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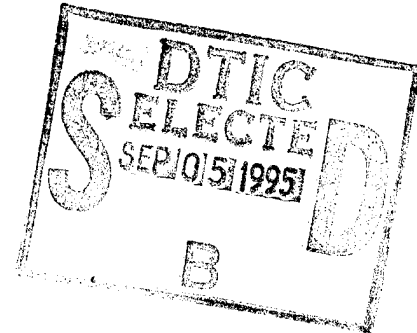
TITLE: Novel Cytochrome P4501B1 as a Mammary Cancer Risk Factor

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13. ABSTRACT (Maximum 200 words) <p>This work aims to elucidate whether cytochrome P450 1B1 (CYP1B1) and the related P450 1A1 (CYP1A1) are determinants or markers for breast cancer. Potential mechanisms include carcinogenic activation of polycyclic hydrocarbons (PAH) and of 17β-estradiol to 4-catecholestrogens. Both processes may be modulated by organochlorine compounds via the Ah-receptor (PCB's), the estrogen receptor (ER), or Ca elevation (hexachlorocyclohexanes). We have established quantitative rtPCR methods to measure levels of CYP1A1 and CYP1B1 mRNA in breast cells, and have generated affinity purified antibodies to measure CYP1B1 protein levels in microsomes (immunoblots) and tissue sections (immunocytochemistry). CYP1B1 expression has been established (mRNA and microsomal protein) in normal human breast epithelial cells, breast fibroblasts, and carcinoma cell lines. The Ah-receptor stimulant 2, 3, 7, 8 tetrachlorodibenzodioxin (TCDD) elevates CYP1B1 expression in epithelial and carcinoma cells, but not in fibroblasts. Unlike CYP1A1, induction of CYP1B1 by TCDD is not dependent on ER. A program for collection of breast tumors has been established and rtPCR has been used to detect CYP1B1 mRNA in these tumors. PAH metabolism in normal human breast cells is variable between individuals and is sensitive to culture conditions. The role of CYP1B1 in this variability is currently under investigation. CYP1B1 has been localized in ductal epithelia of terminal end buds in human and rat mammary gland, but is selectively expressed in stroma in subsequently cultured rat cells.</p>				
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FOREWORD

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Yes For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

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Colin R. Jefcoate 07/28/95
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for
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Introduction

This proposal addresses potential mechanisms for contributions from environmental chemicals to the etiology of breast cancer. In particular, this research examines mechanisms for bioactivation of polycyclic aromatic hydrocarbons (PAH). These ubiquitous environmental pollutants readily concentrate in the breast adipose tissue and generate DNA adducts that can potentially mediate mutagenesis. Mutations in *ras* and *p53* genes that are consistent with PAH mutagenesis have been detected in human breast cancers. A second contribution from environmental chemicals has been indicated by epidemiology studies, which point to organochlorine compounds (OC's) that accumulate in breast adipose as a risk factor. Here three mechanisms have been identified in our proposal; (a) estrogenic/anti-estrogenic activity of DDE, PCB metabolites (b) Ca^{++} -elevating effects of many OC's (c) Ah-receptor activation (dibenzodioxins, planar PCB's). These mechanisms may be synergistic. PAH's require activation to the ultimately carcinogenic form, the bay-region dihydrodiol epoxide, and this step involves P450 cytochromes. This laboratory has recently cloned a novel cytochrome P450, CYP1B1, that is particularly active in this process (1, 2). This form is related to a second P450 form, CYP1A1 that also metabolizes PAH (3). Each form is induced via the Ah-receptor by dibenzodioxins and planar PCB's, and this laboratory has provided evidence that these forms also metabolize 17 β -estradiol to 2- and 4-catecholestrogens (4). CYP1B1 seems to be selectively effective in forming 4-catecholestrogens. This conversion is selectively elevated in endometrial and breast cancers. Recent work has also shown that estrogens exert a potentiating effect on the stimulation of CYP1A1 by Ah-receptor ligands (5).

Our recent characterization of CYP1B1 expression strongly suggests a constitutive regulatory function; that is, the associated enzyme activity either forms, or removes a physiological lipophilic regulatory molecule. Thus, the gene has a very unusual structure (only 2 introns) and produces one of the largest P450 mRNA (5.2 kb), which includes 3 kb of 3'-untranslated sequence (6). CYP1B1 is selectively expressed in hormonally regulated steroidogenic tissues (adrenal, ovary, testis), in the stromal cells of steroid sensitive tissues (mammary, prostate, uterus), and transiently in the embryo in tissue undergoing morphogenesis (1, 2). Thus, CYP1B1 may also be a key determinant of the level of an agent that directly regulates tissue development including cancer cells. We have hypothesized, based on the stromal expression pattern, that CYP1B1 may modulate stromal effects on epithelia, a key regulatory mechanism in the breast.

The initial aim of this work has been to investigate the expression of CYP1B1 in normal human breast cells including selectivity of expression between ductal epithelia, and stromal fibroblasts, *in vivo* and in cell culture. These studies will be paralleled by an examination of the expression in carcinoma cell lines with various phenotypes, and in cells cultured from breast cancers. We are interested in whether CYP1B1 is sensitive to hormonal regulation and induction or suppression by OC's. The first experiments will focus on induction by 2, 3, 7, 8 tetrachlorodibenzodioxin (TCDD), the best stimulant for the Ah-receptor. This will be correlated with measurements of potential CYP1B1-dependent activities — notably metabolism of 7, 12-dimethyl benzanthracene (DMBA) and 17 β -estradiol. We need to establish that human CYP1B1 catalyzes both activities as evidenced by parallel expression in many breast cell types and concomitant inhibition by anti-CYP1B1 antibodies. We will find out whether CYP1B1 is selectively expressed in breast tumors of all or selective types. Induction through OC activation of CYP1B1 transcription is being tested by measuring expression in relation to OC-content of breast fat, and through examination of the effects of OC's on mammary cells *in vitro*. Recent work suggests that estradiol 4-hydroxylase activity is high in breast cancers, suggesting that CYP1B1 will also be elevated (7).

Our approach during the first year has been to develop tools to measure CYP1A1 and CYP1B1 expression at the levels of mRNA, protein, and activity in cultured breast cells, and through histology and *in situ* hybridization in breast tissue *in vivo*. This has involved establishing

rtPCR quantitation of mRNA, improved CYP1B1 antibodies for histology, and culture methodologies for cell lines, tumors, and normal epithelial cells. We have also set up a breast tissue collection process that results in mRNA, protein, and DNA for further characterization for CYP1B1, CYP1A1, and other genes.

Progress Year 1

AIM 1 *Develop high-sensitivity detection methods for P4501A1 and P4501B1 using PCR, in situ hybridization, and immunohistochemistry.*

a) mRNA

In this project we will be examining the expression of CYP1A1 and CYP1B1 in cultured cells and in tissue from mammary glands, for both humans and rats. Since the numbers of available cells or amounts of tissue are relatively small, we have elected to use rtPCR to quantitate the levels of individual mRNA resulting from transcription of these genes. To this end we have identified primers and polymerase chain reaction conditions that provide linear responses between amplified cDNA product (CYP1A1 or CYP1B1 fragments) and log [mRNA]. The primers and conditions for each amplification are shown in Table I, while dilution response plots are shown for human CYP1A1 and CYP1B1 in Fig 1. These plots were obtained by serial dilutions of cDNA from TCDD induced MCF-7 and MDA-MDB-231 cells. This approach is being used to quantitate the relative amounts of constitutive CYP1A1 and CYP1B1 in several human mammary cell lines, and to measure induction factors for each gene with TCDD in these lines. In order to determine absolute amounts of CYP1A1 mRNA and CYP1B1 mRNA, competitive standards are necessary (8). A standard for rodent CYP1B1 mRNA has been constructed (cRNA with sequences complementary to CYP1B1 primers, but with a different intervening sequence). Developing these standards for human CYP1A1 and CYP1B1 is an immediate goal.

Analogous experiments are being conducted with cultured normal human breast epithelial cells from three individuals. mRNA from these cells after six-eight days in culture has been isolated, both with and without induction by TCDD. mRNA has also been isolated from seven human breast carcinomas, and for one of these tumors we have also obtained mRNA from cultured cells. In each case the only remaining step is the rtPCR amplification and quantitation of the amounts of mRNA for CYP1A1, CYP1B1, AND GAPDH (an internal standard).

b) Protein

In this project we are interested not only in transcriptional regulation, but in expression of CYP1A1 and CYP1B1 protein. This is being carried out by immunoblotting of microsomal samples relative to a pure CYP1B1 standard, and by immunohistochemistry on fixed mammary sections (in part in collaboration with Judith Weisz, Hershey Medical Center). For both studies it has been necessary to obtain a relatively large amount of pure CYP1B1 protein both for generating the antibody, and also for affinity purification of anti-CYP1B1 IgG with Sepharose-bound CYP1B1. To this end, we have expressed functional recombinant mouse CYP1B1 (rec. CYP1B1) in *E. coli*, purified the protein, and generated antibodies. These antibodies are effective in inhibiting DMBA metabolism by rec. CYP1B1, but have not been tried against human CYP1B1. We have previously identified uninduced MCF-7 cells as a source of active human CYP1B1 that is inhibitable by anti-CYP1B1 IgG. These activities are low, and an immediate goal is to identify a more active source of human CYP1B1, including expression of rec. human CYP1B1.

These antibodies have been used to immunoblot uninduced and TCDD induced microsomes from human breast epithelial cells and carcinoma-derived fibroblasts. This immunoblot is shown in Fig. 2. This confirms that there is constitutive expression of CYP1B1 in cultured human mammary epithelial cells and in carcinoma-derived mammary fibroblast cells. The expression is

much more inducible by TCDD in epithelia than in fibroblasts. Of particular note is the consistent expression of CYP1B1 as a 52 kDa protein, substantially smaller than the 57 kDa seen for rat CYP1B1 and expected from the sequence for human CYP1B1.

Affinity purified anti-CYP1B1 antibodies have also been used to localize CYP1B1 protein expression in human breast and rat mammary gland (collaboration with Judith Weisz). The protein is expressed selectively in the epithelia of terminal end buds and also in the associated stroma, both in humans and rats. Surprisingly, the expression is much more selective in cells cultured from rat mammary gland. CYP1A1 is seen in the stromal fibroblasts, but is barely detectable in the epithelia.

c) Cellular Activity

We have used DMBA metabolism in cultured human breast cells as a means of monitoring changes in basal and induced expression. This does not allow distinction between CYP1A1 and CYP1B1, each of which may contribute to this activity. We have, however, found that there are substantial differences between epithelia isolated from breasts (mammoplasties) of three individuals. For 2 individuals, initial cultures exhibited barely detectable basal DMBA metabolism that increased progressively with further time in culture. TCDD induced DMBA metabolism did not increase to the same extent (Fig. 3). One possible explanation of the increase in basal activity, but not TCDD induced activity is that there is constitutive activation of the Ah-receptor. These two sets of cells also showed a morphological change in the day six-eight cultures indicating some organization of the cells into channel-like structures (Fig. 4). It is tempting to suggest that the constitutive Ah-receptor activity plays a role in this morphological change. Here we are interested in activation of a set of Ah-receptor linked genes; CYP1A1, CYP1B1, plasminogen activator inhibitor 2 [PAI2], GSH transferase Ya, and quinone reductase (9). Receptor activation will be shown by basal stimulation of this set in a concerted manner in cultures showing the transformation. It will also be critical to examine these cells with cytological markers that distinguish epithelial, myoepithelial, and fibroblasts (10).

We have also succeeded in culturing cells from one estrogen receptor negative tumor. The initial organoids exhibited extremely high activity that declined dramatically after trypsin treatment, and then slowly recovered over the subsequent five days (Fig 5). Cells liberated by trypsin showed much lower activity suggesting that there may be two populations of cells in the tumor. This will be evaluated immunohistochemically as pointed out for normal cells. There are major questions about the lineage of cells cultured from breast tumors, notably that these cells may not represent the predominant tumor cell type. We also expect the phenotype of both normal and tumor cells to be greatly affected by culturing with the extracellular matrix fraction, Matrigel, that is extracted from EHS tumors. We, like others, have already seen large morphogenic changes for breast epithelial cells in this medium, and are the process of assessing the impact on basal and TCDD induced CYP expression. These tumor cells exhibit TCDD inducible DMBA metabolism, which is surprising in view of the resistance of CYP1A1 in ER⁻ cells to this stimulation, notably in MDB231 cells (5).

AIM 2 Regulation of CYP1B1 expression in mammary cells.

a) Dependence of CYP1B1 Expression on Ah-receptor and Estrogen Receptor[ER]

Recent work has shown that the induction of CYP1A1 via the Ah-receptor is also dependent almost completely on activation of the ER (5). We have examined this dependence in several human breast cell lines and in normal epithelial cells by addition of the full ER antagonist ICI 182780. The measurement of mRNA levels by rtPCR confirmed that there was a suppression of TCDD induced expression of CYP1A1 in all cases, except in the MDB231 cells. Here there

was no effect due to the initial absence of the ER and consequent resistance to induction. However, we observed that the constitutive expression was also suppressed by ICI 182780, thus raising the possibility that ER is required for basal expression of CYP1A1, and that the Ah-receptor enhancer activity is actually not substantially affected. This conclusion was reinforced by the observation that MDB231 cells show induction of CYP1B1 comparable to breast cells that retain ER. In addition ICI 182780 does not suppress the expression of CYP1B1 in any of the breast cells. We are currently examining the induction of other Ah-receptor linked genes.

We have also examined the regulation of CYP1A1 and CYP1B1 in rat mammary cells (Fig. 6). We have shown previously that CYP1A1 and CYP1B1 are selectively expressed in, respectively, epithelia and fibroblasts. In the fibroblasts, estradiol effects a doubling of CYP1B1 mRNA levels, but surprisingly ICI 182780 produces a 2-3 fold increase in CYP1B1 microsomal protein. This set of experiments is being completed with equivalent measurements of mRNA and functionality, together with evaluation of this regulation of CYP1A1 in epithelial cells. The results in human cells suggest a bimodal activation and inhibition of CYP1B1 expression by ER that also requires further analysis.

b) Other Regulation of CYP's 1A1 and 1B1

Our earlier work has shown that CYP1B1 in rat mammary fibroblasts is suppressed by both progesterone and by cortisol. A similar suppression of CYP1A1 is seen in rat mammary epithelial cells. A further suppression mechanism is indicated by the capacity of the protein synthesis inhibitor, cycloheximide, to stimulate the expression of CYP1B1 in these fibroblasts (Fig. 7). This points to a labile protein that mediates a suppression process similar to that previously described for CYP1A1 in hepatoma cells (11). We are now determining whether there is any connection between this mechanism and the steroid-dependent changes. The receptor involvement in these steps is also being furthered by means of receptor antagonists. These suppression effects may also be dependent on ER, possibly through elevation of progesterone receptors which probably mediated the suppression effected by progesterone.

AIM 3 Role of CYP1B1 in estrogen activity.

In previous work we have shown that CYP1B1, or an immunologically related form, catalyzed the conversion of 17 β -estradiol to 3,4-catecholestrogen. One problem is that while CYP1B1 seems to be involved in this activity in human breast cells, this does not seem to be the case in rodent tissues expressing even higher levels of CYP1B1. We have, however, collaborated with Joachim Liehr (Texas, Galveston) in showing that human myomas from the uterus express a very similar activity, which is higher in these benign tumors than in endometrial tissue of normal individuals. Liehr has recently shown a large increase in estradiol 4-hydroxylase activity in breast tumors. In both situations we are working to show, by immunoblots, that there is an elevation of CYP1B1 in microsomes isolated from these tumors in proportion to 4-hydroxylase activity.

Our second approach, which is in progress, has been to prepare microsomes from the several human breast cell lines and primary epithelial cell cultures. We have described the measurement of DMBA metabolism above and the generation of anti-CYP1B1 inhibitory antibodies. We are now establishing whether there is a correlation between antibody-inhibitable DMBA metabolism (marker of CYP1B1) and antibody-inhibitable estradiol 4-hydroxylase. These activity measurements will be correlated with CYP1B1 immunoblots on uninduced and TCDD induced microsomes. At the present time microsomes from several lines and day 8 primary cultures have been prepared, and DMBA metabolism is being correlated with estrogen metabolism.

All immunoblots conducted by us to date on human breast microsomes show CYP1B1 as a 5.2 kDa protein: that is 5 kDa more mobile than anticipated, based on the sequence similarity to

CYP1B1. We have constructed human CYP1B1 vectors exactly analogous to the ones used to express mouse CYP1B1 in *E. coli*. Initial attempts have been unsuccessful, but this is not unusual and will be pursued with high priority. Similar vectors will also be constructed to transfect human CYP1B1 into HEPG2 cells which do not express the protein at measurable levels. Our goal is to determine whether the recombinant CYP1B1 expresses both DMBA and estrogen 4-hydroxylase activities.

Elucidating the potential functional roles of estrogen hydroxylation or other constitutive metabolism involving CYP1B1 can be facilitated by identification of inhibitors that block these activities selectively. We have succeeded in showing that 1-ethynyl pyrene is a potent suicide inhibitor of mouse and rat CYP1B1, particularly in cells where lower concentrations can be used. Equivalent experiments with human breast epithelial cells, however, require 1-2mM inhibitor rather than 0.1mM that is effective in mouse cells expressing CYP1B1. This needs further study. One possibility is that the 20 percent sequence change from mouse CYP1B1 to human CYP1B1 is sufficient to leave the inhibitor activation mechanism inactive.

AIM 4 Collection of human breast tumor samples/Correlation with organochlorine measurements.

Human breast tumors may express elevated levels of CYP1B1 (or CYP1A1) due to some endogenous activation process. Elevation of these proteins, particularly in normal breast tissue, but also in tumors, may, in part, be linked to the presence of inducing organochlorine compounds in breast adipose. While collecting seven breast tumors, we have also obtained adipose samples for analysis. Once we have completed rtPCR quantitation of CYP1A1 and CYP1B1 mRNA levels, these adipose samples will be analyzed by GC-MS for levels of various OC. Preliminary measurements on these tumors indicate heterogeneity in the CYP1B1 expression relative to GAPDH mRNA reference measurements.

AIM 5 Epithelial/Fibroblast interactions among breast cells.

Although we have cultured epithelia and fibroblasts from both rat and human mammary tissues co-culture has not been examined. Experiments in this direction will not be conducted in the near future.

Conclusions

Our early studies suggest that there may be substantial differences between human and rat mammary tissue with respect to Ah-receptor regulation of CYP1A1 and CYP1B1. Most notable while CYP1A1 and CYP1B1 are essentially segregated in their expression between, respectively, cultured rat mammary epithelial and stromal fibroblasts, this separation is less clear in human breast cells. We find that both CYP1B1 and CYP1A1 are expressed in cultured human breast epithelial cells. Cultured human breast fibroblasts retain the more selective expression of CYP1B1, but exhibit resistance to TCDD induction through the Ah-receptor. Contrary to the expression in cultured epithelia, we find consistent CYP1B1 expression in both rat and human ductal structures *in vivo*, particularly terminal end buds. There may be regulatory factors present *in vivo* that are absent in culture. We are currently exploring the possibility that changes in cell differentiation brought about by extracellular matrix proteins also modulate CYP1B1 expression.

Preliminary data also suggests that there may be interspecies differences in the dependence of CYP1A1 expression on the estrogen receptor. In rat cells we have found no evidence for such dependence, while we have found clear demonstrations for a near complete requirement for estradiol activation in human breast cells of all types. This seems to apply to basal and induced expression of CYP1A1. This raises the important possibility that estrogens may increase the

amount of CYP1A1-mediated activation of environmental PAH's. In addition, environmental estrogens may potentially synergize with Ah-receptor agonists in this activation.

The clearest conclusion at this early stage is that there is not a universal linkage between estrogen receptor activity and Ah-receptor activation in human breast cells. There is no doubt that CYP1B1 mRNA is stimulated by Ah-receptor activators when ER is blocked or absent. We have yet to show whether there is a parallel capacity to increased functional protein. This finding is important since recent work from Dr. Steve Safe shows that Ah-receptor activation by TCDD is strongly anti-estrogenic for all ER responsive genes examined (12).

Finally, it is clear from our early studies that P450 expression in culture human breast epithelial cells, whether tumorigenic or normal, is highly variable between individuals. In the case of normal cells we cannot be sure whether this reflects the population of cells present due to *in vivo* endocrine life stage or environmental differences, or to genetic differences. We have studied cells from relatively few individuals and more will be necessary before trends become apparent. It is, however, clear that cytochrome P450-dependent DMBA metabolism is extremely sensitive *in vitro* to conditions such as passage number and environment. We will be able to understand this better as we make progress in identifying physiological functions for CYP1A1, CYP1B1, and the controlling Ah-receptor.

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**Personnel Receiving Pay
on
Grant No. DAMD17-94-J-4054**

<u>Personnel</u>	<u>Rank</u>	<u>Percent Time</u>	<u>Appointment Dates</u>
Jefcoate, Colin R.	Professor	5	07/01/94 - 06/30/95
Angus, William	Research Associate	100	02/01/95 - 06/30/96
Artemenko, Irina	Research Associate	100	07/01/94 - 08/31/94
Brake, Paul	Research Assistant	50	07/01/94 - 12/31/94
Carstens, Carsten-Peter	Assistant Researcher	100	07/01/95 - 12/31/95
Ganem, Leonardo	Research Assistant	50	12/01/94 - 06/30/95
Larsen, Michele	Research Associate	100	07/01/94 - 09/30/94
Shen, Xin	Associate Research Specialist	50	07/01/94 - 09/11/94
Shen, Xin	Associate Research Specialist	75	09/12/94 - 06/30/95
Zhang, Leying	Assistant Researcher	100	04/06/95 - 06/30/95

Table 1

Primers

CYP 1A1

Forward AAGCACGTTGCAGGAGCTGATG

Reverse GACATTGGCGTTCTCATCCAGCTGCT

CYP1B1

Forward CGTACCGGCCACTATCACTG

Reverse GCAGGCTCATTGGGTTGGC

Conditions for PCR

	CYP 1A1	CYP1B1
dNTP (20 mM stock)	1.5 μ l / sample (600 μ M)	0.5 μ l / sample (200 μ M)
10x Thermal Buffer #	5.0 μ l / sample	5.0 μ l / sample
MgCl ₂ (25 mM stock)	5.0 μ l / sample (2.5 mM)	5.0 μ l / sample@ (2.5 mM)
DEPC-treated water	35.0 μ l / sample	36.0 μ l / sample
Taq Polymerase *	0.5 μ l / sample (1.25 units)	0.5 μ l / sample (1.25 units)
Primers (25 μ M stock)	1.0 μ l / sample (500 pM)	1.0 μ l / sample (500 pM)
Template cDNA	2.0 μ l / sample	2.0 μ l / sample
Cycles	30	30
Denaturing Temperature/Time	94°C / 1.0 minute	94°C / 1.0 minute
Annealing Temperature/Time	57°C / 1.5 minutes	60°C / 1.5 minutes
Extention Temperature/Time	72°C / 2.0 minutes	72°C / 2.0 minutes
Hot Start	No	Yes\$

10x Thermal buffer contains: 500 mM KCl, 100 mM Tris-HCl (pH 9.0 at 25°C), 1% Triton X-100

* Taq Polymerase is diluted 1:1 in DEPC-treated water

@ MgCl₂ is added to initiate the reaction at 94°C\$ Samples are heated to 94°C for 3 minutes prior to initiation of reaction with MgCl₂

Table 1 Primers and conditions used for polymerase chain reaction amplification of cDNA fragments of CYP1A1 and CYP1B1.

Oligonucleotide primers were designed based on the cDNA sequence of the respective cytochrome P450. Primers were designed such that 5' and 3' oligos were in different exons. RNA was isolated from cultured human breast cancer cell lines using the TRIZOL isolation procedure (GIBCO), and subjected to reverse transcription to obtain cDNA. Aliquots of cDNA were amplified by PCR according to the conditions described.

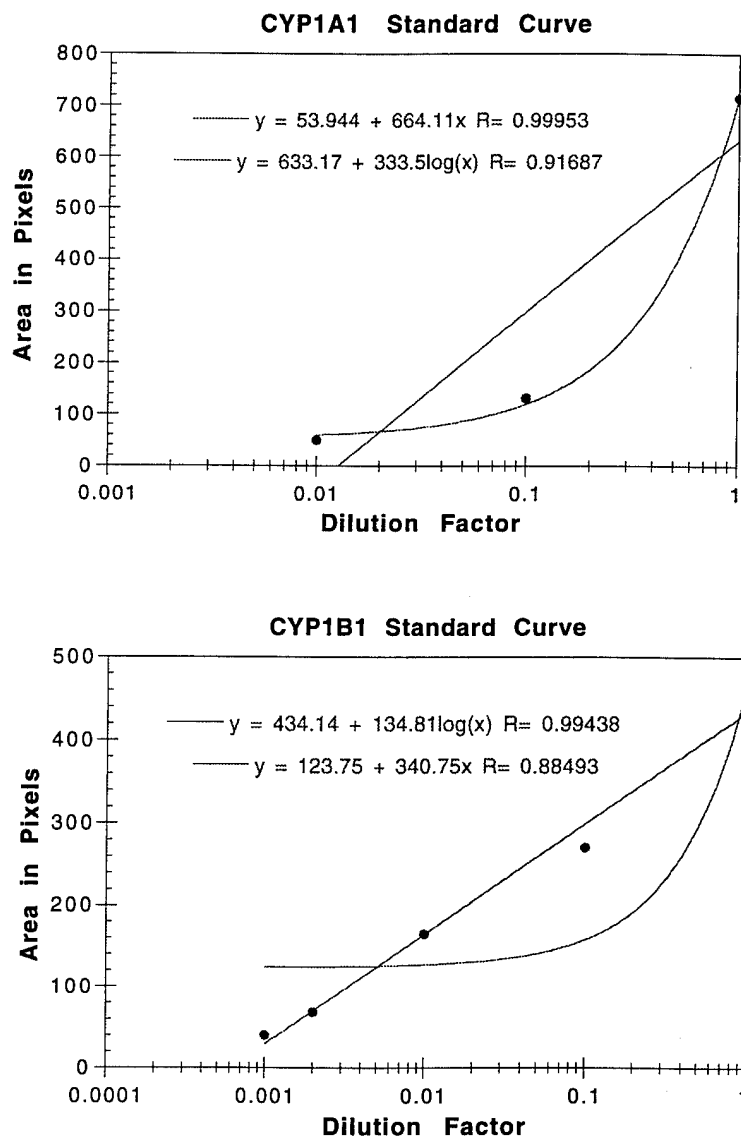


Figure 1 Dilution/response curves for CYP1A1 and CYP1B1 for MCF-7 and MDA-MB-231 mammary cells.

Total RNA (2 μ g) from control and TCDD-treated cells was subjected to reverse transcription (total volume of 50 μ l). A volume of 2 μ l cDNA was serially diluted and subjected to PCR under conditions described in Table 1. PCR product was electrophoresed through 1.5% agarose buffered in 0.5x TBE and visualized by ultraviolet transillumination using ethidium bromide. A photograph of each gel was taken using Polaroid P55 positive/negative film. Developed negatives were scanned, digitized, and quantitated using NIH Image nonFPU software. Standard curves were developed based on the areas, in pixels, under the curve for each dilution. Areas under the curve of dilutions of unknowns were compared to the standard curve and relative induction calculated.

Immunoblot Analysis of CYP1B1 Expression in Normal HMEC and Carcinoma-derived HMF

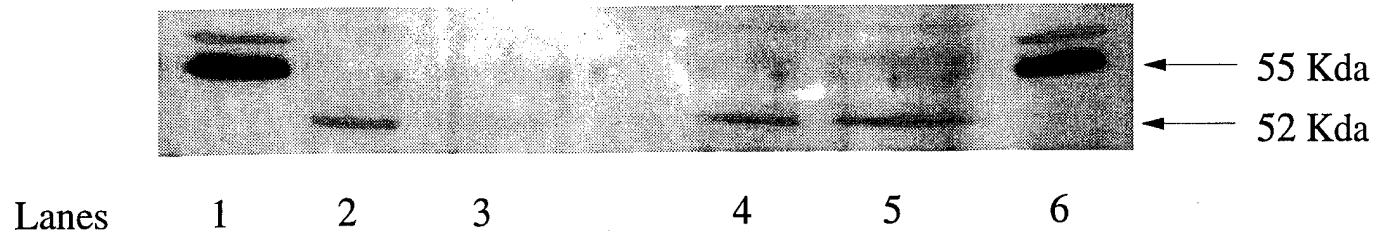


Figure 2. Microsomal CYP1B1 expression in normal human mammary epithelial cells (HMEC) and carcinoma-derived human mammary fibroblasts (HMF) was examined by immunoblot analysis.

Lane 1 and 6: BA-induced CH3/10T1/2 CL8 (5 μ g/lane)

Lane 2: TCDD-induced HMEC (7.5 μ g/lane)

Lane 3: Basal HMEC (15.0 μ g/lane)

Lane 4: TCDD-induced carcinoma-derived HMF (7.5 μ g/lane)

Lane 5: Basal carcinoma-derived HMF (15.0 μ g/lane)

BASAL AND TCDD-INDUCED DMBA METABOLISM IN NORMAL HMEC

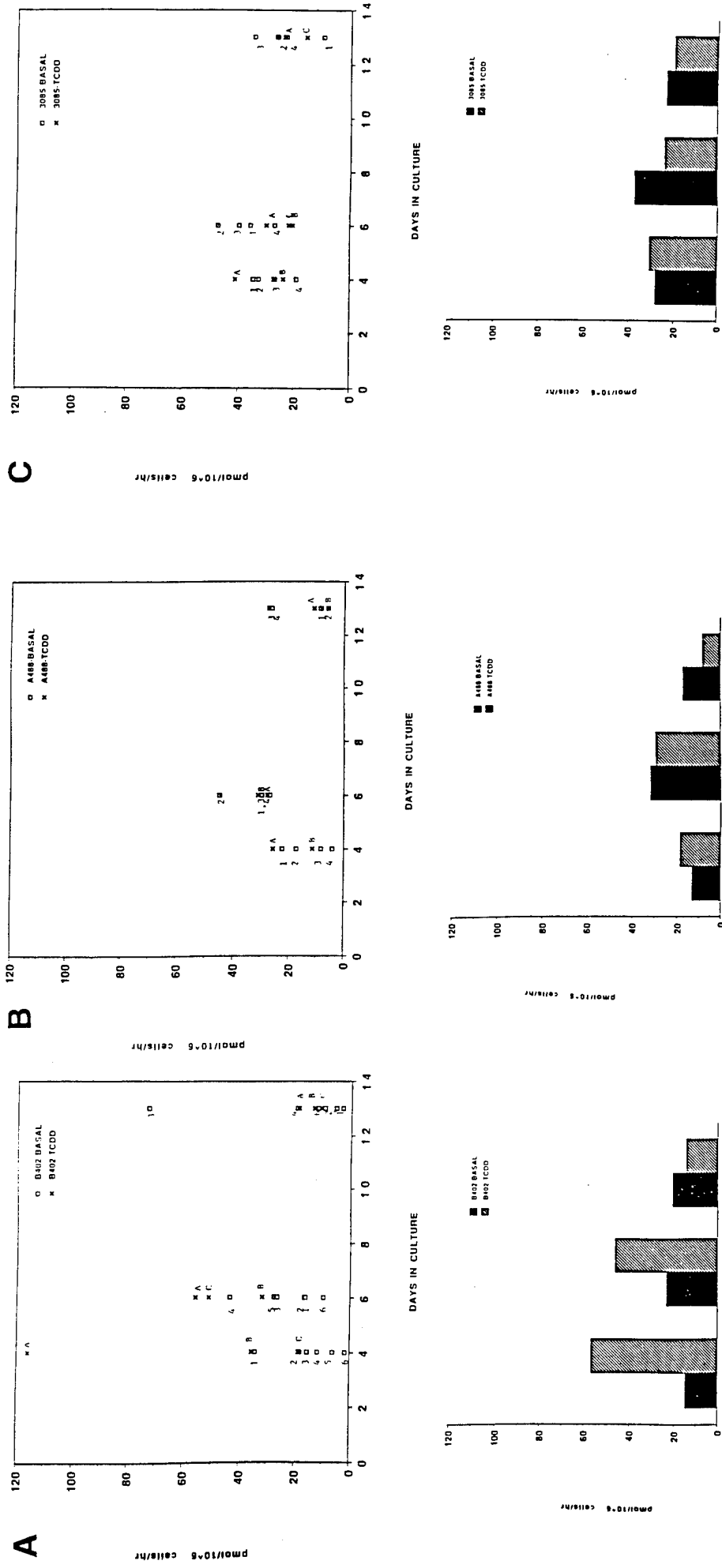


Figure 3. DMBA metabolism (8,9- + 10,11-dihydrodiol formation) in control and TCDD-induced normal HMEC cultures from three patients undergoing reduction mammoplasty surgeries. Organoid cultures were grown for 10 days prior to trypsinization. Harvested cells were replated and DMBA metabolism assayed on the cells in culture on days 4, 6, and 13 post-trypsinization. Data points represent individual wells of replicate cultures. Basal cultures are represented by numerals while TCDD-induced cultures are identified by letters. Bar graphs depict the average metabolite levels at the corresponding days of cultures.

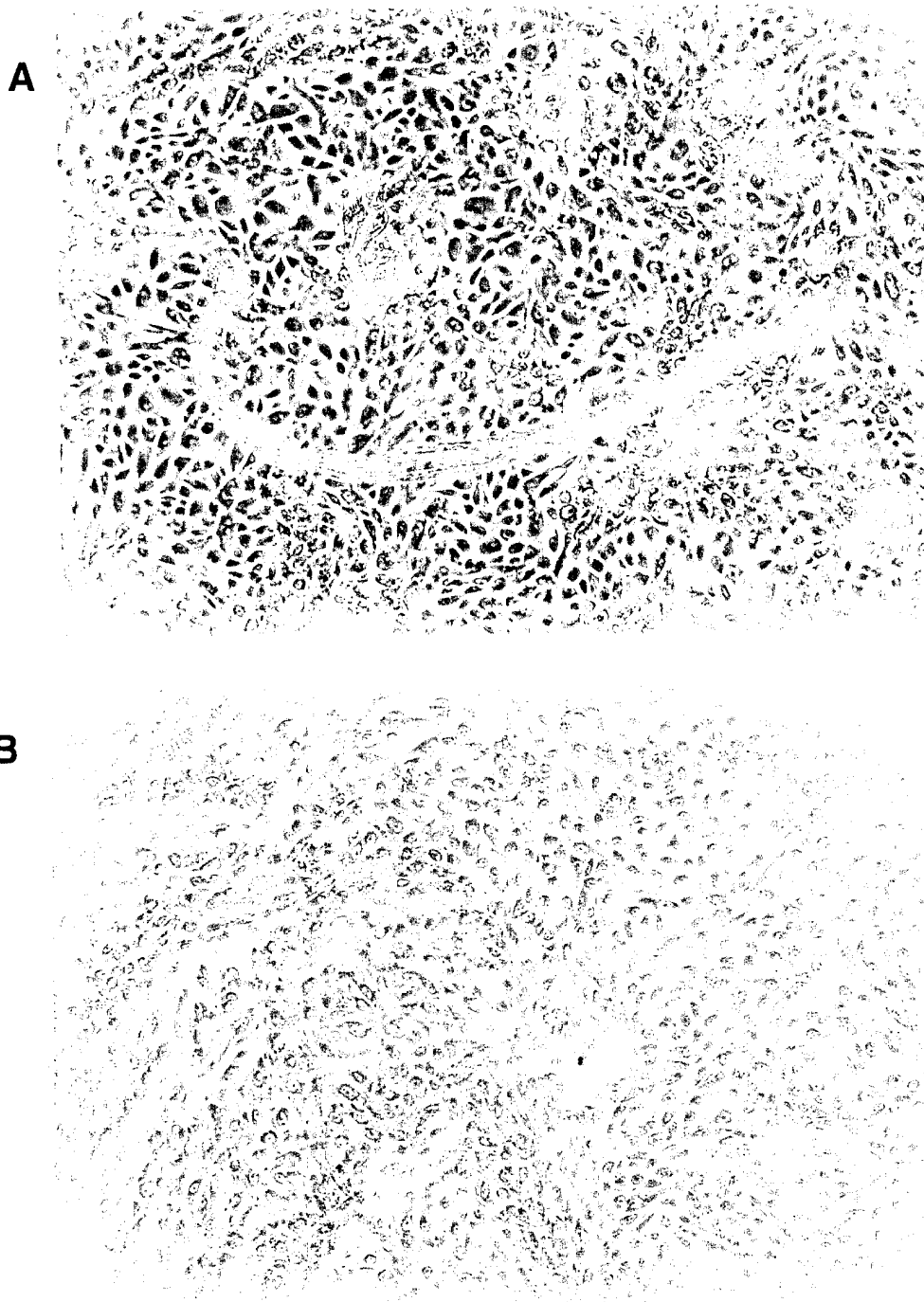
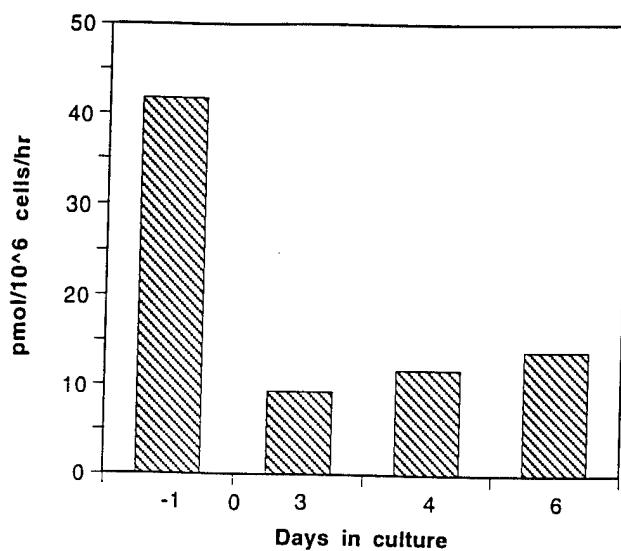


Figure 4. Normal human mammary epithelial cells (HMEC), obtained from reduction mammoplasty surgeries, were cultured on plastic in Clonetics Mammary Epithelial Growth Media (MEGM). The A488 cells (A) reproducibly (3 independent cultures) demonstrate the formation of ductal-like channels between days 6-8 while B402 (B) cells fail to form similar structures.

A Primary Human Carcinoma-Derived Epithelial Cells (Original Plating)



B Replated Human Carcinoma-Derived Epithelial Cells

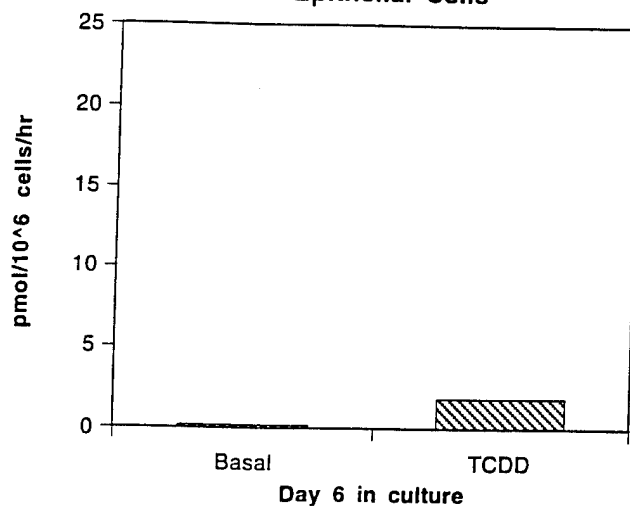


Figure 5. The following data was obtained from primary human carcinoma-derived epithelial cells isolated from a poorly differentiated, necrotic, estrogen negative, infiltrating ductal carcinoma. (A) Cellular DMBA basal metabolism was assayed 8 days after plating (day -1) prior to trypsinization. Cells were exposed to trypsin for passaging (day 0) until 50% of the cells had detached. The remaining attached cells were assayed on day 3, 4 and 6 (post-trypsinization). (B) The cells passaged at day 0 were replated and assayed for basal and TCDD-induced DMBA metabolism on day 6.

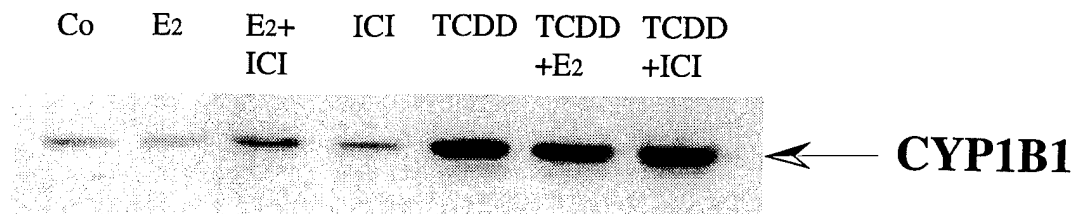


Figure 6. Expression of CYP1B1 protein as measured by Western immunoblot analysis of microsomes from estrogen-, ICI 182,780-, and/or TCDD-treated rat mammary fibroblasts. Primary cultures of rat mammary fibroblasts, 15th passage, were treated with 0.1% DMSO (Co), 10⁻⁷ M estradiol (E2), 10⁻⁷ M ICI 182,780 (ICI), and/or 10⁻⁸ M TCDD for 24 h before isolation of microsomes. Microsomal proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, incubated with anti-CYP1B1, and visualized by the enhanced chemiluminescence method. Protein loadings for all samples was 10 μ g.

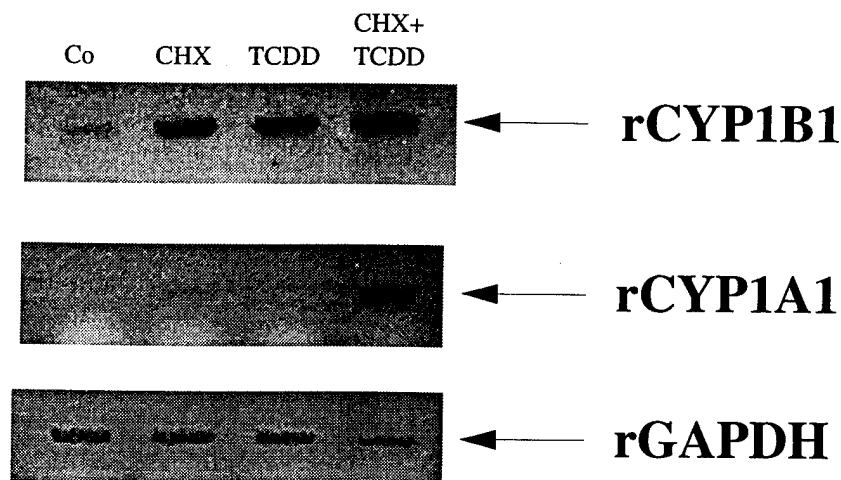


Figure 7. Expression of rat CYP1B1 and CYP1A1 as measured by Southern analysis of RT-PCR products of RNA from rat mammary fibroblasts treated with CHX and/or TCDD. Primary cultures of rat mammary fibroblasts, 12th passage, were treated with 0.1% DMSO (Co), 10⁻⁵ M CHX, and/or 10⁻⁸ M TCDD for 12 h before isolation of RNA. RT-PCR was performed and PCR products (5 μ l/sample) were separated through 1.5% agarose and transferred by capillary action to Nytran nylon membranes, the cDNA immobilized by UV cross-linking, and hybridized with ³²P-labeled cDNA probes for CYP1B1, CYP1A1, or GAPDH.

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CYP1B1 EXHIBITS CELL-TYPE SPECIFIC EXPRESSION IN RAT MAMMARY CELLS. P B Brake, M Christou, and C R Jefcoate. Environmental Toxicology Center, University of Wisconsin, Madison, WI, USA.

We are characterizing a new class of polycyclic aromatic hydrocarbon (PAH)-metabolizing cytochrome P450 recently cloned from the rat adrenal [Bhattacharyya *et al.* (1994) *J. Biol. Chem.* submitted] which, based on its sequence relationship to CYP1A1, has been designated CYP1B1. This 543 amino acid protein is constitutively expressed and/or induced by PAHs such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in a number of rodent tissues including liver, kidney, lung, uterus, adrenal, ovary, and testis. In the rat mammary, CYP1B1 exhibits cell-type specific expression. It is constitutively expressed and highly induced by TCDD (10-fold) in fibroblasts, presumably via the cytosolic Ah receptor. Conversely, mammary epithelia selectively express induced CYP1A1. Both CYP1B1 and CYP1A1 are stimulated by estradiol (2-fold) and suppressed by glucocorticoids (2-fold) in mammary fibroblasts and epithelial cells, respectively. Involvement of the estrogen receptor in regulation of CYP1B1 is currently under investigation by use of the anti-estrogen, ICI 182,780. Cycloheximide (CHX) treatment of fibroblasts induced CYP1B1, with a superinduction occurring when TCDD was co-administered. Interestingly, CYP1A1 was induced in these same cells following CHX treatment. This suggests that both CYP1B1 and CYP1A1 are under the influence of a negative regulatory factor(s) present in mammary fibroblasts. Thus, CYP1B1 exhibits cell-specific expression in the rat mammary, representing the influences of endogenous (hormonal) and exogenous (PAH) factors. (Supported by NIH Grant P30 CA14520 and DAMD Grant 17-94-J-4054.)

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CELL-SPECIFIC REGULATION OF CYP1B1 IN RAT CELLS.

PB Brake, M Christou, and CR Jefcoate. Environmental Toxicology Center, University of Wisconsin, Madison, WI.

This laboratory has cloned a new class of polycyclic aromatic hydrocarbon (PAH)-metabolizing cytochrome P450 from mouse embryo fibroblasts [Savas *et al.* (1994) *J. Biol. Chem.* **269**: 14905.] and rat adrenals [Bhattacharyya *et al.* (1994) *J. Biol. Chem.* submitted] which, based on the sequence relationship to CYP1A1, has been designated CYP1B1. The 57 kDa CYP1B1 protein has been shown to be hormonally regulated in the intact rat adrenal gland by adrenocorticotrophic hormone (ACTH) [Otto *et al.* (1991) *Endocrinology* **129**, 970.]. Activity and expression of CYP1B1 is elevated 4-fold in primary cultures of rat adrenocortical (RAC) cells following 24 h of ACTH treatment as measured by selective 7,12-dimethylbenz[*a*]anthracene metabolism and Northern blot analysis, respectively. Maximal stimulation of CYP1B1 by ACTH has been duplicated in RAC cells by the cyclic adenosine 3',5'-monophosphate (cAMP) analogue, 8-bromo-cAMP, and by the adenylyl cyclase agonist, forskolin, suggesting that cAMP mediates induction of CYP1B1 by ACTH. RAC cells respond to PAHs such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) with a lower induction of CYP1B1 (1.8-fold). Conversely, CYP1B1 is induced 6-fold in rat embryo fibroblast cells by TCDD, presumably through the cytosolic Ah receptor, but is insensitive to cAMP treatment. In cultures of rat mammary cells, CYP1B1 exhibits cell-type specific expression. It is constitutively expressed in fibroblast cells and is highly induced by TCDD. Mammary epithelial cells, however, do not appear to express CYP1B1, and instead selectively induce CYP1A1 to high levels following TCDD treatment. CYP1B1 and CYP1A1 are suppressed by glucocorticoids in mammary fibroblast and epithelial cells, respectively. CYP1B1 expression is increased about 2-fold in mammary fibroblast cells after treatment with 17 β -estradiol. Involvement of the estrogen receptor in regulation of CYP1B1 is currently under investigation by use of the anti-estrogen, ICI 162,780. Thus, CYP1B1 exhibits cell-specific regulation, representing the influences of both endogenous (hormonal) and exogenous (PAH) factors. The CYP1B1 response to hormonal stimuli suggests that this protein may have an important physiological role in the steroidogenic and steroid-responsive cells where it is constitutively expressed (adrenal, testis, ovary, mammary, and uterus). Supported by NIH Grant CA16265 and DAMD Grant 17-94-J-4054.

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