ISOLATION AND CHARACTERIZATION OF AN ARYL HYDROCARBON RECEPTOR NEGATIVE T47-D HUMAN BREAST CANCER SUBLINE

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Abstract

T47-D human breast cancer cells were cultured in media containing 1 μM benzo[a]pyrene (BaP) for 3.5 months. Ten resistant clones were individually isolated and screened for inducibility of ethoxyresorufin *O*-deethylase (EROD) activity by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). One of the BaP resistant clones (C5) exhibited only minimal EROD induction and its responsiveness to TCDD was further investigated. Results from a sucrose density gradient analysis indicate the presence of liganded nuclear Ah receptor in the wild type cells. In contrast, C5 cells treated with [^{3}H]TCDD failed to accumulate significant levels of the 6S nuclear Ah receptor complex. Ah receptor (AhR) mRNA levels were also investigated and only the wild type cells constitutively expressed the AhR mRNA.

Introduction

Approximately 75% of breast carcinomas are estrogen receptor positive, and one successful approach for the treatment of these types of tumors has utilized antiestrogens such as tamoxifen[1)]. T47-D human breast cancer cells express the estrogen receptor (ER) and estrogen induces a broad spectrum of responses in these cells. T47-D cells also express the AhR and treatment of these cells with AhR agonists such as TCDD, results in the rapid formation of liganded nuclear AhR complex which acts as a transcriptional enhancer by interacting with dioxin responsive elements (DREs) in the 5' flanking region of TCDD-inducible genes.

Several studies have demonstrated that TCDD and related compounds inhibit a broad spectrum of estrogen-induced responses both *in vivo* and in human breast cancer cells. Furthermore, studies in our laboratory indicate that TCDD has antimitogenic as well as antiestrogenic activity in T47-D cells [2)]. Thus, there appears to be an interaction between AhR- and ER-dependent signal transduction pathways.

This study reports the isolation of a BaP resistant T47-D subline (C5) that exhibits a unique TCDD-nonresponsive phenotype. TCDD fails to induce EROD activity in these cells and only very low levels of nuclear AhR complex are detectable in the nucleus following treatment with [^{3}H]TCDD. Furthermore, results from Northern blot analysis indicate that the *Ah R mRNA* levels are only detectable in wild type T47-D cells. In contrast, the C5 cells have retained responsiveness to estrogen and this AhR$^-$: ER$^+$ phenotype can be utilized

as a model for investigating the mechanism of TCDD-induced antiestrogenic and antimitogenic effects.

Materials and Methods

Chemicals and Biochemicals TCDD, [^{3}H]TCDD (32 Ci/mmol), 2,3,7,8-tetrachlorodibenzofuran (TCDF), and ethoxyresorufin were prepared in this laboratory. All other chemicals and biochemicals were either reagent or molecular biology grade and were purchased from commercial sources. T47-D cells were purchased from American Type Culture Collection (Rockville, MD).

Selection and isolation of BaP resistant T47-D Cells T47-D cells were maintained in media containing 1 μM BaP for 3.5 months. The surviving resistant cells were replated at a low density (10,000 cells/cm^2) and allowed to replicate and form colonies. The colonies were individually isolated using glass cloning rings and plated separately in 6 well plates. One cell line (C5) with the lowest inducibility of EROD activity by TCDD was selected for further characterization.

Cell Maintenance Both the wild type and BaP resistant cells were maintained in Dulbecco's DME/F-12 medium supplemented with sodium bicarbonate (2.2 g/liter), fetal bovine serum (5%) and Sigma's antibiotic - antimycotic solution (10 ml/liter). Cells were grown in 150 cm^2 culture flasks in a 95% air 5% $C0_2$ atmosphere at 37°C.

Ethoxyresorufin O-Deethylase Activity At 70% confluency, cells were dosed with DMSO or 1 nM TCDD. After 24h, cells were harvested manually by scraping, resuspended in Tris sucrose buffer (38 mM Tris, 0.2 M sucrose, pH 8.0), and EROD activity was determined by methods previously described[3].

Determination of Nuclear Ah Receptor Levels At 80% confluency, cells were harvested by trypsinization, washed with 30 ml of media and resuspended in 10 ml of culture media. Cells were then treated with 1 nM [^{3}H]TCDD. To obtain a baseline, cells treated with [3H]TCDD were cotreated with a 200-fold molar excess of TCDF. Following a 2 h incubation at 37°C, nuclear extracts were obtained and sucrose density gradient analysis was performed as previously described[4].

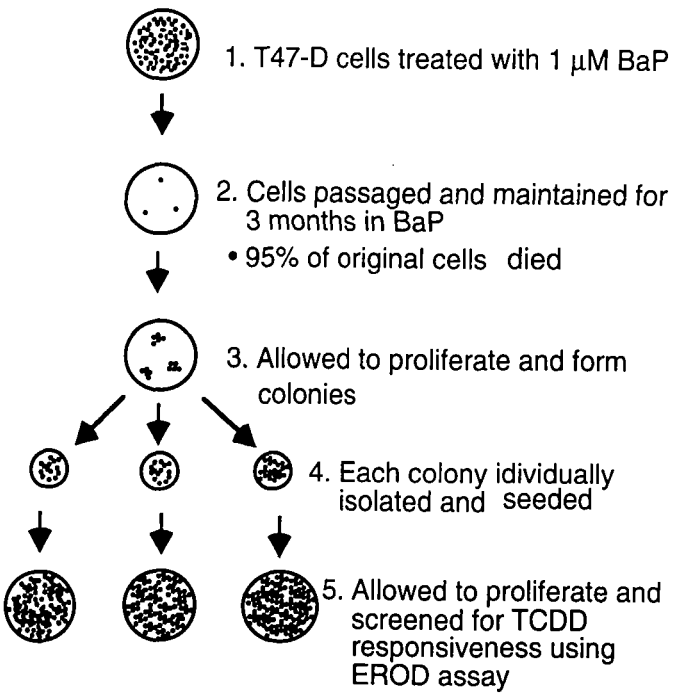
Northern Analysis The ß-tubulin (human) cDNA probe was purchased from American Type Culture Collection. The murine Ah R cDNA was a generous gift from Dr. C. Bradfield, Northwestern University[5] Total RNA was isolated by guanidinium thiocyanate/ acid phenol extraction method and mRNA levels were determined as previously described[6].

Results and Discussion

The objective of this study was to isolate an Ah-nonresponsive T47-D human breast cancer subline that retained estrogen responsiveness. The Ah-nonresponsive cell lines were selected from the wild type T47-D cells using 1 μM BaP in the culture media for 3.5 months. BaP was chosen for selection because its genotoxic metabolites are thought to be CYP1A1 dependent and AhR mediated. Thus, treatment with BaP results in the enrichment of cells that are defective in AhR dependent induction of *CYP1A1* gene expression. Previous studies have utilized similar approaches to isolate Ah nonresponsive MCF-7 and Hepa 1c1c7 cells[6,7]. The MCF-7 cells were also selected with BaP and, like the BaP resistant T47-D cells described in this study, were nonresponsive to TCDD. In contrast, the BaP resistant MCF-7 cells express the AhR; however, the liganded AhR fails to bind the DRE and induce *CYP1A1* mRNA.

Figure 1 illustrates the procedure used to isolate the BaP resistant T47-D cells. Treatment with 1 μM BaP for 3.5 months resulted in the death of approximately 99% of the wild type cells. Surviving BaP resistant cells formed colonies on the plate, were isolated individually and cultured separately until they reached confluency.

Figure 1. Selection of BaP Resistant T47-DCells



TCDD-induced EROD activity was investigated in ten of the resistant cell lines. Following treatment with 1 nM TCDD for 24 h, one clone (C5) exhibited an approximately 10-fold reduction in EROD activity compared to wild type cells (Table 1), and was selected for further characterization.

Table 1

TCDD inducibility of ethoxyresorufin O- deethylase activity in wild type T47-D and BaP resistant C5 cells[a]

Cell type	Treatment	EROD Activity (pmol/min/mg)
T47-D	DMSO	4.5±1.1
	TCDD (1 nM)	302.8±12.6
C5	DMSO	not detectable
	TCDD (1 nM)	[b]32.5±0.9

a The cells were treated with DMSO or 1 nM TCDD for 24 h and EROD activity was determined. The results are expressed as means ± S.D. for three separate determinations for each treatment.

b Significantly lower than T47-D cells treated with TCDD ($p < 0.05$)

Sucrose density analysis was performed to determine AhR complex formation in both wild type and C5 T47-D cells treated with 1 nM [^{3}H]TCDD for 2 h. The BaP resistant C5 cells exhibited significantly reduced binding compared to the wild type T47-D cells (Table 2), indicating the lack of a ligand binding form of the AhR.

Table 2

Nuclear Ah receptor levels in T47-D and C5 cells[a]

Cell Type	Receptor level (fmol/mg)
T47-D	67.7±2.3
C5	[b]11.7±0.5

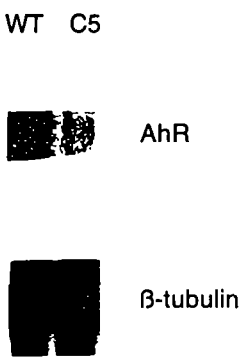
a Cells were treated for 2 h with 1 nM [^{3}H]TCDD and receptor levels were determined by sucrose density gradient analysis.

b Significantly lower than T47-D cells treated with TCDD ($p < 0.05$)

AhR mRNA levels were determined in both cell lines using Northern blot analysis and the results (Fig 2) indicate that the wild type cells constitutively express the AhR mRNA. In contrast, AhR mRNA was only minimally expressed in C5 cells. These data indicate that the BaP resistant C5 cells are Ah-nonresponsive and this phenotype correlates with the failure of these cells to express the AhR mRNA. In contrast, these cells have retained their estrogen responsiveness (data not shown) and therefore are ideally suited for further studies on TCDD mediated mechanisms of antiestrogenicity and antimitogenicity in T47-D cells.

Figure 2

Constitutive AhR mRNA Levels in Wild Type and C5 cells[a]



a Total RNA was isolated from untreated cells and AhR mRNA levels were determined and standardized against ß-tubulin

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