Relation between Cytochrome P450IA1 Expression and Estrogen Receptor Content of Human Breast Cancer Cells

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Multidrug resistance (MDR) in an MCF-7 human breast cancer cell line (MCF7/Adr^R) is associated with decreased drug accumulation and overexpression of *P*-glycoprotein as well as alterations in the levels of specific drug-metabolizing enzymes, including decreased activity of the phase I drug-metabolizing enzyme aryl hydrocarbon hydroxylase (AHH) and increased expression of the anionic form of the phase II drug-metabolizing enzyme glutathione *S*-transferase. Since the development of MDR in this MCF-7 cell line is also associated with a loss of estrogen receptors (ER), we have examined the expression of cytochrome P450IA1, the gene encoding AHH activity, in other breast cancer cell lines not selected for drug resistance but expressing various levels of ER. These studies show that a relationship exists between 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-inducible AHH activity and the ER content in a series of breast cancer cell lines. In these cell lines expression of AHH activity is regulated, at least in part, at the level of P450IA1 RNA. While TCDD-specific binding proteins (Ah receptors) were found in each of the breast cancer cell lines, there was no apparent relation between the level of nuclear TCDD-binding proteins and the level of TCDD-inducible P450IA1 expression. Previous studies from our laboratory have described an inverse relationship between levels of the anionic form of glutathione *S*-transferase and ER in breast cancer. The findings of the present study suggest that the ER status of breast cancer cells is associated with distinct patterns in the expression of both phase I and phase II drug-metabolizing enzymes, and that these biochemical changes result in differential sensitivities of ER-positive and ER-negative cell lines to the procarcinogen benzo(a)pyrene and the antineoplastic agent ellipticine. (Molecular Endocrinology 3: 157–164, 1989)

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INTRODUCTION

Significant clinical responses may be achieved in patients with breast cancer by either chemotherapy or hormonal therapy. The tumors most likely to respond to hormonal manipulations can be identified by the presence of estrogen receptors (ER). Understanding the mechanisms involved in regulating ER expression and determining the effect, if any, of alterations in ER status on sensitivity to various agents are important areas of clinical research.

Once bound to estrogen, the ER interacts with cellular DNA to regulate the expression of a number of proteins, including the progesterone receptor (1), dihydrofolate reductase (2), and several secreted proteins (3–6). In addition to proteins whose levels are acutely affected by estrogen, it is also apparent that the levels of a number of other proteins are related to the ER content of breast cancer cells. For example, studies of breast cancer cell lines have shown that there is an inverse correlation between the levels of protein kinase-C and ER in these cell lines (7, 8). Similarly, an inverse relation has been found between the expression of ER and epidermal growth factor receptors (EGFR) in both breast cancer cell lines (9) and primary breast cancer specimens (10–12). It is, therefore, apparent that a different pattern of gene expression exists in ER-positive and ER-negative breast cancer cells.

Our laboratory has previously reported the isolation of an adriamycin-resistant MCF-7 human breast cancer cell line (MCF-7/Adr^R) which has developed the phenotype of multidrug resistance (MDR). MDR in MCF-7/Adr^R is associated with the amplification and overexpression of the *P*-glycoprotein gene (13). MDR in

these cells is also associated with increased expression of the anionic isozyme of the phase II drug-conjugating enzyme glutathione *S*-transferase (GST) (14). This anionic GST (GSTπ) is also induced in rat hyperplastic liver nodules, an animal model of carcinogenesis (15). The finding that the development of MDR in MCF-7/Adr^R is associated with the loss of ER in these cells (16) prompted studies examining the relationship between the expression of ER and GSTπ in human breast cancer. This study demonstrated that GSTπ is preferentially expressed in several ER-negative breast cancer cell lines as well as in ER-negative primary breast cancers (17).

In previous studies (15, 18) we have reported that the development of MDR in MCF-7/Adr^R cells, like the development of xenobiotic resistance in rat hyperplastic nodules, is associated with the diminished expression of the phase I drug-metabolizing enzyme arylhydrocarbon hydroxylase (AHH). The gene encoding this enzyme, P450IA1, is one of several enzymes induced in mouse liver and several cell lines by polycyclic hydrocarbons and haloaromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (19). Studies have shown that the expression of P450IA1 is regulated, at least in part, at the level of transcription (20). The studies presented in this report demonstrate that there is a marked difference in the expression of the phase I drug-metabolizing enzyme AHH in ER-positive and ER-negative breast cancer cells, and that this is associated with altered sensitivity to various cytotoxic agents.

Results

AHH Activity of Breast Cancer Cell Lines

Six breast cancer cell lines were used in these studies. Wild type MCF-7, ZR-75B, and T47D are ER-positive cell lines and have previously been reported to contain 195,000, 72,000, and 24,500 receptors/cell, respectively (9). MDA-MB231 and HS578T are each reported to lack detectable levels of ER (9). MCF-7/Adr^R is an MCF-7 subline which was selected for resistance to adriamycin and displays the phenotype of MDR (13, 14). MDR in this cell line is associated with the stable loss of ER (16).

MDR in MCF-7/Adr^R is also associated with a loss of TCDD-inducible AHH activity, the enzyme encoded in these cells by the P450IA1 gene (15, 18). TCDD is a potent inducer of AHH activity. The maximal induction of AHH activity in wild type MCF-7 cells occurs after incubation of cells with 100 nM TCDD for 24 h (18, 21). To explore the possible relationship between AHH expression and ER status, we have assayed the activity of this enzyme in all of the breast cancer cell lines described above both in the absence of TCDD and under the conditions known to cause maximal induction of AHH activity in wild type MCF-7 cells (Table 1). None of the cell lines tested displayed detectable AHH activity in the absence of TCDD. After 24-h incubation in the presence of 100 nM TCDD, each of the ER-positive cell lines (wild type MCF-7, T47D, and ZR-75B) displayed significant increases in AHH activity, with wild type MCF-7 cells having the greatest activity. In contrast, the ER-negative cell lines displayed either significantly less TCDD-inducible AHH activity than the ER-positive cell lines (MDA-MB231 and HS578T) or undetectable AHH activity (MCF-7/Adr^R).

Table 1. AHH Activity and ER Content of Breast Cancer Cell Lines

Cell Line	ER Content (receptors/cell)	AHH Activity (pmol/min·mg)	
		−TCDD	+TCDD
WT MCF-7	195,000[a]	ND	23.9 ± 2.3
T47D	24,500[a]	ND	6.5 ± 1.5
ZR-75B	72,000[a]	ND	5.1 ± 1.0
MDA-MB231	<1000[a]	ND	1.5 ± 0.6
HS578T	<1000[a]	ND	0.4 ± 0.3
MCF-7/Adr^R	<1000[b]	ND	ND

AHH activity was measured, as described in the *Materials and Methods* after growth of cells for 24 h in medium containing either no TCDD (−TCDD) or 100 nM TCDD (+TCDD). ND, Not detectable (<0.2 pmol/min·mg). The results are expressed as the mean ± SD of three separate determinations, each done in duplicate.

[a] Ref. 9.

[b] Ref. 16.

To compare the TCDD concentration required for maximal induction of AHH activity, each of the cell lines as incubated with a range of TCDD concentrations for 24 h (Fig. 1). AHH activity was inducible to high levels in wild type MCF-7 cells over a wide range of TCDD concentrations, with half-maximal stimulation occurring between 1 and 10 nM TCDD. AHH activity was also inducible over a wide range of TCDD concentrations in the ER-positive cell lines T47D and ZR-75B, although the maximal enzyme activity induced was less than that in wild type MCF-7 cells. The concentration of TCDD required for half-maximal stimulation of these cell lines is similar to that displayed by wild type MCF-7 cells. Jaiswal *et al.* (21) have previously shown that MCF-7 cells have higher levels of TCDD-inducible AHH activity than T47D cells. Other studies have shown that T47D cells form hydroxylated derivatives of benzo(a)pyrene (22), further suggesting that these cells contain significant AHH activity.

In comparison to the ER-positive cell lines, all of the ER-negative cell lines displayed lower levels of AHH activity at all concentrations of TCDD. MCF-7/Adr^R cells had undetectable AHH activity after incubation for 24 h with concentrations of TCDD up to 500 nM TCDD. HS578T cells displayed very low levels of AHH activity (< 6% of wild type MCF-7) and only detectable after induction with TCDD concentrations of 100 nM or greater. Although the MDA-MB231 cell line displayed AHH activity at all concentrations of TCDD used, the activity was significantly below that of each of the ER-positive cell lines. Previous studies have reported the

MDA-MB231 cell line to have no AHH activity when incubated with TCDD at concentrations ranging from 10^{-11}–10^{-9} M for 3 days (23), and very low activity (5% of wild type MCF-7) when induced with 100 nM TCDD for 24 h (21). These results clearly show that a relationship exists between TCDD-inducible AHH activity and ER levels in these breast cancer cell lines.

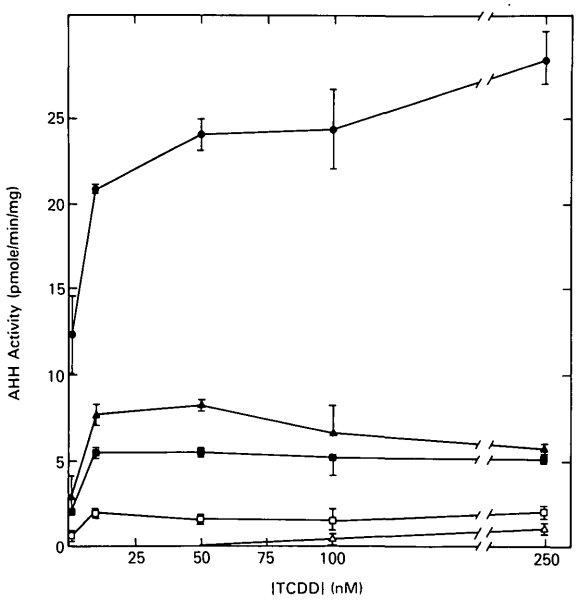

Fig. 1. AHH Activity in Breast Cancer Cells after Incubation at Various TCDD Concentrations

Cells were incubated at 37 C in various concentrations of TCDD. After 24 h of incubation the cells were harvested, and AHH activity was assayed as described in *Materials and Methods*. *Solid symbols* represent ER-positive cell lines (●, wild type MCF-7; ▲, T47D; ■, ZR-75B), while *open symbols* represent ER-negative cell lines (□, MDA-MB231; △, HS578T). MCF-7/Adr^R cells had no detectable AHH activity.

AHH RNA in Breast Cancer Cells

To study the mechanism responsible for the decrease in TCDD-inducible AHH activity in ER-negative breast cancer cell lines, levels of P450IA1 RNA were measured in each cell line both in the absence of TCDD treatment, and after treatment with 100 nM TCDD for 24 h (Fig. 2). In the absence of TCDD, no detectable level of P450IA1 RNA was measured in any of these breast cancer cell lines. After incubation with TCDD, the level of P450IA1 RNA increased in some of the cell lines. Indeed, the level of P450IA1 RNA correlated well with the level of AHH activity in these cells (see Table 1). Northern blot analysis of RNA synthesized by cell lines grown in the presence of TCDD (Fig. 3) confirmed that the mRNA measured by RNA slot-blot analysis was the same size (~ 2.6 kilobases) as that previously reported for P450IA1 RNA (18). These findings suggest that the relative expression of AHH activity in breast cancer cell lines may be due, at least in part, to differential regulation of P450IA1 gene transcription.

Binding of [^{3}H]TCDD to Breast Cancer Cell Nuclear Extracts

To study the mechanism responsible for the decrease in TCDD-inducible AHH activity and RNA in ER-negative cell lines, the levels of TCDD-specific binding proteins (Ah receptors) were determined in nuclear extracts of MDA-MB231, HS578T, and MCF-7/Adr^R cells. These levels were compared to those present in the ER-

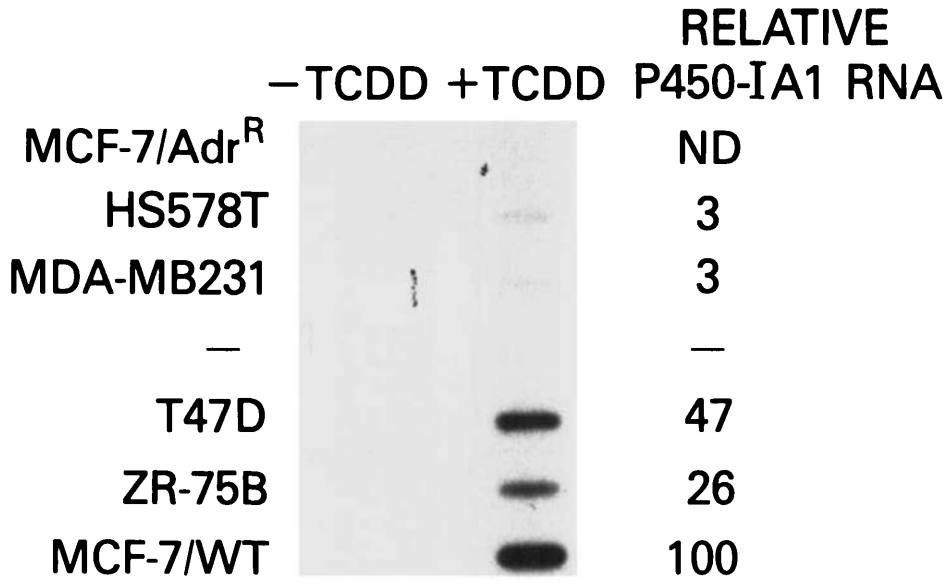

Fig. 2. Quantitation of P450IA1 Transcripts in Breast Cancer Cells by RNA Slot-Blot Analysis

Total cellular RNA was prepared from breast cancer cells grown in the absence of TCDD (−TCDD) or in the presence of 100 nM TCDD for 24 h (+TCDD) as described in *Materials and Methods*. The RNA slot blot was prepared using 10 μg total cellular RNA from each cell line and was hybridized with a radiolabeled cytochrome P450IA1 cDNA. The *far right column* indicates the P450IA1 RNA level relative to that in wild type MCF-7 cells (100%), as determined by densitometry.

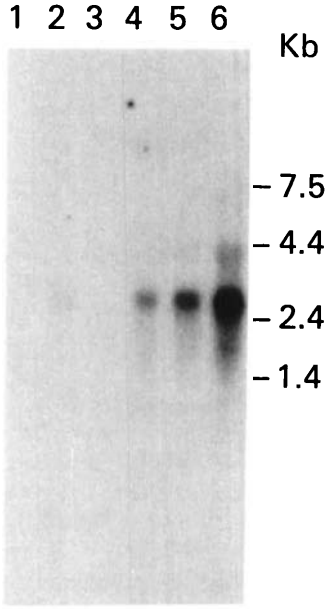

Fig. 3. Northern Blot Analysis of P450IA1 RNA in Breast Cancer Cell Lines

Total cellular RNA was prepared from breast cancer cells grown in the presence of 100 nM TCDD for 24 h as described in *Materials and Methods*. Northern blots were prepared as described in *Materials and Methods*, using 10 μg RNA/lane. The blot was hybridized to radiolabeled P450IA1 cDNA. The lanes represent RNA samples prepared from MCF-7/Adr^R (lane 1), HS578T (lane 2), MDA-MB231 (lane 3), ZR-75B (lane 4), T47D (lane 5), and wild type MCF-7 (lane 6) cells. The migration position of molecular mass markers from a RNA ladder (Bethesda Research Laboratories) are indicated. Kb, Kilobases.

positive cell lines WT MCF-7 and ZR-75B, as well as to the levels in the Ah receptor-positive mouse hepatoma cell line Hepa c1-9 (24–26). Incubation of the five human breast cancer cell lines with 5 nM [^{3}H]TCDD resulted in nuclear binding peaks at a position corresponding to fractions 8–10 (Fig. 4). The binding to this peak was eliminated by incubation in the presence of excess unlabeled TCDD. These results were similar to those obtained with the mouse Hepa c1-9 nuclear preparations (fraction 7). The amount of [^{3}H]TCDD-specific binding in each of the cell lines is shown in Table 2. The ER-negative cell line HS578T had levels of specific nuclear binding similar to those of the ER-positive cell line ZR-75B and the mouse cell line Hepa c1-9. These values were approximately 50% greater than those in WT MCF-7 cells. Levels of specific nuclear binding in the ER-negative cell line MDA-MB231 and MCF-7/Adr^R were routinely 30–50% lower than those in HS578T, ZR-75B, and Hepa 1c-9. There does not appear to be a significant correlation between the amount of TCDD-specific nuclear binding and the decrease in TCDD-inducible AHH activity in the ER-negative breast cancer cell lines.

Cytotoxicity Studies

AHH is a phase I mixed function oxidase implicated in the metabolism of a number of carcinogens and drugs (27–29). AHH metabolizes such compounds to phenolic metabolites, which are generally more cytotoxic than the parent compounds. For example, the procarcinogen benzo(a)pyrene forms potent hydroxylated cytotoxins when incubated in the presence of AHH (19, 27, 29).

Our laboratory has recently shown that the loss of TCDD-inducible AHH activity in MCF-7/Adr^R is associated with an inability to form polar metabolites of benzo(a)pyrene and a decreased sensitivity to this compound (18). The decreased sensitivity of this cell line to benzo(a)pyrene is not associated with decreased up-

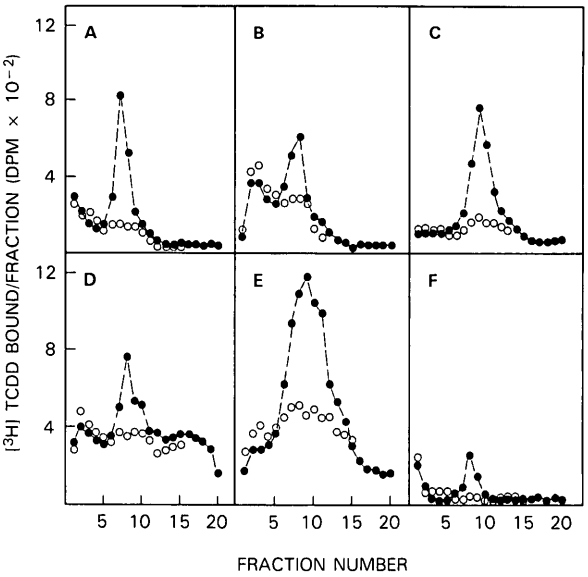

Fig. 4. Sucrose Density Gradient Analysis of [^{3}H]TCDD-Specific Binding in Hepa c1-9 and Human Breast Cancer Cells

Nuclear extracts were prepared from WT MCF-7 (A), ZR-75B (B), MDA-MB231 (C), HS578T (D), MCF-7/Adr^R (E), and Hepa c1-9 (F) cells which were incubated with 5 nM [^{3}H]TCDD in the presence (●) or absence (○) of 0.25 μM unlabeled TCDD for 2 h at 37 C. Specific binding was determined by sucrose density gradients as described in *Materials and Methods*.

Table 2. Specific Binding of [^{3}H]TCDD to Nuclear Extracts of Human Breast Cancer Cells

Cell Line	Specific [^{3}H]TCDD Binding in Nuclear Extracts	
	fmol/mg protein	Relative binding[a]
WT MCF-7	42.0	1.00
ZR-75B	60.4	1.44
MDA-MB231	19.4	0.46
HS578T	71.4	1.70
MCF-7/Adr^R	26.0	0.62
Hepacl-9	66.7	1.59

Near-confluent cells were incubated for 2 h at 37 C in medium containing 5 nM [^{3}H]TCDD. The quantity of TCDD-specific binding (femtomoles per mg protein) was determined as described in *Materials and Methods*.

[a] Ratio of specific nuclear binding in each cell line to that obtained in WT MCF-7 cells.

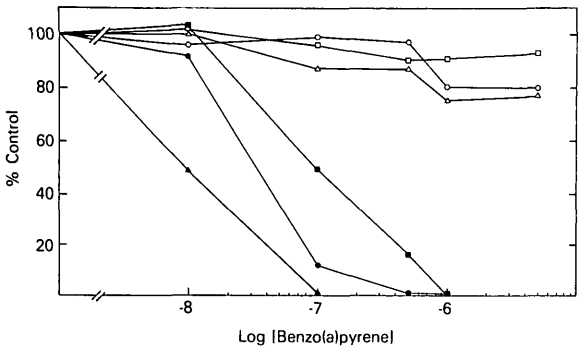

Fig. 5. Sensitivity of Breast Cancer Cell Lines to Benzo-(a)pyrene

Colony-forming assays were performed in triplicate as described in *Materials and Methods*. O, MCF-7/Adr^R cells. The other symbols represent cell lines as described in Fig. 1. Results are expressed as the percentage of colonies formed relative to cells grown in the absence of drug. There was less than 10% error between replicate samples.

take or accumulation of the compound. We, therefore, studied whether the down-regulation of AHH activity that we observed in ER-negative breast cancer cell lines is also associated with resistance to benzo(a)pyrene (Fig. 5). Whereas each of the ER-positive cell lines have IC_{50} values for benzo(a)pyrene below 10^{-7} M, the ER-negative cell lines have IC_{50} values above 5×10^{-6} M. Therefore, each of the ER-positive breast cancer cell lines studies is markedly more sensitive to benzo(a)pyrene than the ER-negative breast cancer cell lines.

We have previously shown that the loss of AHH activity in MCF-7/Adr^R cells is associated with resistance to ellipticine (18), an antineoplastic drug whose potency is increased after metabolism to a hydroxylated species by AHH or a similar mixed function oxidase (30, 31). We, therefore, studied the sensitivity of each of the breast cancer cell lines to ellipticine and the more potent 9-hydroxy metabolite of this compound. As shown in Fig. 6A, ER-negative breast cancer cell lines are significantly more resistant to the cytotoxic effects of ellipticine than are ER-positive breast cancer cells. In contrast, all of the breast cancer cell lines display a similar sensitivity to the hydroxylated form of this drug (Fig. 6B). Thus, in contrast to results with the parent drug, there is no apparent relationship between relative sensitivity to 9-hydroxyellipticine and the ER status of these breast cancer cell lines.

DISCUSSION

Our laboratory has recently shown that the phenotypes of MDR in MCF-7/Adr^R and xenobiotic resistance in rat hyperplastic nodules are associated with similar patterns of genetic and biochemical changes (15, 32). For example, in both of these model systems there is decreased expression of the phase I drug-metabolizing enzyme AHH (18) and increased expression of the anionic form of the phase II drug-metabolizing enzyme GSTπ (14, 15). The development of resistance in both rat hyperplastic nodules and MCF-7/Adr^R is also associated with overexpression of the putative drug efflux pump termed *P*-glycoprotein (13, 32).

Furthermore, our laboratory has recently shown that levels of GSTπ inversely correlate with ER levels in both breast cancer cell lines and biopsy specimens of primary breast tumors (17). In this study we have investigated a series of six breast cancer cell lines possessing various levels of ER and shown that a relationship also exists between ER levels and TCDD-inducible AHH activity in breast cancer. Since the procarcinogen benzo(a)pyrene and the anticancer drug ellipticine are converted by AHH to hydroxylated derivatives which are potent cytotoxic species, we also studied the sensitivity of the breast cancer cell lines to these agents. These studies demonstrate that the down-regulation of AHH activity in ER-negative breast cancer cell lines is associated with increased resistance to benzo(a)pyrene and ellipticine relative to that of the ER-positive cell lines. The observation that the cell lines used in this study are equally sensitive to 9-hydroxyellipticine sug-

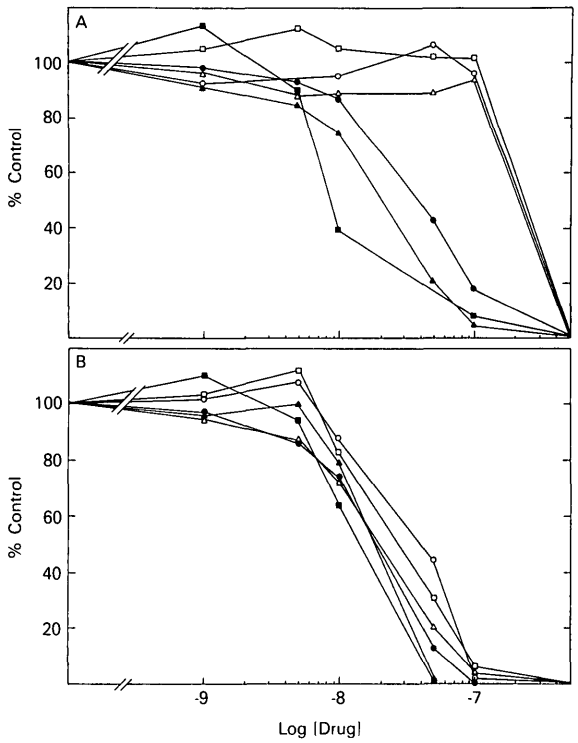

Fig. 6. Sensitivity of Breast Cancer Cell Lines to Ellipticine and 9-Hydroxyellipticine

Colony-forming assays were performed in triplicate as described in *Materials and Methods*. The symbols represent cell lines as described in Fig. 5. Results are expressed as the percentage of colonies formed relative to that of cells grown in the absence of drug. There was less than 10% error between replicate samples. A and B represent results from cells grown in the presence of ellipticine and 9-hydroxyellipticine, respectively.

gests that it is the loss of formation of this more potent derivative in ER-negative cell lines that is responsible for the difference in sensitivity to ellipticine.

The induction of AHH activity by TCDD is mediated by the interaction of this molecule with a cytosolic receptor, referred to as the Ah receptor (30). The TCDD-Ah receptor complex translocates to the nucleus and interacts with cellular DNA to regulate the expression of a number of genes, including P450IA1 (27, 33–35). In the case of P450IA1, there is evidence which indicates that the gene is regulated, at least in part, at the level of transcription. The alterations in the levels of AHH activity in human breast cancer cells are also associated with corresponding differences in the levels of P450IA1 RNA. This suggests that the decreased expression of AHH activity in ER-negative breast cancer cells is due to decreased transcription of the P450IA1 gene.

Animal cell lines expressing decreased levels of AHH activity have been isolated (36–40). Decreased AHH expression in these cell lines may be caused by a number of mechanisms, including decreased levels of Ah receptors (41), decreased ability of the occupied receptor to translocate to the nucleus (41, 42), and structural mutation in the P450IA1 gene (41, 43). There also exist dominant mutations, in which down-regulation of AHH activity may involve a trans-acting repressor protein (37, 41). The lower AHH activity of the ER-negative cell lines used in this study relative to that of the ER-positive cell lines is apparently not due to a loss of Ah receptors. Radiolabeled TCDD binding studies indicate that nuclear TCDD-specific binding proteins are present in each of the five breast cancer cell lines examined. Furthermore, there is no apparent relation between the levels of nuclear TCDD-binding proteins and the levels of TCDD-inducible AHH activity in the ER-positive and ER-negative breast cancer cell lines. Thus, the loss of ERs in the breast cancer cell lines studied in this report is not associated with the loss of Ah receptors. Mechanisms other than the decreased expression of Ah receptors must be responsible for the loss of TCDD-inducible P450IA1 expression in these cells. Whether the mechanism(s) responsible for decreased TCDD-inducible AHH activity is similar in all of the ER-negative cell lines that we have studied will require further analysis.

Previous studies have revealed that the expression of ER in breast cancer is inversely correlated with the expression of several other gene products, including the EGF receptor (9–12), protein kinase-C (7, 8), and the anionic isozyme of GST (17). The studies presented in this report demonstrate that polycyclic hydrocarbon-inducible P450IA1 expression is related to the ER content of human breast cancer cells. Furthermore, the decreased expression of AHH in ER-negative cells is associated with resistance of these cells to the procarcinogen benzo(a)pyrene and the antineoplastic agent ellipticine. This suggests that in addition to decreased responsiveness to hormonal agents, ER-negative breast cancer cells may have altered sensitivity to agents that are substrates for drug-metabolizing enzymes.

The results of this study provide further evidence that a set of common intracellular factors may regulate the expression of several genes in breast cancer cells. Although relatively little is known concerning factors that control the regulation of these proteins, transcriptional regulation of P450IA1 may be regulated by a number of DNA-binding proteins (29, 44). Understanding the factors that regulate the expression of drug-metabolizing enzymes in human breast cancer cells may provide important insights into the factors responsible for the different patterns of gene expression observed in ER-positive and ER-negative breast cancer cells. Identifying these factors may provide an understanding of the differences in tumor cell biology associated with ER in human breast cancer.

MATERIALS AND METHODS

Materials

NADPH was purchased from Sigma Chemical Co. (St. Louis, MO). Benzo(a)pyrene, 3-hydroxy-benzo(a)pyrene, and TCDD were obtained from the NCI Carcinogen Reference Standard Repository. Ellipticine and 9-hydroxyellipticine were obtained from the Drug Development Branch, NCI (Bethesda, MD).

Cell Culture

The breast cancer cell lines ZR-75B, T47D, MDA-MB231, and HS578T were kindly provided by R. B. Dickson, NCI. The selection of MCF-7/Adr^R cells was previously described (14). All cell lines were maintained in zinc option Improved Minimum Essential Medium (Gibco, Grand Island, NY) containing 5% fetal bovine serum (Gibco). Cells were grown as monolayers in an atmosphere of 5% CO_2.

Toxicity Studies

The sensitivity of cells to various agents was studied by colony forming assays, which were performed by plating 1000 cells/well in triplicate in six-well Linbro dishes as previously described (13).

AHH Assay

AHH activity was measured in cell sonicates by a method similar to that described by Nebert and Gelboin (19), using benzo(a)pyrene as a substrate. Cells were grown in medium in the absence or presence of TCDD for 24 h. The medium was then removed, and cells were washed twice with ice-cold PBS. After the washing procedure, cells were harvested, and cell pellets were stored at −70 C until used. Immediately before the assay, cell pellets were thawed and sonicated in an equal volume of 0.05 M Tris-HCl, pH 7.57. Reaction mixtures contained Tris-HCl (50 μmol; pH 7.57), $MgCl_2$ (3 μmol), NADPH (0.5 mg), benzo(a)pyrene (100 nmol), and protein (0.05–0.5 mg) in a total volume of 1.0 mL. All solutions containing benzo(a)pyrene were kept in the dark during all procedures. The reaction mixtures were incubated at 37 C for 45 min. The reaction was stopped by the addition of 1.0 mL acetone, and extraction was performed with 3.0 mL hexane for 10 min. A 1.0-mL aliquot of the organic layer was then extracted with 1.0 mL 1.0 N NaOH. The fluorescence of the alkaline extract was measured immediately at 396 nm excitation and 522 nm emission in a Perkin-Elmer 650-15 Fluorescence Spectrophotometer (Perkin-Elmer, Norwalk, CT). Fluorescence was compared to the fluorescence of a standard solution of 3-hydroxy-

benzo(a)pyrene. One unit of AHH is defined as that amount of protein that catalyzes in 45 min the formation of phenolic products with fluorescence equivalent to 1 pmol 3-hydroxybenzo(a)pyrene. The reaction was linear with respect to both protein concentration and time.

Protein concentrations were determined with Pierce Protein Assay reagent (Pierce, Rockford, IL), using BSA as a standard.

[^{3}H]TCDD Binding Studies

The procedure for measuring the binding of [^{3}H]TCDD to cells in culture using sucrose density gradients has been previously described (24–26). This procedure was modified as follows. Near-confluent cells were incubated at 37 C for 2 h in the presence of 5 nM [^{3}H]TCDD. After incubation, cells were harvested, suspended in 25 mM HEPES, pH 7.6, containing 1 mM dithiothreitol and 1 mM $MgCl_2$, and homogenized with 30–60 strokes of a Teflon Dounce homogenizer (Kontes Co., Vineland, NJ). Nuclear extracts were prepared, treated with 0.1 mg/ml dextran-coated charcoal for 15 min, and layered (300-μl samples) on sucrose density gradients (5–20%) containing 0.4 mM KCl. After centrifugation at 265,000 × g for 1 h at 4 C, 25 200-ml samples were collected from each gradient, and the radioactivity for each sample was determined in a Beckman LS scintillation counter (Beckman, Palo Alto, CA). Specific binding is that binding which was eliminated by the presence of a 500-fold excess of unlabeled TCDD.

RNA Studies

Total cellular RNA was purified from cells using guanidinium isothiocyanate and cesium chloride gradient centrifugation as previously described (45). Slot-blot analysis was performed by applying RNA directly to nitrocellulose filters as described by the manufacturer (Schleicher and Schuell, Keene, NH). RNA Northern blot analysis was performed after electrophoresis of RNA on a 20 mM 3-(*N*-morpholino)propanesulfonic acid-1% agarose gel, staining with ethidium bromide to check for equality of RNA loading, and then transferring the size-fractionated RNA to nitrocellulose filters (46).

The probe used in these studies contained 1000 basepairs DNA 5′ to the *Eco*RI site of the human cytochrome P450IA1 cDNA (47), which was generously provided by D. Nebert. Blots were hybridized to radiolabeled probe prepared by a nick translation kit (Bethesda Research Laboratories, Gaithersburg, MD). Filters were prehybridized, hybridized, and washed as described previously (48).

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