the arithmetic mean of 3 experiments and are expressed as ± SEM.

binding of a radioligand. Fig. 16 (*stippled* bars) shows a significant decrease ($p=0.0437$) in the level of ER in BP^R cells compared to WT cells. Moreover, acute exposure (24 h, *solid* bars) to BP or TCDD caused a significant down-regulation ($p=0.0461$) of ER in WT cells, but had no effect on BP^R cells.

DISCUSSION

We have cultured MCF-7 cells in escalating concentrations of BP leading to the establishment of a resistant subline. Ah-responsiveness was initially characterized by quantitation of *CYP1A1* mRNA levels. These studies showed that *CYP1A1* gene expression is severely repressed in the BP^R cell line. From cytotoxicity assays showing that WT and BP^R cells are equally sensitive to BPDE, we were able to demonstrate that resistance is mediated through decreased phase I activation of BP. Furthermore, our data strongly indicates that AhR signaling is inhibited in BP^R cells via down-regulation of the AhR. This conclusion is drawn from experiments showing significantly lower AhR mRNA and protein by semi-quantitative RT-PCR, immunoblot and band shift analyses.

Previously, Moore *et al.* [475] isolated a BP-resistant MCF-7 clone and characterized loss of Ah-responsiveness by showing that these cells were less responsive to TCDD as determined by *CYP1A1* mRNA induction, *CYP1A1* promoter-CAT activity, EROD activity and XRE binding studies, and by showing that resistant cells metabolized BP at a slower rate. Basal levels of AhR and Arnt were not examined, although velocity sedimentation analysis after exposure to [^{3}H]TCDD for 2 h indicated that AhR levels were about 50% higher in the BP^R clone [475]. In a subsequent study this group reported that, relative to WT cells, there was a significant reduction (by about 40%) of *AhR* mRNA levels in BP-resistant T47-D cells, whereas *Arnt* mRNA levels were unchanged [476]. Zhang *et*

al. [477] showed that *AhR* mRNA levels were similarly reduced in a group of BP-resistant mouse Hepa1c1c7 clones. The authors suggest that lower *AhR* mRNA levels result from altered expression or mutation of a transcription factor that causes an open chromatin structure in the vicinity of the *AhR* gene since: (i) fusion of a BP-resistant clone with a rat hepatoma cell line restores *AhR* mRNA expression, and (ii) the reversion frequency of a clone was increased 20-fold with exposure to a chemical mutagen, suggesting that the gene was not silenced by an epigenetic mechanism [477]. Taken together with our results, it is appears that down-regulation of AhR at the level of transcription is a common mechanism by which cells can adapt to prolonged BP exposure.

AhR and ER signal transduction pathways interact with one another on at least two levels. Firstly, TCDD and other AhR agonists suppress a broad range of estrogen-induced responses in laboratory animals [302, 305] and mammalian cells in culture [310, 312, 478]. Secondly, Ah-responsiveness has been shown to correlate with ER expression in a panel of human breast cancer cell lines [341]. For example, MCF-7 cells are ER^+ and Ah-responsive, whereas MDA-MB-231 cells do not express ER and are Ah-nonresponsive. In addition, transient transfection of an ER expression vector converts MDA-MB-231 cells to an Ah^+ phenotype [347]. We quantitated ER protein levels to determine if there was correlation between ER status and Ah-responsiveness in the BP-resistant MCF-7 subline. We found BP^R cells to be ER^+, although expression was reduced by 33% compared to WT cells. Down-regulation of cellular ER content may be one mechanism by which AhR agonists function as antiestrogens. *In vivo* studies have shown that 2 day TCDD exposure decreases hepatic and uterine ER in murine and rat models by up to 42% [303, 304, 317]. To determine whether short-term exposure also decreases ER *in vitro*, we exposed MCF-7 cells to TCDD and BP for 24 h. These experiments showed a 25% reduction of ER expression in WT cells,

but ligand did not alter ER expression in the BP^R cell line. It is not known at the present time whether this level of ER suppression is associated with the antiestrogenic nature of AhR agonists, or is sufficient to affect Ah-responsiveness.

Adriamycin-resistant MCF-7 cells are cross-resistant to benzo[*a*]pyrene [342, 458]. AHH activity is undetectable in this cell line, and TCDD failed to induce either endogenous *CYP1A1* expression or a transfected construct containing the normal mouse *CYP1A1* promoter fused to a *CAT* gene [342]. These results indicated that the defect was due to an alteration in AhR signaling and not a mutation in the regulatory region of the *CYP1A1* gene. We found no significant differences in AhR expression between WT and Adr^R cells. One possibility is that AhR activity is down-regulated in Adr^R cells by a post-translational mechanism. For example, phosphorylation of residues within the AhR DNA-binding domain may be a critical determinant for receptor signaling [280, 283, 479]. Our results show that the transformed AhR complex was able to bind a XRE in electrophoretic mobility shift assays. Thus, AhR DNA-binding activity does not appear to be aberrant in Adr^R cells. This data is in agreement with previous studies which have indicated that AhR expression or XRE binding may not be predictive of Ah-responsiveness [341, 480, 481].

TCDD induces a rapid loss of AhR in cultured cell lines [221, 482, 483]. In mouse hepatoma cells (Hepa-1), it has been shown that less than 20% of the total cellular AhR content is present after 6 h treatment with TCDD, and this effect is prolonged for at least 3 days [484, 485]. Our experiments show that AhR is depleted to 15% of normal levels in WT cells after 4 h TCDD exposure. In contrast, 45% of AhR remained in Adr^R cells over this time-course. P-glycoprotein has been reported to act as an energy-dependent drug efflux pump for a wide range of xenobiotics, including benzo[*a*]pyrene [486]. However, drug

uptake studies showed that P-glycoprotein overexpression does not decrease the level of intracellular TCDD in Adr^R cells. Okey and coworkers [484] have shown that a decrease in AhR protein following exposure to TCDD was not accompanied by a decrease in mRNA. It is plausible that AhR becomes more vulnerable to proteolytic cleavage during receptor activation. The HSP90-binding domain colocalizes with the ligand-binding domain of AhR and steroid hormone receptors. In the process of ligand activation, HSP90 becomes disassociated from the receptors. Furthermore, the presence of ligand depletes cellular receptor levels, which is accompanied by a change in the half-life of the receptor proteins. This same effect occurs when cells are treated with the HSP90 inhibitor geldanamycin [348]. For example, a 75% depletion in AhR was found to occur within one hour of exposure to geldanamycin in HeLa and Hepa 1c1c7 cells [355]. In the case of the glucocorticoid receptor, there is evidence that receptor degradation following geldanamycin treatment involves the ubiquitin-proteasome pathway [378].

The DNA binding activity of AhR from cell extracts may not accurately reflect the ability of AhR to bind DNA *in vivo*. For example, we have shown by confocal microscopy that the pattern of AhR subcellular distribution is altered in the Adr^R subline. This in turn may contribute to loss of Ah-responsiveness if AhR colocalizes with a high concentration of inhibitory factors such as HSP90. Members of the HSP90 family are cytosolic proteins that have been shown to interact with unliganded or liganded receptors by a dynamic process, and to inhibit their native DNA binding activity [389, 390]. Based on results showing that AhR expression and DNA-binding activity are similar in WT and Adr^R cells, but rates of AhR protein turnover and *CYP1A1* transactivation are decreased in the latter, our data suggests that AhR may be protected from ligand-binding in Adr^R cells.

In summary, these studies have established the following conclusions: (i) BP-resistance is associated with down-regulation of *AhR* mRNA in MCF-7 cells, (ii) the Ah-nonresponsive phenotype of BP-resistant MCF-7 cells is not associated with a loss of ER, although a 33% reduction of expression was found, (iii) despite an Ah-nonresponsive phenotype, AhR expression and DNA-binding activity are normal in adriamycin-resistant MCF-7 cells, and (iv) TCDD-induced AhR depletion occurs about 3-fold less efficiently in Adr^R cells compared to the parental cell line.

CHAPTER IV

ROLE OF HSP90 IN MEDIATING CROSS-TALK BETWEEN THE AHR AND ER SIGNAL TRANSDUCTION PATHWAYS

PREFACE TO CHAPTER IV

The AhR and members of the steroid hormone receptor and thyroid hormone nuclear receptor families are ligand-activated transcription factors. Although similarities exist with respect to function, the different classes of receptor proteins are structurally divergent. It is for this reason that interference between the three receptor families is an unexpected finding. Presently, mechanisms underlying receptor interference and cooperation are largely unknown.

In this chapter, HSP90 was investigated as a potential mediator of cross-talk between the AhR and ER signal transduction pathways. Elucidation of the interference mechanism is an important objective for several reasons. For instance, this mechanism may prove to be a universal means by which Ah/steroid/thyroid receptors interact with one another.

In addition, insight into the mode of interference between TCDD and the ER signaling pathway could have a direct clinical application. About one-third of primary mammary tumors are ER-positive and respond to endocrine therapy. Thus far, the nonsteroidal antiestrogen tamoxifen has been the endocrine treatment of choice for all stages of breast cancer. However, concerns about the side effects associated with tamoxifen and the development of drug resistance during long-term therapy has led to the search for novel antiestrogenic agents. TCDD is a potent antiestrogen but the high toxicity of this compound precludes its use as an antineoplastic drug. With a better understanding of the AhR-ER interaction, it may be possible to mimic the antiestrogenic effect of TCDD without the deleterious side effects associated with CYP activation, thereby generating a novel endocrine therapeutic agent.

ABSTRACT

Tetrachlorodibenzo-*p*-dioxin (TCDD)-mediated gene transactivation via the Ah receptor (AhR) has been shown to be dependent upon estrogen receptor (ER) expression in human breast cancer cells. We have investigated the 90 kDa heat shock protein (HSP90) as a mediator of cross-talk between the AhR and ER signal transduction pathways. The effect of HSP90 overexpression on receptor activity was determined by transient transfection assays using a HSP90 expression vector. Ligand-inducible gene expression was inhibited when the HSP90 expression vector was cotransfected with a TCDD-responsive reporter plasmid. However, overexpression of HSP90 did not block induction of an estrogen-responsive reporter plasmid. To determine whether ER facilitates AhR signaling through its ability to squelch HSP90, two vectors which express protein products that bind HSP90 were transfected into MDA-MB-231 cells. Introduction of: (i) He11, an ER deletion mutant that does not bind DNA, and (ii) the ligand-binding domain of human AhR, both led to increased basal and TCDD-inducible expression of *CYP1A1* mRNA. Finally, the subcellular distribution of HSP90 was investigated in human breast cancer cell lines. These studies showed HSP90 to be primarily cytoplasmic in ER-positive cell lines, whereas in matched ER-negative cell lines HSP90 was equally distributed between the cytoplasm and nucleus. Taken together, these results demonstrate that HSP90 can regulate AhR activity *in vivo*, and that Ah-responsiveness is dependent upon cellular ER content through a mechanism that involves HSP90.

INTRODUCTION

The AhR and Arnt are members of the PAS family of transcription factors. Ligand binding to cytosolic AhR initiates nuclear translocation and heterodimerization with Arnt. This complex modulates the transcription of Ah-responsive genes by binding to cognate DNA sequences, known as xenobiotic responsive elements, which are found in the vicinity of target genes. Although TCDD and related AhR agonists do not associate with the ER [313], these compounds exhibit antiestrogenic properties in animal models [303, 487] and in cell culture [310, 312]. Vickers *et al.* [341] demonstrated a second level of cross-talk between AhR and ER when they showed that the Ah-responsiveness (i.e. the ability of TCDD to induce *CYP1A1* expression) of human breast cancer cells is related to their ER content. Moreover, several ER-negative cell lines that express AhR are Ah-nonresponsive and these include MDA-MB-231 and adriamycin-resistant MCF-7 cells, whereas ER-positive wild-type MCF-7 cells are Ah-responsive, along with MDA-MB-231 cells transiently transfected with human ER (hER) [347, 458]. Presently, mechanisms of interference and cooperation between the AhR and ER signal transduction pathways are not clear.

The AhR and ER can be recovered from untreated target cells extracted in low-salt medium in a 8-9S form that does not bind DNA [390, 488]. The inactive 8-9S form is a complex of receptor with a set of proteins including a HSP90 dimer. In agonist treated cells, the receptors can be extracted under high salt conditions as a smaller 4-5S form, free of any detectable HSP90, and with DNA-binding activity [390, 488]. HSP90 proteins have a critical role in AhR and ER signal transduction. First, receptor activation is defective in a strain of

Saccharomyces cerevisiae that produce reduced levels (5%) of HSP90 [382, 384]. Second, the HSP90-binding domain colocalizes with the ligand-binding domain in each receptor [387, 468], which suggests that ligand may activate the receptor by displacing HSP90. Third, HSP90 is capable of blocking the binding of either receptor to its respective responsive element in *in vitro* assays [361, 389, 390]. Thus, it is generally believed that HSP90 acts to inhibit the intrinsic DNA binding activity of steroid hormone and Ah receptors either by steric interference and/or by passive interference with the dimerization step.

Here, we propose a model in which ER facilitates AhR signaling through its ability to squelch HSP90. This is based on experiments showing restoration of Ah-responsiveness in MDA-MB-231 cells transfected with receptor deletion mutants that can bind HSP90. Although it has been shown that HSP90 can inhibit the DNA-binding and transactivation activities of AhR and ER in cell-free systems, it has not been demonstrated whether HSP90 can repress AhR and ER transactivation *in vivo*. To address this question, we transfected HSP90 into two ER-positive, Ah-responsive cell lines. These studies indicated that overexpression of HSP90 does not affect ER signaling, but it does repress AhR-mediated gene transactivation. Finally, the subcellular localization of HSP90 and AhR in matched ER-positive and -negative human breast cancer cell lines was investigated.

MATERIALS AND METHODS

1. Cell culture

MCF-7 and T47-D human breast cancer cell lines and adriamycin-resistant MCF-7 cells [457] were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 International Units/ml penicillin and 50 μg/ml streptomycin. MDA-MB-231 cells were maintained in α-MEM with 10% fetal bovine serum and penicillin/streptomycin. The S30 cell line was established previously via stable transfection of a hER expression vector into MDA-MB-231 cells [489]. These cells were maintained in phenol red-free α-MEM supplemented with 10% charcoal-stripped fetal bovine serum, penicillin/streptomycin and 500 μg/ml of the antibiotic G418. For drug treatments, TCDD (Midwest Research Institute) was dissolved in acetone at a concentration of 20 μg/ml. 17β-estradiol (Sigma) was dissolved in ethanol at a concentration of 1 mg/ml, diluted with medium for a 10^{-5} M stock solution, and stored in small aliquots at -20°C.

2. Reagents

Anti-AhR antibody was a gift from Dr. Allan Okey, HSP90 expression vectors and BF4 antibody from Dr. Maria-Grazia Catelli, pRNH241c plasmid from Dr. Ronald N. Hines, *He11* cDNA from Dr. Sylvie Mader, and *CYP1A1* cDNA from Dr. Alan Anderson.

3. Transient transfection assays

The pGL3-1A1 plasmid was constructed by excising a fragment encompassing human *CYP1A1* promoter/exon 1 sequences -1140 to +59 from pRNH241c and subcloning it into the *SmaI* site of the pGL3-Basic luciferase vector (Promega). The HSP90 expression vectors utilized here were described by Kang *et al.* [370]: 90WT contains a copy of chicken HSP90 cDNA ligated into the pSVK3 expression vector; and 90NLS is a similar expression vector but with the addition of the nucleoplasmin NLS inserted into the HSP90 coding sequence. MCF-7 and T47-D cells in 12-well culture dishes were transfected with 250 ng of reporter plasmid (pGL3-1A1), 50 ng of expression vector (pSVK3, 90WT or 90NLS), 2 ng of the pRL-CMV *Renilla* luciferase control reporter vector (Promega) and 0.6 μl of Lipofectamine reagent by methods provided by the manufacturer (GibcoBRL). The following day, cells were treated with either vehicle or 10 nM TCDD for 24 h. Extracts were prepared from transfected cells and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's guidelines. Luciferase activity was calculated as pGL3-1A1 activity/pRL-CMV activity.

For CAT assays, cells seeded in 6-well plates were cotransfected with 2 μg of expression vector (pSVK3, 90WT or 90NLS), 2 μg of the vit-tk-CAT estrogen-responsive reporter plasmid [490], and 8 μl of Lipofectamine reagent. After a 5 h incubation, the medium was replaced with phenol red-free RPMI 1640 supplemented with 10% charcoal-stripped fetal bovine serum. The following day, cells were either left untreated or exposed to 100 nM estradiol for 24 h. Whole cell extracts were obtained to perform CAT enzyme assays. The acetylated and nonacetylated forms of [^{14}C]-chloramphenicol were separated by thin layer

chromatography, and quantification was performed using a phosphoimager. CAT activity was standardized to the protein content of the cellular extracts.

4. Stable transfections

He11 cDNA was ligated into the *EcoRI* site of pBluescript (Stratagene) and then subcloned into the *BamHI* and *KpnI* restriction sites of the pCEP4 episomal eukaryotic expression vector (Invitrogen). The human homologue of the murine AhR ligand-binding domain [474] was PCR-amplified from MCF-7 cDNA and subcloned into pCEP4. The upstream primer (CATCTAAGCTTGCCGCCATGAATTTCCAAGGGAAG) was designed to provide a *HindIII* restriction site and the downstream primer (AGTCCCTCGAGGTTAGGGATCCATTATGGCA) to provide a stop codon and *XhoI* site at its terminus. The pCEP4, pCEP4-He11 and pCEP4-AhR/LBD expression vectors were transfected into MDA-MB-231 cells using Lipofectamine reagent. Stable transfectants were selected by culturing cells in 500 μg/ml of the antibiotic Hygromycin B (Boehringer Mannheim).

5. Northern blot analysis

Total RNA was isolated from cells in log growth phase using the RNAzol B (Tel-Test) method by the manufacturer's guidelines. RNA samples (25 μg/lane) were resolved through 1.1% agarose/formaldehyde/ethidium bromide gels for 3 h at 100 V and photographed. The RNA was transferred to Zeta-Probe membranes (Bio-Rad) by capillary transfer overnight and hybridized with ultraviolet light. The oligonucleotide probe, consisting of a 1200 base pair fragment from a *PstI* digestion of rat *CYP1A1* cDNA, was radiolabeled with [α-

^{32}P]dCTP by random primer extension (Oligo Labeling Kit; Pharmacia). Hybridization was performed in 40% formamide, 4X standard saline citrate, 4X Denhardt's reagent, 0.2 mg/ml salmon sperm DNA, 1.2% sodium dodecyl sulfate and 10% dextran sulphate at 42°C overnight.

6. Immunohistochemistry

Cells that had been plated the previous day on coverslips were fixed with 4% paraformaldehyde/PBS for 15 min at 37°C. Membranes were made permeable with a 0.3% Triton X-100/PBS solution for 5 min followed by blocking for 30 min with 5% BSA/PBS. The specimens were co-incubated with a 1:200 dilution of monoclonal (mouse) anti-HSP90 antibody (SPA-830, StressGen) and a 1:1000 dilution of polyclonal (rabbit) anti-AhR antibodies in 5% BSA/PBS for 2 h at 37°C. The secondary antibodies consisted of rhodamine-conjugated rat anti-mouse IgG (1:100 dilution) and fluorescein-conjugated rat anti-rabbit IgG (1:100 dilution) and was applied for 1 h at 37°C. HSP90 and AhR cellular localization was determined using a Zeiss LSM410 confocal microscope.

To confirm the correct localization of 90WT and 90NLS, transfected cells prepared as above were incubated with BF4 monoclonal (rat) IgG followed by fluorescein-conjugated goat anti-rat IgG. The BF4 antibody is specific for avian HSP90 [370] and did not recognize human HSP90.

7. Immunoblot analysis

Cells in exponential phase growth on 100 mm plates were washed with PBS, detached with a cell scraper, and sedimented. The cells were resuspended in 0.5 ml lysis buffer [10 mM Tris-HCl (pH 8.0), 60 mM KCl, 1 mM EDTA, 1 mM

dithiothreitol, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 0.01 mg/ml leupeptin, 0.01 mg/ml pepstatin, 0.01 mg/ml aprotinin] per $2x10^7$ cells and incubated on ice for 10 min. Samples were spun down at high speed and the protein content of the supernatant was quantitated by the Bradford assay (Bio-Rad). Protein samples (25 μg/lane) were resolved through 10% polyacrylamide-sodium dodecyl sulfate gels and electrophoretically transferred to nitrocellulose (BioBlot-NC; Costar) by standard techniques. The blots were stained with Ponceau S to verify the efficiency of transfer and the gel loading. If areas of the gel did not transfer properly or if differences in gel loading were apparent, the blot was not used. Membranes were blocked overnight in BLOTTO buffer [50 mM Tris (pH 7.5), 0.2% Tween-20, 150 mM NaCl, 5% dry milk powder] at 4°C and then washed 4 times in TTBS+ buffer [50 mM Tris, 0.5% Tween-20, 300 mM NaCl (pH 7.5)] for a total of 40 min. Immunoreactive staining was carried out with a 1:1000 dilution of anti-HSP90 antibody or 1:1000 dilution of anti-β-actin antibody (Boehringer Mannheim) in BLOTTO buffer for 2 h at room temperature. Membranes were washed as previously and incubated with 1:10,000 horseradish peroxidase-conjugated secondary antibody in BLOTTO buffer for 1 h at room temperature. Following another wash cycle, specific proteins were detected by the enhanced chemiluminescence (ECL) system from Amersham as described by the manufacturer. Bovine HSP90 (SPP-780, StressGen) was used a positive control.

8. Densitometry

Radiographic and ECL exposures were scanned into a Power Macintosh computer with a UMAX VistaS-12 scanner and Adobe Photoshop 3.0.5 software.

Images were then quantified with the use of National Institutes of Health Image 1.61b7 software. Quantification was performed as follows. A rectangular tool was produced that surrounded the largest band of interest. The size of this tool was held constant for all measurements within a specific set of samples. The mean value of the intensity within the tool was then determined for: (i) the band of interest, (ii) the area directly above the band, and (iii) the area directly below the band. The intensities of the area above and below the band of interest were then averaged and subtracted from the band of interest.

RESULTS AND DISCUSSION

1. *In vivo* inhibition of AhR signaling by HSP90

TCDD has been proposed to act as an antiestrogen by enhancing estradiol metabolism [311, 318-322] or by decreasing ER transcription [324]. Alternatively, Kharat and Saatcioglu [316] have suggested that the antiestrogenic nature of TCDD results from the ability of liganded AhR to block ER transactivation of gene expression. In DNA binding experiments, these researchers showed that cotreatment of MCF-7 cells with estradiol and TCDD completely blocked ERE-specific DNA binding by the ER, and reciprocally, blocked the XRE-specific DNA binding activity of the AhR [316]. The validity of these 3 models is uncertain as Safe and coworkers have reported contradictory data for each of them [313-315, 325, 329]. It has been shown that ER protein levels are decreased by up to 50-75% in TCDD-treated animals [303, 491] and cells in culture [307, 313, 315]. However, this finding does not appear to account for interference between the AhR and ER signal transduction pathways since TCDD remains antiestrogenic in cells where

the levels of ER cannot be down-regulated due to constitutive expression from an ER expression vector [316, 326].

HSP90 is a potential mediator of cross-talk since it interacts with both the AhR and ER and is known to repress receptor signaling *in vitro*. HSP90 is expressed in vast excess relative to intercellular receptor levels, however, this chaperone associates with numerous proteins, including several proteins which are also expressed in great amounts (such as actin and tubulin). Therefore, although HSP90 expression is abundant, it is possible that most of HSP90 is sequestered within the cell. The working hypothesis for the studies presented here was that drug treatment could lead to an increase of unbound HSP90 which would be inhibitory to receptor signaling. To determine whether overexpression of HSP90 interferes with the transactivation functions of AhR and ER *in vivo*, we performed transient transfection assays with ER-positive, Ah-responsive MCF-7 and T47-D cells.

For the first experiment an expression vector, either the control plasmid (pSVK3) or a vector containing HSP90 cDNA (90WT or 90NLS), was cotransfected into cells along with an estrogen-responsive reporter construct. The following day transfected cells were treated with estradiol for 24 h. Results from these studies showed that there was no significant difference between estradiol-induced CAT activity in HSP90-transfected cells relative to cells carrying the empty expression vector (Fig. 17). In the second experiment, cells were cotransfected with an expression vector (pSVK3, 90WT or 90NLS), a TCDD-responsive reporter plasmid (pGL3-1A1), and a control reporter vector (pRL-CMV). Following a 24 h exposure to 10 nM TCDD, cells were lysed on the culture

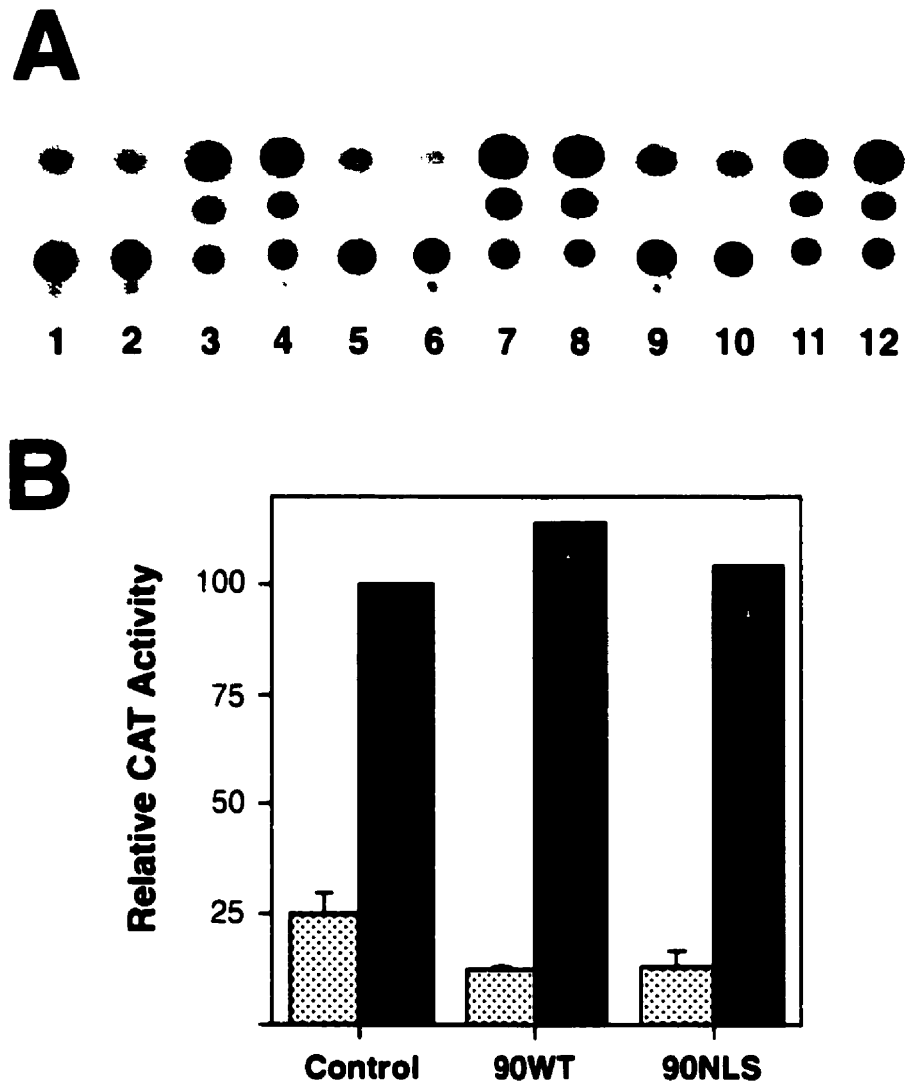

Figure 17. Effect of HSP90 overexpression on estrogen receptor-mediated gene transactivation. (A) Representative assay. MCF-7 cells were transiently transfected with an expression vector (pSVK-3, lanes 1-4; 90WT, lanes 5-8; 90NLS, lanes 9-12) and an estrogen-responsive reporter plasmid (vit-tk-CAT). The following day, transfected cells were either left untreated (lanes 1, 2, 5, 6, 9, 10) or exposed to 100 nM estradiol (lanes 3, 4, 7, 8, 11, 12) for 24 h. CAT enzyme assays were performed as described in "Materials and Methods." (B) Overexpression of HSP90 does not inhibit estrogen receptor signaling *in vivo*. Untreated, *stippled* bars; estradiol-treated, *solid* bars. Results shown are the average of four separate experiments in MCF-7 cells, each performed in duplicate, and are expressed as percentage acetylated substrate ± standard error.

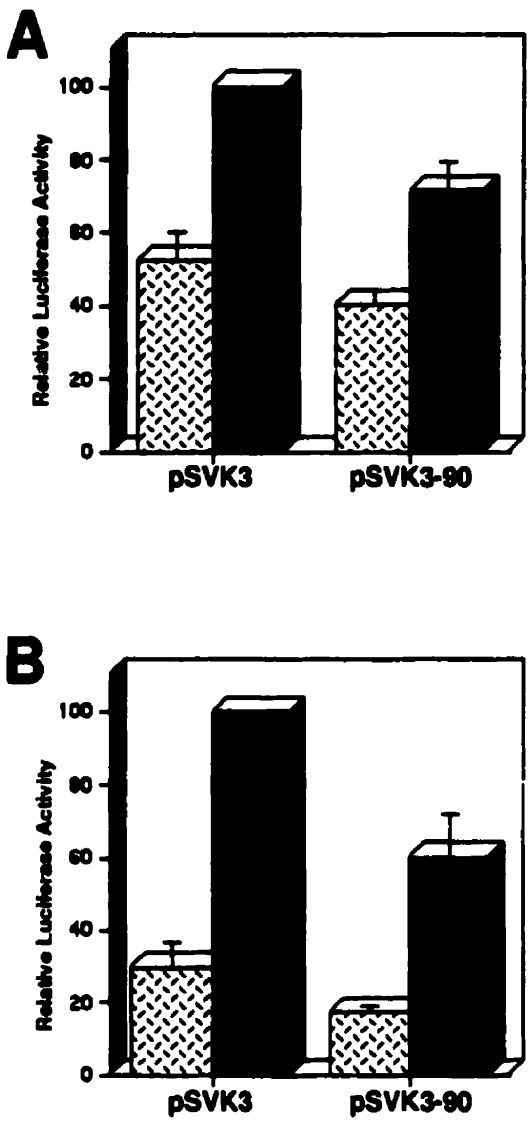

Figure 18. Overexpression of HSP90 inhibits Ah receptor signaling *in vivo*. Assays were performed with (A) MCF-7 cells, and (B) T47-D cells. Cells plated in 12-well dishes were co-transfected with an expression plasmid (pSVK3, 90WT, or 90NLS), a 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-responsive reporter plasmid (pGL3-1A1), and pRL-CMV to control for differences in transfection efficiencies and cell number from sample to sample. The next day, transfectants were treated with vehicle alone (*stippled* bars) or 10 nM TCDD (*solid* bars) for 24 h. Luciferase activity was detected in whole cell extracts using a luminometer equipped with dual automatic injectors. Results shown are the average of three separate experiments each performed in triplicate and are expressed as a function of pGL3-1A1 activity/pRL-CMV activity ± standard error.

dish and the samples collected for luciferase activity determination. Overexpression of wild-type HSP90 resulted in a significant decrease of pGL3-1A1 luciferase expression. As shown in Fig. 18, TCDD-induced luciferase activity was limited to about 70% and 60% of control levels in MCF-7 and T47-D cells, respectively. Conversely, HSP90 that is targeted to the nucleus (90NLS) failed to significantly decrease induction of pGL3-1A1 (Fig. 18).

Although all steroid hormone receptors bind to HSP90, there appears to be differences among them with respect to the outcome of this association. The AhR is more similar to the glucocorticoid receptor in that it must be bound to HSP90 in order to bind ligand [358, 361]. Deletions of amino acids within the LBD of the GR yield derivatives that constitutively activate transcription [492]. Thus, the LBD of the GR is solely capable of repressing receptor action, presumably in an indirect manner via interaction with HSP90. Interestingly, this motif retains its repressive function both in rearranged receptor derivatives and when fused to the unrelated adenovirus E1A [385] and c-Myc transcription factors [386]. Similar evidence exists for the AhR: Pongratz *et al.* [361] were able to show that a HSP90-free form of the AhR did not form a stable complex with TCDD but bound DNA constitutively. In contrast, the role of HSP90 in ER signaling is not as apparent. It has been shown that HSP90 assists the ER in assuming a proper DNA binding conformation [493], although it is not required for ligand binding [387]. In addition, White and coworkers have shown that ER chimeras that do not associate with HSP90 were functional hormone-dependent transcriptional activators *in vivo* [494], and that an amino acid substitution of one residue in the LBD of the ER led to a high level of constitutive activity, in spite of a stable interaction with HSP90 [391]. These observations can be related to the fact that

the GR (and presumably the AhR) form stable associations with HSP90 whereas the ER forms a relatively weak association detectable only at non-physiological low-salt concentrations [493, 495].

The 90NLS expression vector was utilized in these experiments to determine whether the subcellular localization of HSP90 impacted upon receptor signaling. Differential localization of HSP90 was confirmed by transient transfection of 90WT and 90NLS plasmids into MCF-7 and T47-D cells followed by immunofluorescent staining with an avian HSP90-specific antibody as described in "Materials and Methods" (not shown). In electrophoretic mobility shift assays using purified HSP90 and ER, Sabbah *et al.* [389] have reported that binding of ER to the ERE was inversely dependent upon the concentration of HSP90 present in the reaction, and that HSP90 was capable of dissociating preformed ER-ERE complexes. Based on these results is was postulated that during heat shock ER-dependent transcription may be repressed due to increased HSP90 transcription and translocation of HSP90 into the nucleus [389]. However, our results indicate that under *in vivo* conditions overexpression of HSP90 in either the cytoplasm or nucleus does not inhibit ER signaling (Fig. 17). It is likely that HSP90 mediates ER folding as a molecular chaperone, but has less of a role in repressing the intrinsic DNA binding activity of the ER. For the AhR, only 90WT was found to inhibit receptor signaling (Fig. 18). This data suggests that HSP90 can block the initial binding of TCDD to the AhR in the cytoplasm, but fails to compete for preformed TCDD-AhR complexes in the nucleus.

2. Restoration of Ah-responsiveness by expression of HSP90-binding proteins

ER-negative MDA-MB-231 cells can be converted to an Ah-responsive phenotype through transient transfection of a hER expression vector [347]. We have introduced two expression vectors into MDA-MB-231 cells to determine whether ER facilitates AhR signaling through its association with HSP90. The vectors were constructed to overexpress HSP90-binding proteins in the form of receptor deletion mutants. Previously it has been reported that the LBD of the AhR constitutes a minimal structural requirement for HSP90 binding [474], whereas the LBD and an additional sequence element at the C-terminus of the DBD appear to be necessary for HSP90 binding by ER [387, 393]. The pCEP4-He11 expression vector contains a hER cDNA insert with deletion of the core DBD region (Δ185-251) [490]. As shown in Fig. 19, basal and TCDD-induced expression of *CYP1A1* are increased ~7.5- and 24.7-fold, respectively, in pCEP4-He11-transfected cells relative to TCDD-induced *CYP1A1* expression in cells harboring the empty expression vector.

This data indicated that ER is not transactivating a critical gene in the AhR signal transduction pathway. To exclude the possibility that the TAF1 or TAF2 transactivation regions within ER are squelching an inhibitory factor that is also associated with AhR, we constructed an expression vector containing a short region of hAhR spanning the overlapping ligand- and HSP90-binding domains. The design of this vector was based on work by Whitelaw *et al.* [474] who delineated a region of murine AhR located between amino acids 230 and 421 that co-immunoprecipitates with HSP90. Stable transfection of MDA-MB-231 cells with pCEP4-AhR/LBD was found to increase TCDD-induced *CYP1A1* mRNA levels ~5.6-fold relative to control cells (Fig. 19). A probable explanation for lower

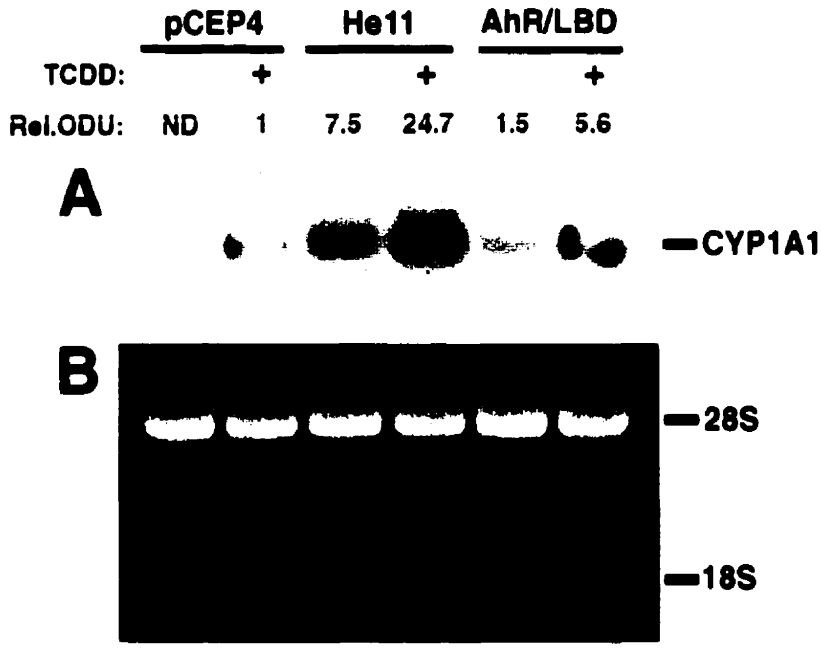

Figure 19. Overexpression of HSP90-binding proteins restores Ah-responsiveness in MDA-MB-231 cells. (A) Analysis of *CYP1A1* expression in MDA-MB-231 cells. Stable transfectants were selected for retaining the pCEP4 episomal eukaryotic expression vector, pCEP4-He11, or pCEP4-AhR/LBD. Cells were treated either with vehicle alone or 10 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) for 24 h prior to RNA isolation. Northern blot analysis was performed as described in "Materials and Methods." Exogenous mRNA expression was verified by reprobing the membrane with radiolabeled He11 and AhR/LBD oligonucleotides. (B) Photograph of total RNA run on an agarose/ethidium bromide gel to assess RNA integrity and quantity. 18S and 28S indicate the location of ribosomal RNA bands. This data is representative of two experiments using different RNA preparations. Similar results were also achieved by cotransfecting the above expression vectors with the TCDD-responsive pGL3-1A1 reporter plasmid in transient transfection assays. ND, not determined; Rel. ODU, relative optical density units.

CYP1A1 levels in pCEP4-AhR/LBD- versus pCEP4-He11-transfected cells is that the former protein product has less affinity for HSP90: although the LBD of AhR fulfills the minimal requirement for HSP90 binding, it has recently been shown that involvement of the bHLH domain is necessary for high affinity binding [396]. However, by restricting our experiments to the LBD, we have minimized the likelihood that factors apart from HSP90 are responsible for increasing Ah-responsiveness in this model. Therefore, this data strongly suggests that Ah-responsiveness can be restored in MDA-MB-231 cells by squelching HSP90.

Previously, Wang *et al.* [471] cotransfected mutant ER expression plasmids and TCDD-responsive reporter constructs into ER-negative Hs578T cells. These studies showed that expression of C-terminal-deleted ER (He15, Δ282-595) did not restore Ah-responsiveness, whereas TCDD-induced CAT activity was increased 23-fold in cells expressing N-terminal-deleted ER (He19, Δ1-178). Significantly, the He15 and He19 protein products possess differential affinities for HSP90: only He19 forms 8-9S complexes when expressed in COS-7 cells [387]. Therefore, this data supports the hypothesis that AhR signaling can be enhanced via expression of HSP90-binding proteins.

In the original study showing that transfection of ER restores Ah-responsiveness in MDA-MB-231 cells, Thomsen *et al.* [347] utilized an ER expression vector that differed from the wild-type *mAhR* sequence by a guanine to valine substitution at codon 400 [496]. This alteration was subsequently found to destabilize the structure of the ligand-binding domain [496, 497] and to allow the receptor to form stable associations with HSP90 [392]. The He11 nucleotide used in our experiments also possesses this mutant genotype. Therefore, some caution must taken when extrapolating the results presented here to those events

that occur *in vivo*. Nonetheless, utilization of a protein with greater affinity for HSP90 strengthens our hypothesis that squelching of HSP90 can lead to restoration of Ah-responsiveness in MDA-MB-231 cells.

Safe [498] has previously reported that the Ah-nonresponsiveness of ER-negative MDA-MB-231, MDA-MB-435 and Adr^R MCF-7 cells is associated with expression of a variant Arnt protein which contains deletion of amino acid residues 330 to 789. This alteration was not a consequence of genomic mutation but was postulated to arise through a defective splicing mechanism in high passage (>50) cells [498]. Cells that were low passage (<20) expressed a normal Arnt protein. It is possible, therefore, that a variant Arnt contributes to Ah-nonresponsiveness in ER-negative cell lines. However, there are two points which contradict this assertion. First, the data reported by Safe's group may relate to cell culture techniques that result in novel cell sublines since this observation was not made in early passage cells. Although Arnt protein was not examined in our MDA-MB-231 cells, investigation of Arnt in adriamycin-resistant MCF-7 cells did not uncover any alterations in size (Fig.s 11 and 13). Second, Safe reported that transient transfection of wild-type Arnt into MDA-MB-231 cells resulted in a less than 2-fold induction of XRE-CAT activity in the presence of TCDD [498], whereas transfection of ER results in a 10-fold induction of *CYP1A1* promoter-CAT activity [347]. Therefore, expression of full-length Arnt is not sufficient to fully restore Ah-responsiveness in MDA-MB-231 cells.

Thomsen *et al.* [347] showed that *CYP1A1* promoter-CAT activity was not significantly increased by TCDD in MDA-MB-231 cells transfected with a human progesterone receptor expression plasmid, indicating that not all HSP90-binding proteins have the ability to restore Ah-responsiveness. This finding may be

related to the localization of PR relative to ER, He11, He15 and AhR/LBD. Steroid receptors actively shuttle between the cytoplasm and nucleus through the accessibility of nuclear localization signals on their surfaces [499]. However, these motifs vary structurally and in number between the various family members. For the cytoplasmic GR, two NLSs have been characterized: one between the DBD and LBD regions, and the other within the LBD [500]. It is thought that binding of ligand to the GR causes release of HSP90, unmasking of its NLS(s), and subsequent translocation to the nucleus [501]. In the case of the PR, three putative NLSs have been identified in the second zinc finger of the DBD, the hinge region, and a weaker site in the LBD [502, 503]. These different signals cooperate to elicit a strong nuclear localization. In contrast, only one NLS has been identified in the ER, which is located between the LBD and DBD regions [504]. Although the ER is primarily found in the nucleus, a portion of ER is found in the cytoplasm, as evidenced by the finding that about 15% of the ER content of MCF-7 cells resides in the cytosolic fraction [505]. We have shown that expression of 90NLS does not affect Ah-responsiveness in MCF-7 and T47-D cells. In a similar fashion, it may be that nuclear squelching of HSP90 by the PR does not significantly modify AhR signaling. It remains to be determined, however, whether a greater amount of ER is cytoplasmic relative to PR, or if these receptors possess different affinities for HSP90.

3. Correlation of HSP90 subcellular localization with ER status

Two HSP90 homologues are expressed in higher eukaryotes, which are termed α and β in humans (reviewed in [348]). Only a minor proportion of the total cellular content of HSP90 is normally situated in the nucleus. During heat

shock a reversible time-dependent nuclear translocation of HSP90 occurs [351]. It has been shown that estrogen treatment increases cellular HSP90α mRNA and protein levels [506, 507], although it is not known whether estradiol or TCDD alters the subcellular localization of HSP90. To address this question, MCF-7, adriamycin-resistant MCF-7, MDA-MB-231, and S30 cells were incubated with either 100 nM estradiol or 10 nM TCDD for 0 h, 1 h and 2.5 h on coverslips, fixed, and then probed with an anti-HSP90 antibody that recognizes HSP90α and β proteins. In each instance drug exposure did not cause a change in HSP90 subcellular distribution as determined by confocal microscopy (data not shown). These results indicated that estradiol and TCDD did not invoke a nuclear translocation of HSP90, which has been shown to coincide with a transient paralysis of estradiol-mediated gene transactivation [508]. However, it was discovered that the subcellular localization of HSP90 correlated with the ER status of the cell line (Fig. 20). HSP90 is primarily cytoplasmic in ER-expressing cell lines MCF-7 and S30 (panels 1A and 4A), whereas ER-negative adriamycin-resistant MCF-7 and MDA-MB-231 cells have an equal distribution of HSP90 between the cytoplasm and nucleus (panels 2A and 3A). No differences in HSP90 expression were found between the matched ER^+/ER^- cell lines by immunoblot analysis (data not shown).

To determine whether ER expression had an effect on AhR distribution, cells were co-incubated with anti-AhR antibodies. As shown in Fig. 20, AhR is visualized throughout the cytoplasm in MCF-7 cells (panel 1B), in contrast to adriamycin-resistant MCF-7 cells (panel 2B) where there is an uneven distribution of AhR that is more associated with the cell periphery. MDA-MB-231 cells have a similar pattern of expression as seen in adriamycin-resistant MCF-7

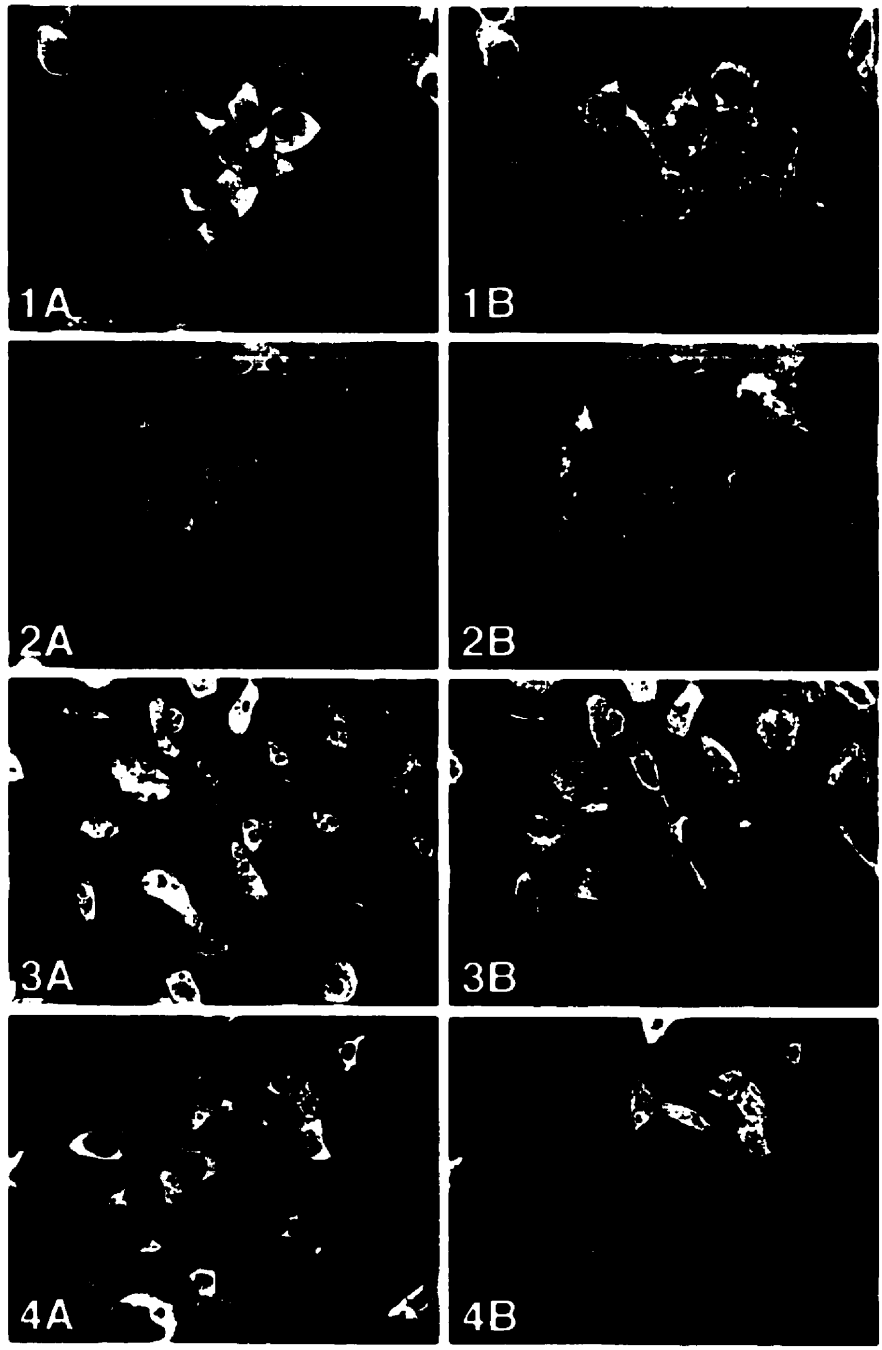

Figure 20. Cytoplasmic HSP90 localization is associated with estrogen receptor (ER) status in matched ER-negative and -positive human breast cancer cells. Cells plated the previous day on coverslips were fixed and co-incubated with anti-HSP90 and anti-Ah receptor antibodies as described in "Materials and Methods." Subcellular localization of (A) HSP90 and (B) Ah receptor were detected by indirect immunofluorescence staining and laser confocal microscopy. (1) ER^+ wild-type MCF-7 cells; (2) ER^- adriamycin-resistant MCF-7 cells; (3) ER^- MDA-MB-231 cells; (4) ER^+ S30 cells.

cells (panel 3B versus 2B), and transfection of hER into this cell line appears to restore the phenotype observed in MCF-7 wild-type cells (panel 4B versus 1B). It will be interesting to determine whether the subcellular localization of AhR has an effect on its transactivation function.

In conclusion, data presented here provides evidence for the mechanism of cooperation between AhR and ER signaling pathways. We have utilized receptor deletion mutants to squelch HSP90 resulting in restoration of Ah-responsiveness in ER-negative human breast cancer cells. In addition, overexpression of HSP90 was found to modulate AhR signaling *in vivo*. Transfection of HSP90 did not have a similar effect on ER activation. It is possible that these results reflect the greater stability of the AhR/HSP90 association relative to ER/HSP90. Finally, we have examined the subcellular localization of HSP90 in human breast cancer cell lines. HSP90 was found to be primarily cytoplasmic in ER-positive cells, whereas in ER-negative cells HSP90 was distributed equally between the cytoplasm and the nucleus.

Chapter V

General Discussion

Claims to Original Research

Suggestions for Further Work

5.1 General Discussion

Although the majority of human mammary tumors are thought to arise as the result of exposure to environmental carcinogens, specific xenobiotics have not as yet been associated with breast cancer. Activation of the AhR has been implicated in both mammary tumor initiation and promotion in animal models. Therefore, AhR ligands such as PAHs and halogenated aromatic molecules may have a role in the genesis of this disease. To determine an association between these compounds and breast cancer, improved epidemiological methods are required to assess exposure, which occurs years before diagnosis of the cancer. Furthermore, this exposure has to be evaluated in light of several confounding factors, including components of the diet that have synergistic or negative effects on AhR-mediated carcinogenesis.

Another means to investigate the role of the AhR in human mammary tumorigenesis is to determine the effects of receptor activation on human cells in cell culture models. The objective of this mode of research is to correlate *in vitro* results with clinical observations. This latter approach was the basis for the studies presented in this thesis.

Tumorigenic cells often possess gross alterations in their genotype. The mutations can be detected by cytogenetic analysis, or analyzed at the level of individual loci. For tumors of different origin, trends exist in patterns of oncogene activation and tumor suppressor gene inactivation. To determine whether BP-transformed human mammary epithelial cells manifest genotypic changes associated with breast cancer, transformants were analyzed cytogenetically, and genes often mutated in breast cancer were screened for alterations. To this end, MCF-10A cells were exposed to BP and *in vitro*

transformed clones were isolated and expanded based on their loss of contact inhibition and altered morphology.

The *p53, H-ras,* and *c-myc* genes were analyzed by Southern blot, sequencing, and/or SSCP analyses. Mutations were not detected in any of the gene regions tested. However, the transformants possessed novel cytogenetic aberrations not present in parental cells. The alteration common to all the clones was an isochromosome 8q replacing one of their normal complements of chromosome 8. This was a particularly interesting finding since chromosome 8 is cytogenetically abnormal in about one third of breast cancer patients, and loss of heterozygosity occurs on this chromosome in about one half of human mammary tumors.

The *c-myc* proto-oncogene is localized at position 8q24. In agreement with cytogenetic analysis, *c-myc* mRNA expression was found to be increased in the transformants. Moreover, the highest level of c-Myc protein expression was found in a clone which possessed a loss of chromosome 5q. Previous studies have shown that LOH for polymorphic markers on chromosome 5q was correlated with increased c-Myc expression. Since *c-myc* overexpression is correlated with high proliferative capacity, phenotypic traits of transformation including foci formation may be a direct consequence of c-Myc overexpression.

Based on studies showing that chromosome 8 alterations occur with equal frequency in small and large tumors, and that c-Myc overexpression is associated with high proliferative capacity but less so with nodal status or metastasis, it has been postulated that chromosome 8 is involved in the early stages of tumorigenesis. Thus, the data presented here is significant because it reinforces the hypothesis that alteration of chromosome 8 is associated with transformation in human mammary epithelial cells, and it demonstrates that a ubiquitous environmental carcinogen that binds to the AhR can induce this process.

A significant limitation to the effectiveness of chemotherapy is the appearance of drug resistance. Elucidation of the cellular mechanisms responsible for resistance could lead to the development of novel antineoplastic treatments. To further understand drug resistant phenotypes, the basis for loss of Ah-responsiveness was examined in two xenobiotic-resistant cell lines that were established by exposing MCF-7 breast cancer cells to increasing concentrations of either BP (BP^R cells) or adriamycin (Adr^R cells).

Initially, the Ah-nonresponsive phenotype of the BP^R cell line was characterized. From cytotoxicity studies it was determined that BP^R cells could withstand 100-fold greater concentrations of BP compared to WT cells. Enhanced survivability of BP^R cells was correlated with a steep reduction (about 50-fold) in the ability of these cells to transactivate *CYP1A1* mRNA expression in the presence of either BP or TCDD. Although this data indicated that the mechanism of resistance was probably not associated with such pathways as enhanced DNA repair or phase II enzyme activity, it could not be determined whether this was the result of down-regulation of phase I metabolism of BP or increased efflux of drug. To resolve this question, cytotoxicity studies were performed with the activated metabolite of BP, BPDE. From these experiments it was determined that MCF-7 WT and BP^R cells were equally sensitive to BPDE. Therefore, these data suggest that resistance is mediated via decreased bioactivation of BP.

Components of the AhR signal transduction pathway were analyzed for alterations in genetic structure, expression, and activity. For BP^R cells, it was found that AhR mRNA and protein levels were decreased about 3- and 4-fold, respectively. The decrease in AhR protein likely accounts for the sharp decline in AhR DNA binding activity observed in the BP^R cells.

By far the cytochrome P450 enzymes with the highest activity towards BP are members of the CYP1A subfamily. CYP1A1 and CYP1A2 are not expressed in

breast tissue under normal conditions. However, *CYP1A1* transcription is induced within minutes after exposure to ligands of the AhR. Since BP requires activation in order to be transformed into a mutagen, inhibition of *CYP1A1* transactivation is a logical mechanism of resistance for this class of compounds.

In contrast to the BP^R subline, Adr^R cells are Ah-nonresponsive despite expression of normal levels of AhR. Although the AhR derived from Adr^R cell nuclear extracts is functionally capable of binding to a XRE in *in vitro* gel shift assays, it does not respond to TCDD *in vivo*. In addition to the lack of *CYP1A1* induction, TCDD-induced AhR depletion was found to occur about 3-fold less efficiently in the Adr^R subline. Based on these results it was postulated that AhR is protected from ligand-binding in Adr^R cells since ligand causes dissociation of HSP90 and consequent degradation of the receptor.

By immunofluorescent methods, it was determined that the subcellular distribution of AhR is altered in Adr^R cells relative to the parental cell line. It is possible, therefore, that ligand is prevented from binding to the AhR if the receptor colocalizes with a high concentration of inhibitory factors, such as HSP90.

The loss of Ah-responsiveness in Adr^R cells may also be associated with their ER content. In general, ER-negative cell lines such as Adr^R MCF-7 are Ah-nonresponsive. BP^R cells were found to be ER-positive, although ER levels were decreased in this cell line by about one third relative to WT cells. Although it remains to be determined, this reduction of ER may also contribute to the Ah-nonresponsive phenotype of BP^R cells.

Since HSP90 is known to modulate the activity of the AhR and ER, we hypothesized that this heat shock protein is mediating the interference and cooperation observed between these disparate signal transduction pathways. To determine whether ER expression facilitates AhR signaling by squelching HSP90,

two vectors that express protein products that bind HSP90 were transfected into ER-negative MDA-MB-231 breast cancer cells. Expression of: (i) He11, and ER deletion mutant that lacks its DNA-binding domain, and (ii) the ligand/HSP90-binding domain of hAhR, both led to increased basal and TCDD-inducible expression of *CYP1A1* mRNA. Thus, this data suggests that less unbound HSP90 may be available in ER-positive cells to act as a repressor for AhR signaling.

Previously it has been demonstrated that HSP90 could directly inhibit the DNA-binding activity of the Ah and estrogen receptors in cell-free systems. To further understand the role of HSP90 in regulating receptor function *in vivo*, an expression vector containing a copy of chicken HSP90 cDNA was transiently cotransfected into breast cancer cell lines with a reporter construct. These experiments determined that overexpression of HSP90 had no effect upon 17β-estradiol-induced expression of a chloramphenicol acetyltransferase gene under the control of an estrogen-responsive promoter. In contrast, exogenous expression of HSP90 was able to inhibit TCDD-inducible expression of reporter vector consisting of a portion of the *CYP1A1* promoter upstream of a luciferase gene. These data show that AhR signaling can be regulated by decreased or increased levels of HSP90, and that HSP90 has an alternate effect on the ER signal transduction pathway.

The cell lines utilized in Chapters II, III, and IV were chosen to most appropriately address the specific questions posed. Although different cell lines were utilized, they all share a human mammary epithelial cell origin. In Chapter II, the purpose was to investigate the early genetic changes associated with mammary carcinogenesis and therefore a non-transformed HMEC line (MCF-10A) was selected. In Chapter III, the mechanism for BP-resistance was investigated. For this purpose, MCF-7 cells were utilized since this cell line has been extensively characterized for resistance to other chemicals. Finally, in

Chapter IV, MDA-MB-231 cells were used since this ER-negative HMEC line was previously shown to gain an Ah-responsive phenotype upon transfection with an ER expression vector.

In conclusion, the data presented here sheds new light on the role of AhR-mediated processes in human mammary epithelial cells. We show evidence that a ubiquitous environmental AhR ligand can transform HMEC and elicit genetic alterations observed in clinical investigations. Furthermore, we have provided preliminary data for a mechanism of cross-talk between the AhR and ER signal transduction pathways. Elucidation of this process may lead to a greater understanding of receptor signaling in general and possibly to novel anti-estrogenic agents.

5.2 Claims to Original Research

The following novel findings and observations have been demonstrated in this thesis:

1. Three BP-transformed MCF-10A clones were established and characterized. Each of the clones has a unique karyotype which includes an isochromosome 8q aberration not observed in parental cells (Chapter II). These cell lines provide a useful model for studies of *in vitro* transformation of HMEC.

2. A novel drug-resistant human mammary carcinoma cell line was established by exposing MCF-7 cells to increasing concentrations of BP over a several month period. Resistance was determined to be mediated via decreased metabolic activation of BP (chapter III).

3. Expression of *AhR* mRNA was found to be significantly down-regulated in BP-resistant MCF-7 cells (Chapter III). This data supports findings in other breast cancer cell lines, and indicates that suppression of *AhR* mRNA levels may be a common mechanism by which cells adapt to prolonged BP exposure.

4. The AhR signal transduction pathway was characterized in Ah-nonresponsive adriamycin-resistant MCF-7 cells (Chapter III). Expression and DNA-binding activity of the AhR and Arnt proteins were not found to be altered in the resistant cells. However, Adr^R cells were found to possess differences with respect to: (i) the rate of TCDD-induced AhR protein turnover, and (ii) the subcellular localization of AhR.

5. The subcellular localization of AhR and HSP90 correlated with the ER status of human breast cancer cells. In ER-positive cell lines, AhR and HSP90 were visualized throughout the cytoplasm. In contrast, AhR was distributed unevenly and more towards the cell periphery in ER-negative cells, and HSP90 was distributed equally between the cytoplasm and the nucleus (Chapter IV). The localization of AhR and/or HSP90 may be a factor in the regulation of AhR activity.

6. The Ah-nonresponsive phenotype of ER-negative breast cancer cells was reversed via introduction of HSP90-binding proteins (Chapter IV). This data suggests that ER can facilitate AhR signaling by squelching HSP90.

7. It was previously shown that HSP90 can suppress ER DNA binding activity under *in vitro* conditions, but it was not known whether this was occurring in intact cells. By cotransfecting human mammary carcinoma cells with a HSP90 expression vector and an estrogen-responsive reporter vector, it was demonstrated that HSP90 overexpression does not inhibit ER signal transduction. Conversely, HSP90 overexpression was found to suppress induction of a TCDD-responsive reporter vector. These observations indicate that HSP90 can differentially regulate AhR and ER activities *in vivo*.

5.3 Suggestions for Further Work

1. Expression of c-Myc was found to be elevated in BP-transformed MCF-10A clones. To directly determine the involvement of c-Myc in transformation of these cells, it would be necessary to show that suppression of c-Myc restores a WT morphology, and that increased expression of c-Myc in WT cells replicates the phenotype observed in the transformed clones.

2. Several clinical studies point to an association of chromosome 8 with the initial stages of human mammary tumorigenesis. This hypothesis can be investigated via transformation studies involving microinjection of chromosome 8 fragments into normal cells to search for oncogenes, or deletion analyses to detect the presence of putative tumor suppressor genes on chromosome 8.

3. The BP-transformed clones described here may represent an intermediate step before tumorigenic transformation. Therefore, further manipulations of the clones may lead to a tumorigenic phenotype. This could be achieved either through another round of exposure(s) to BP, transfection of an oncogene, or knockout of a tumor suppressor gene. Alternatively, the BP-exposed cells already obtained can be reselected for transformed cells by another means, such as expansion of colonies that grow in soft agar.

4. *AhR* mRNA levels are significantly decreased in BP-resistant MCF-7 and T47-D human breast cancer cells, although the mechanism underlying this reduction is not known. Greater understanding of xenobiotic resistance and the regulation of AhR activity could be attained by analyzing *AhR* transcription and mRNA stability in BP-resistant cells.

5. A decrease in the level of ER protein expression was observed in BP-resistant MCF-7 cells. This down-regulation of ER is proportional to the decrease found in TCDD-treated animals. Since ER is known to facilitate AhR signal transduction, it would be interesting to determine whether enhanced Ah-responsiveness could be achieved by restoring wild-type ER levels in the resistant subline.

6. It is possible that the subcellular distribution of AhR is a determinant for its transactivation capability. For example, we have shown that at high concentrations of HSP90 there is an inhibition of AhR signaling. Alternatively, in the presence of low levels of HSP90 the AhR cannot bind ligand. Colocalization studies could be performed to determine which intracellular structures the AhR is associated with in MCF-7 WT and Adr^R cells, and whether the AhR colocalizes with specific factors that can modulate its function.

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