Expression of CYP1B1 but not CYP1A1 by primary cultured human mammary stromal fibroblasts constitutively and in response to dioxin exposure: Role of the Ah receptor

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The expression of CYP1B1 in human mammary fibroblasts (HMFs) was characterized as a potential modulator of their individual function as well as effects on adjacent mammary epithelia. We have used these characteristics to explore the diversity of fibroblast cells isolated from different breast locations in breast cancer patients (tumors, peripheral to tumor and skin). These parameters have also been used to examine differences between two donors. The results have shown that while none of these HMFs expressed a detectable CYP1A1 protein basally or in response to TCDD, they all expressed CYP1B1 constitutively at similar levels (0.5–0.9 pmol/mg microsomal proteins) and were induced by TCDD (up to 5-fold) consistent with mediation by the Ah receptor (AhR). DMBA metabolism by HMFs exhibited high proportions of 5,6-, 10,11- and 3,4-dihydrodiols, a profile that is typical of human CYP1B1 regioselectivity. RT-PCR followed by Southern blot analyses demonstrated that CYP1B1 mRNA expression in HMFs parallels levels of respective microsomal proteins. The AhR is expressed in these HMFs as two cytosolic forms (~106 and 104 kDa) and a substantial proportion of the 104 kDa form was localized to the nucleus even prior to TCDD treatment. In all HMFs isolated directly from collagenase digested breast tissues the AhR is expressed at levels 10-fold lower than in breast epithelial cells. However, HMFs that were isolated after serial passaging of mammary epithelial cultures had shown much higher levels of the AhR expression and more dramatic TCDD-induced down-regulation (>80% in 24 h) associated with more efficient nuclear translocation. These differences suggested the presence of two functionally distinct subtypes of HMFs: interstitial stromal fibroblasts that are readily released by collagenase digestion of breast tissues, and lobular stromal fibroblasts which are more tightly associated with the breast epithelia.

Introduction

The stroma of the mammary gland accounts for >80% of the resting breast volume (1). This stroma consists of fibroblasts, blood vessels and a macromolecular network composed of glycoproteins and proteoglycans known collectively as extra cellular matrix (ECM*). There is accumulating evidence that stromal fibroblasts play a crucial role both in normal development and in carcinogenesis of the mammary gland. During the maturation of the mammary gland, the interaction between stroma and epithelia is critical for normal development (2). The matrix metalloproteinases, gelatinase A and stromalinysin-1, and serine proteinase, a urokinase-type plasminogen activator, are expressed at low levels during lactation and are up-regulated during mammary gland involution to facilitate tissue remodeling. These enzymes are synthesized mainly in the fibroblast-like cells of the periductal stroma (3).

The estrogen sensitivity of mammary epithelia is mediated by stromal fibroblasts and only estrogen receptor (ER) expression in the mesenchyme is necessary for mammary development (4). This has been demonstrated by analysis of mammary development through the combination of epithelia and mesenchyme from wild-type and ER knockout mice (5). On hormonal stimulation, stromal fibroblasts that express ER respond by expression and release of growth factors [tumor growth factor (TGF)α, TGFβ, hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) I, FGFII, keratinocyte growth factor (KGF), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF) I and IGFII] and ECM (6). Each of these stromal factors contribute to the regulation of epithelial growth in a paracrine fashion (7).

Breast tumors are a complex and heterogeneous mix of epithelia, stromal cells, matrix proteins and vascular elements. The growth and dissemination of breast cancer requires the complex interaction between these various tumor elements. Most human breast carcinomas are associated with aberrant stromal expression of ECM degrading proteases and epithelial infiltration of the surrounding stroma (8). Stromal fibroblasts also contribute to the growth and progression of breast carcinomas by the secretion of angiogenic factors. Evidence for hypoxic-induced up-regulation of VEGF, a major angiogenic factor in human mammary fibroblasts was presented, which suggests a paracrine influence by fibroblasts on endothelia within hypoxic regions of the tumor (9).

Human breast is a target for many environmental chemicals including polycyclic and polyhalogenated aromatic hydrocarbons (PAHs). PAHs exert their pleotropic toxic responses, including carcinogenesis (10), by binding to the Ah receptor (AhR), which is a basic helix-loop-helix (b-HLH) protein (11). Ligand binding results in activation of AhR and subsequent nuclear translocation, where it heterodimerizes with another bHLH partner, the Ah receptor nuclear translocator protein (ARNT) (12). The AhR–ARNT dimer binds to specific regulatory elements, xenobiotic responsive elements (XREs),

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upstream of the responsive genes and enhances their expression (13). In addition to induction of cytochrome P450s and some phase II drug metabolizing enzymes, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a prototype PAH, elicits multiple effects on growth factors, cytokines and plasminogen activator components through this Ah receptor-mediated signal transduction pathway (14–19).

This laboratory has cloned and characterized a novel form of rodent cytochrome P450, CYP1B1, which is preferentially expressed in fibroblasts under control of the AhR (20,21). In most mouse organs (liver, lung and kidney), activation of the AhR by TCDD results in increased expression of CYP1A1 mRNA, and there is only a minor induction of CYP1B1 mRNA (20). The human CYP1B1 cDNA was cloned from a human keratinocyte cell line as a dioxin-responsive gene (18,22). The gene characterization (23) and the functional analysis of its promoter has revealed the distinct regulation of CYP1B1 gene from the closely related CYP1A1 and CYP1A2 genes (24). The cell-specific expression of CYP1B1 has been identified in human cell lines and tissues (25) and, although CYP1A1 and CYP1B1 are expressed at similar levels in human breast epithelia after TCDD treatment, only CYP1B1 is present under basal conditions in these cells (26). Furthermore, fibroblasts from a wide range of species and tissue sources lack the expression of CYP1A1 and exclusively express CYP1B1 under the control of the AhR (20,27,28). The lack of CYP1A1 mRNA expression in human skin fibroblasts was attributed to the presence of a putative repressor that competes with AhR for binding to CYP1A1 regulatory elements (29). On the other hand, in mouse embryo fibroblasts, the AhR is required for the basal expression of CYP1B1 (30). Thus, CYP1B1 expression in fibroblasts provides a measure of AhR activity, even in the absence of an external chemical inducer.

Recent studies have shown that human CYP1B1 activates many structurally diverse chemicals, including PAHs and their dihydriodiol derivatives, heterocyclic and aryl amines, and nitroaromatic hydrocarbons to genotoxic metabolites (31). In addition to its metabolic activation of carcinogens, several lines of evidence suggest that CYP1B1 has a key physiological regulatory function. These factors are the unusual cell specificity of expression in cells of mesodermal origin including steroidogenic cells (adrenal, testis, ovary), expression in the embryo, and the most recent linkage of a genetic deficiency as the cause of human congenital glaucoma, which is a defect in mesodermal development (32). The physiological substrate remains to be identified; however, human CYP1B1 catalyzes the conversion of E2 to 4-hydroxy E2 (33). This high affinity but low turnover reaction, although not effectively conserved across species, may have pathophysiological consequences.

In the present study we characterize the mRNA and protein expression of human CYP1B1 in cultured primary mammary fibroblasts as a potential modulator of their function. In particular, we have examined the role of Ah receptor activation in regulating this expression. The study uses the criteria of activation of the Ah receptor by TCDD and the expression of CYP1B1 to explore the diversity of normal fibroblasts from different donors. The effect of the tissue source of fibroblasts on these parameters is also examined in matched sets of fibroblasts derived from breast tumor (TF), normal peripheral tissue surrounding the tumor (PF) or from breast skin (SKF) of two different breast cancer patients.

Materials and methods

Materials

All tissue culture media and supplements were purchased from Sigma (St Louis, MO). Fetal bovine serum (FBS) and Trypsin were obtained from Gibco BRL (Grand Island, NY). The horseradish peroxidase conjugated goat anti-rabbit IgG was purchased from Promega (Madison, WI). The enhanced chemiluminescence (ECL) detection system was purchased from Amersham (Arlington Heights, IL). The BCA protein assay kit was purchased from Pierce Chemicals (Rockford, IL). All materials used for SDS-PAGE were obtained from Bio-Rad (Richmond, CA), and nitrocellulose membranes were purchased from Schleicher and Schull (Keene, NH). Purified human recombinant CYP1B1 and CYP1A1 proteins, expressed in human lymphoblasts, were obtained from Gentest (Woburn, MA).

Primary antibodies

Affinity-purified rabbit polyclonal antibodies raised to recombinant mouse CYP1B1 and purified mouse CYP1A1 proteins were generated in this laboratory as previously described (34,35). Rabbit polyclonal anti-CYP1A2 and anti-epoxide hydrolase antibodies were kind gifts from Dr James Hardwick (Northwestern Ohio University, Rootstown, OH) and Dr Charles Kasper (McArdle Laboratory for Cancer Research, WI), respectively. The anti-Ah receptor and anti-ARNT polyclonal antibodies were generously provided by Dr Richard Pollenz (University of South Carolina, Charleston, SC).

Cells and cell culture

Primary cultures of normal mammary fibroblasts were obtained through the University of Wisconsin Comprehensive Cancer Center (UW-CCC) where they were isolated from mammomastic surgery specimens of three individuals (all Caucasians, two females, one male). All fibroblasts were cultured as described above, and three cell lines were cultured in 1:1 mixture of Dulbecco’s minimum essential medium and Ham F12 (DMEM/F12) media, supplemented with 10% fetal bovine serum (FBS), 4.5 mg/ml glucose, 100 U/ml penicillin and 100 µg/ml streptomycin.

The matched fibroblast cultures were derived from three distinct locations: (i) tumor tissues (TF); (ii) normal (macroscopically and histopathologically confirmed) breast tissues in the periphery of a tumor (PF); and (iii) breast skin (SKF) from two breast cancer patients (G16-3: age 59, lobular invasive carcinoma; and G149: age 46, ductal infiltrating carcinoma). These two sets of matched fibroblasts were obtained from Dr Helene Smith’s laboratory (Geraldine Brush Cancer Institute, UC/SF, San Francisco, CA) where they were established as described previously (37). These cells were maintained in DMEM/F12 media supplemented with 10% FBS, 10 µg/ml insulin, non-essential amino acids, penicillin and streptomycin. All cultures were maintained at 37°C in a humidified incubator that contained 5% CO2 and 95% air.

Verification of fibroblast origin by immunofluorescence

To verify whether the cells being studied were indeed fibroblasts, all fibroblast lines were cultured directly on glass cover slips and grown to ~80% confluency. Cells on the cover slips were fixed for 5 min in pre-chilled acetone/methanol mixture (1:1) at ~20°C, and then incubated at 4°C for 20 min in a fresh change of these solvents. Cells were then dried completely before being rinsed in PBS. Cells were blocked in 3% BSA in PBS, including 0.05% Na azide. Mouse monoclonal antibodies against vimentin and cytokeratin 18 were used for primary staining and fluorescein isothiocyanate (FITC)-labeled goat anti-mouse as secondary antibody, as described previously (38). The fluorescence of the positive staining of the slides was captured and photographed by Nikon Optiphot equipped with an MRC Laser Sharp Confocal Imaging System (Bio-Rad).

Treatment

These primary fibroblasts were used for up to eight passages, and we had verified that they maintain their phenotypic characteristics for up to passage 15. Typically, cells were treated when they were ~80% confluent, with 10 nM TCDD or an equivalent volume of dimethyl sulfoxide (DMSO), to a final 0.1% concentration (vehicle control), for 24 h or as specified in the figure legends.

Treated cells were harvested by mechanical scraping in cold PBS, and cell pellets were washed in PBS and used to prepare microsomes that measured CYP1B1 and CYP1A1 proteins by immunoblotting analysis and DMBA metabolism. Microsomes were prepared as described (35), and protein concentration was determined by the BCA method (39).

Enzyme activity in microsomal preparations

DMBA metabolism was measured in microsomal preparation of control or TCDD-treated cells as described (35). The reaction mixtures contained 100 mM potassium phosphate buffer (pH 7.6), 6 mM MgCl2, NADPH-generating system (14.2 mM glucose 6-phosphate, 1.2 mM NADP and 0.06 U/ml glucose-6-phosphate dehydrogenase). Samples (in duplicates) were pre-incubated 1 min at 37°C and the reaction was initiated by the addition of 1.5 µM 7,12-dimethylbenzo[a]anthracene (DMBA) (HPLC purified) and allowed to
incubate under conditions of subdued lighting. The analysis of the human recombinant CYP1B1 and CYP1A1 microsomal standards, 0.075 and 0.050 mg microsomal protein/ml, respectively, were assayed as above, with the addition of 0.5 mg/ml human epoxide hydratase. The reaction was terminated after 15 min by addition of an ethyl acetate–acetone mixture (2:1 ratio) that contained 1 mM DTT, and the metabolites were extracted and prepared for analysis on HPLC as described earlier (40). Metabolites were separated and quantitated by reverse phase HPLC.

Analysis of the Ah receptor nuclear translocation in normal human fibroblasts

Primary cultures of normal fibroblasts were grown to ~80% confluency. The growing medium was removed and duplicate plates of each cell type were treated with either 1 nM TCDD or an equivalent volume of DMSO (0.1% final concentration) for 1 h at 37°C. Cells were harvested by trypsinization and washed three times with ice-cold PBS and counted. Cell pellets were resuspended and incubated on ice for 30 min in lysis buffer (150 mM NaCl, 1% NP-40 and 0.05% SDS in 25 mM MOPS buffer pH 7.4, 0.05% Na azide, 1 mM EDTA, 10% glycerol, 5 mM EGTA, 20 mM Na molybdate), supplemented with protease inhibitors (5 µg/ml leupeptin, 0.15 U/ml aprotinin, 10 µg/ml TLCK, 1 mM PMSF, 5 µg/ml soy bean trypsin inhibitor) and phosphatase inhibitors (1 mM Na orthovanadate and 1 mM Na fluoride). Cell lysates were centrifuged at 2000 r.p.m. for 5 min in a microcentrifuge to pellet cytoplasmic fractions. Supernatants were saved as cytosolic fractions, and nuclei were washed in lysis buffer four times to eliminate cytosolic contamination. Then nuclear pellets were lysed by sonication in lysis buffer that contained inhibitors. Samples were sonicated four times in an ice bath, at 15 s each with 15 s intervals, to eliminate sample heating. Approximately 100 µg protein of cytosolic fraction or nuclear extract of each cell type was subjected to SDS–PAGE and Western blot analysis.

Gel electrophoresis and immunoblotting analysis

Proteins were separated on 7.5% SDS–PAGE and electro-transferred to nitrocellulose membranes as described (41,42). Immuno-reactive proteins were visualized using the ECL detection system as instructed by the manufacturer. CYP1B1 and CYP1A1 were immunodetected in microsomal preparations using their specific antibodies. The specificity of these antibodies was confirmed by using the purified recombinant CYP1B1 and CYP1A1. The microsomal epoxide hydrolase, which is not affected with TCDD-treatment (40), was co-detected on the same blot as a loading control. The expression of CYP approteins in these cells were quantified relative to purified recombinant human CYP1B1 (specific activity of 74 pmol/mg protein) or CYP1A1 (specific activity of 104 pmol/mg protein) standards. To generate CYP1B1, immunoblotting quantitation standard curves of 0, 0.2, 0.4 and 0.8 µg of recombinant human CYP1B1 protein (equivalent to 0, 14.8, 29.6, 59.2 fmol P4501B1) were analyzed. Each of these protein points was spiked with 10 µg of microsomal proteins from cells that do not express CYP1B1 as a carrier to enhance their migration and resolution on SDS-gel. To avoid variability that results from gel running, blotting procedure, developing and exposure conditions, the samples to be quantified were analyzed on the same gel. Standard curve dilutions that were used for the respective quantitation. Images from blots were scanned by PDSI Scanning Densitometer (Molecular Dynamics, Sunnyvale, CA) and band intensities were quantified using the volume integration option of the ImageQuant software (Molecular Dynamics). Regression lines of the curve that related the intensity of CYP1B1 band against amount of CYP1B1 (fmol) were calculated using linear regression analysis. The following two equations are representative of what was used for the calculations of values presented in Figures 1 and 4, respectively: (y = 42.5 + 25.9x, R² = 0.997), (y = 28.5 + 10.7x, R² = 0.988).

RNA isolation and RT-PCR analysis

RT-PCR was used to measure the level of expression of CYP1B1 mRNA. Total cellular RNA was prepared from control or treated cells by an improved method (22). Total RNA from fibroblasts were PCR-amplified along with serially diluted human CYP1B1 cDNA using the Perkin–Elmer thermal cycler according to standard conditions. Approximately 5% of the PCR products were electrophoresed on 1.5% agarose gels and transferred to nylon membranes. The DNA was UV-cross linked to nylon membranes, which were hybridized with 32P-labeled cDNA probe for human CYP1B1 (full length cDNA 2x106 c.p.m./ml, 16 h at 42°C). Probed membranes were exposed to a Phosphoimager screen (Molecular Dynamics) and band intensities were quantified using the volume integration option of the ImageQuant data analysis package (Molecular Dynamics). Standard curves were generated with known amounts of CYP1B1 cDNA (in fmol), which were PCR amplified, and the signals that they generated (band intensity of the PCR products). Such standard curves (equation of the representative dilutions in Figure 2A was: y = 174.5 + 0.876x, R² = 0.992) were used to calculate the relative amounts of CYP1B1 cDNA present in the RT products of 1 µg total RNA from tested fibroblasts.

Results

Cellular characterization

Primary cultures of normal mammary fibroblasts were isolated from reduction mammaplasty surgery specimens of three individuals (A488, A786, 5819). A488 and A786 represent the fast sediment fibroblasts associated with the stromal components that were isolated during the separation from epithelial organoids, and following collagenase digestion of normal breast tissues. Further tyrpinization of the epithelial organoids resulted in generation of 5819 fibroblasts. Therefore, fibroblasts A488 and A786 were of interstitial stromal origin, whereas...
The expression of microsomal CYP1A1 and CYP1B1 was examined in five normal human mammary fibroblasts (HMFs) primary cultures. In addition to the three normal fibroblasts from mammoplasty procedures (A488, A786 and 5819), we included in the comparison the two peripheral normal fibroblasts from the two matched sets (163-PF and 149-PF) from tumor patients. We cultured these fibroblasts in the exact culture conditions recommended by the manufacturers (matched sets in 10 µg/ml insulin; mammoplasty fibroblasts in high glucose). For A488 mammoplasty cells and 163-PF cells no significant difference in expression of basal or TCDD-induced levels of CYP1B1 was observed between the two growth conditions (data not shown). A488 fibroblasts were examined at an early (fifth) and a late (15th) passage. No significant difference was observed in constitutive or TCDD-induced expression of CYP1B1 protein between early and late passages. Examining the expression of CYP1B1 in primary mammmary fibroblasts and epithelial cells from a single donor (A488), revealed that both the basal and induced levels were ~10 times lower in fibroblasts than in the epithelial cells (data not shown).

Expression of constitutive and TCDD-induced CYPs protein and mRNA in normal HMFs

The CYP1A1 protein expression was not detected in HMFs as compared with a normal human mammary epithelial sample, which was included as a positive control, at a detection level of 1 fmol (Figure 1A). CYP1A2 protein expression was also examined in these cells by immunoblotting with the use of polyclonal antibodies specific for CYP1A2. No CYP1A2 protein was detectable in these HMFs, whereas microsomal samples from control rat liver stained positive (data not shown). Western blot analysis of microsomal preparations of basal or TCDD-induced fibroblasts from five individual donors showed substantial levels of constitutive microsomal CYP1B1 protein expression, which was induced ~4-fold by TCDD. The quantitation of these levels, relative to purified recombinant human CYP1B1 standards, showed that the constitutive expression in these individuals ranged from 0.5 to 0.9 pmol of CYP1B1/mg microsomal proteins, with low variability among the different individuals (Figure 1B). Treatment of these cells with TCDD for 24 h induced the expression of CYP1B1 protein to levels ranging between 1.2 and 3.7 pmol of CYP1B1/mg microsomal proteins (Figure 1B).

Examining the mRNA expression of some selected fibroblasts (Figure 2A and B) revealed a similar picture to the protein expression. Cells from each donor showed similar induction but there were substantial parallel variations of basal and induced CYP1B1 mRNA for these cells (A488 > A786 > 5819). This was similar to the pattern seen for microsomal protein expression for these three donors.

DMBA metabolism by normal HMFs

The regioselective profile for DMBA metabolism by microsomes from basal and TCDD-treated normal HMFs in compar-

### Table 1. Summary of the tissue source and the growth conditions of the human fibroblasts used in the study

<table>
<thead>
<tr>
<th>Designated name</th>
<th>Tissue source</th>
<th>Location</th>
<th>Specific growth media requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>A488</td>
<td>Reduction mamoplasty</td>
<td>Interstitial</td>
<td>High glucose</td>
</tr>
<tr>
<td>A786</td>
<td>Reduction mamoplasty</td>
<td>Intersitial</td>
<td>High glucose</td>
</tr>
<tr>
<td>5819</td>
<td>Reduction mamoplasty</td>
<td>Lobular stroma</td>
<td>High glucose</td>
</tr>
<tr>
<td>PF*</td>
<td>Normal tissues peripheral to breast tumor</td>
<td>Tumor periphery stroma</td>
<td>High insulin</td>
</tr>
<tr>
<td>TF*</td>
<td>Breast tumor</td>
<td>Tumor stroma</td>
<td>High insulin</td>
</tr>
<tr>
<td>SKF*</td>
<td>Skin overlying breast tissues</td>
<td>Subcutaneous stroma</td>
<td>High insulin</td>
</tr>
</tbody>
</table>

*Isolated from either G163 or G149 breast cancer patients.
Ah receptor regulation of CYP1B1 in human mammary fibroblasts

Table II. Microsomal DMBA metabolism in human mammary fibroblasts and epithelial cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dihydrodiols</th>
<th></th>
<th>Phenols</th>
<th>Total DMBA metabolism (pmol/mg/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5,6-</td>
<td>8,9-</td>
<td>10,11-</td>
<td>3,4-</td>
</tr>
<tr>
<td>Primary HMFa</td>
<td>0.36</td>
<td>&lt;0.50bc</td>
<td>0.27</td>
<td>&lt;0.50b</td>
</tr>
<tr>
<td>Constitutive</td>
<td>(19)</td>
<td>(&lt;27)</td>
<td>(14)</td>
<td>(&lt;27)</td>
</tr>
<tr>
<td>TCDD-induced</td>
<td>1.79</td>
<td>1.47</td>
<td>1.05</td>
<td>1.19</td>
</tr>
<tr>
<td>(29)</td>
<td>(24)</td>
<td>(17)</td>
<td>(19)</td>
<td>(10)</td>
</tr>
<tr>
<td>Primary HMECc</td>
<td>0.29</td>
<td>0.34c</td>
<td>0.19</td>
<td>1.55b</td>
</tr>
<tr>
<td>Constitutive</td>
<td>(10)</td>
<td>(10)</td>
<td>(7)</td>
<td>(53)</td>
</tr>
<tr>
<td>TCDD-induced</td>
<td>17</td>
<td>68</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>(11)</td>
<td>(45)</td>
<td>(8)</td>
<td>(5)</td>
<td>(25)</td>
</tr>
<tr>
<td>Recombinant human cytochromes P450</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP1B1f</td>
<td>50</td>
<td>33</td>
<td>27</td>
<td>17</td>
</tr>
<tr>
<td>(17)</td>
<td>(11)</td>
<td>(9)</td>
<td>(6)</td>
<td>(54)</td>
</tr>
<tr>
<td>CYP1A1g</td>
<td>189</td>
<td>870</td>
<td>73</td>
<td>79</td>
</tr>
<tr>
<td>(11)</td>
<td>(51)</td>
<td>(4)</td>
<td>(5)</td>
<td>(26)</td>
</tr>
</tbody>
</table>

aMicrosomes were isolated from primary human mammary fibroblasts of donor A488, 0.620 mg microsomal proteins of control or TCDD-treated fibroblasts were used per reaction.
bQuantitative metabolite detection is not possible because of the presence of a contaminating background peak.
c8,9- and 10,11-DMBA dihydrodiols, peaks were poorly resolved at this low level of activity.
dPercentage metabolite distribution.
eMicrosomes were isolated from primary human mammary epithelial cells of donor B402.
fRecombinant human CYP1B1 (Gentest); 74 pmol/mg.
gRecombinant human CYP1A1 (Gentest); 104 pmol/mg.

ison to human mammary epithelial cells (HMEC) and recombinant human CYP1B1 and CYP1A1 is summarized in Table II. Based on the DMBA metabolite profile generated by recombinant human CYP1B1 and CYP1A1, the HMF basal microsomal DMBA metabolism exhibited a profile resembling that of CYP1B1, where it produced similar proportions of 5,6-, 10,11- and 3,4-dihydrodiols. TCDD treatment induced this activity by 3- to 5-fold without changing the ratio of these metabolites, which is in agreement with results obtained by immunoblotting. This is consistent with the exclusive expression of CYP1B1 and the complete lack of CYP1A1 expression. HMEC basal microsomes exhibited a CYP1B1-specific DMBA metabolites profile, but unlike fibroblasts, TCDD preferentially induced the production of a predominant proportion of 8,9-dihydrodiols, which characterized CYP1A1-mediated DMBA metabolism.

Expression of the Ah receptor signaling components in HMFs

The expression of the AhR and its partner ARNT proteins in normal HMF cells was examined in the context of their regulation of TCDD induction of CYP1B1. ARNT expression was consistently high in all cells. The AhR levels were also similar among the different sources of fibroblasts but exhibited distinguishing characteristics according to their source. TCDD induced complete down-regulation of the AhR within 24 h in fibroblasts isolated from epithelial cultures (5819) and fibroblasts peripheral to tumors (163-PF and 149-PF). However, the A488 and A786 fibroblasts, which were isolated from the initial collagenase digestion of breast tissues, responded to TCDD by down-regulating AhR by only 50% in 24 h. No effect for TCDD was observed on ARNT levels in all fibroblasts (Figure 3A).

There was very little inter-individual or tissue source differences in the receptor levels, and TCDD treatment for 24 h resulted in a similar down-regulation of the AhR in each set

Fig. 3. Analysis of the AhR and ARNT expression and the TCDD-induced nuclear translocation and down-regulation of the AhR in normal HMF. (A) Detection of AhR and ARNT in total cellular protein extracts of control or 24-h TCDD-treated HMF. Proteins were isolated from Trizol lysate following RNA isolation as described in Materials and methods. Protein (40 µg) was loaded on each lane and subjected to SDS–PAGE and immunoblotting. Membranes were probed with anti-actin antibody for loading control. (B) Nuclear translocation of AhR following 1 h of treatment with 1 nM TCDD. Treated cells were lysed and fractionated into cytosolic (SN) or nuclear (NE) fractions as described in Materials and methods. Approximately 100 µg protein of SN or NE of each cell type was analyzed by SDS–PAGE and Western blot analysis. Trizol protein extract from MCF-7 cell line was co-analyzed as a positive control.
of fibroblasts (Figure 4A). Although consistent among the two individuals (G163 and G-149), ARNT showed a 10-fold lower level of expression in peripheral fibroblasts compared with skin- and tumor-derived fibroblasts (Figure 3B).

Since AhR down-regulation is preceded by receptor nuclear translocation, nuclear translocation of AhR in response to TCDD was examined in the two categories of isolated HMFs. A period of only 1 h after TCDD treatment was allowed before cellular fractionation and isolation of nuclei so as to allow a reasonable partitioning, but no depletion of the receptor. This time period was sufficient for maximal TCDD-induced nuclear accumulation of AhR in rodent cell lines (45). Even in the absence of TCDD, there was a substantial fraction of the cellular Ah receptor in the nuclei of HMFs. One hour of TCDD treatment resulted in a slight increase in this level in A488 fibroblasts (interstitial stromal-origin) and more than doubled the receptor level in 5819 fibroblasts (lobular stromal-origin) (Figure 3B). Probing these blots for cytosolic proteins marker (GAPDH) confirmed that the constitutive nuclear AhR was not an artifact from cytosolic contamination (Figure 3B). Moreover, the AhR in these preparations resolved in a doublet (~106 and 104 kDa) in the non-nuclear fractions (cytosolic), and only the 104 kDa species of the doublet prevailed in the nuclei. The pattern of nuclear translocation in these fibroblasts correlates with their ability to down-regulate the Ah receptor in response to TCDD treatment. Paradoxically, the A488 fibroblasts expressed higher levels of basal and induced CYP1B1 than 5819 cells, which suggests that these AhR nuclear translocation and down-regulation differences may not correlate with transcriptional regulation.

Comparison of CYP expression in matched fibroblast cultures from three distinct tissue locations

Matched sets of fibroblasts derived from breast tumor, normal peripheral tissues surrounding the tumor or from breast skin of two patients were examined for their basal and TCDD-induced expression of CYP1A1 and CYP1B1. Similar to the reduction mammoplasty HMFs examined earlier, fibroblasts from the three different tissue sources completely lacked detectable CYP1A1 protein (data not shown). All fibroblasts from the three different tissue sources of each donor expressed basal levels of CYP1B1 and similar TCDD-induction. The expression patterns varied substantially between the fibroblasts from the two individuals (Figure 4B). Although basal CYP1B1 expression in the fibroblasts from different tissue source of individual G163 showed a substantial variability, these levels were quite consistent in patient G149. Overall, G149 was less inducible than G163, and averaged a 1.5-fold induction factor compared with 3.5-fold (Figure 4C).

Discussion

Previous work from this laboratory has shown that rodent embryo, mammary and uterine fibroblasts express basal and TCDD-inducible CYP1B1 under the control of the Ah receptor, whereas CYP1A1 is scarcely detectable (20,21,28,30,46). Here we show that these same characteristics are conserved in fibroblasts from the human breast. In this respect, human mammary fibroblasts (HMF) differ from human mammary epithelial cells (HMEC), which also express basal and inducible CYP1B1, but additionally express CYP1A1 (26). The levels of basal and TCDD-induced CYP1B1 in HMF are each ~10-fold lower than in epithelial cells. The level of CYP1B1 mRNA expression showed a similar variation to that of micromosomal CYP1B1 protein for the same cell source. This level of expression is also ~10 times lower than in rat mammary fibroblasts (46).

Based on the regioselectivity in metabolizing DMBA, rodents produce a highly characteristic DMBA metabolite profile for each of CYP1A1 and CYP1B1, which is unlike humans whose profile for the two CYPs is not as distinct (26,34). Whereas rodent CYP1A1-mediated DMBA metabolism was characterized by the production of high ratios of 8,9-dihydrodiols, and CYP1B1 selectively produced high levels of 10,11- and 3,4-dihydrodiols (34), human CYP1B1-mediated DMBA metabolism produced relatively higher proportions of the 5,6- and 10,11-dihydrodiols, relative to CYP1A1, but unlike rodents, humans showed similar ratios of 3,4-dihydrodiols (5%) for both CYP1A1 and CYP1B1 (26). HMF differs from HMEC with respect to its DMBA metabolism. Under both basal and TCDD-treatment conditions, HMF produced a CYP1B1-
specific profile; in contrast, HMEC exhibited a CYP1B1-specific profile under basal conditions, whereas TCDD treatment switched the selectivity to a CYP1A1-specific profile. The much greater stimulation of this activity in HMEC is caused by the induction of the much more active CYP1A1. These differences in DMBBA metabolism were fully consistent with results obtained by immunoblotting, which showed that HMF exclusively expresses CYP1B1 under both basal and induced conditions. It further supports the significant role of HMF in estrogen-mediated breast cancer, since human CYP1B1 also metabolizes estradiol to the potentially reactive 4-hydroxy metabolite (33).

We have attempted to determine whether CYP1B1 expression is dependent on the location of fibroblasts in the breast and whether they differ between the tumor-bearing and normal healthy breast. We also tested whether CYP1B1 expression would distinguish fibroblasts that are readily released by collagenase from breast tissue (interstitial stroma) versus fibroblasts generated during culture of the associated mammary epithelium (lobular stroma) (44). The levels of basal and TCDD-induced expression showed only subtle variations according to tissue or location sources. Even though they can be substantially distinguished by the release of specific growth factors (37,47), the peripheral, tumor and subcutaneous HMF from two donors exhibited larger differences between the donors than between the tissue sources. For one donor, peripheral tumor and subcutaneous HMF each showed 3- to 5-fold induction, whereas for a second donor, the increases were only ~50%.

AhR in the HMF generally showed TCDD-induced down-regulation, which is consistent with previous results in rodent cells (48). AhR and ARNT, the two key regulators of CYP1B1 expression, exhibited some reproducible differences in their functional characteristics according to the source of HMF. First, peripheral HMF showed five times lower levels of ARNT than tumor or subcutaneous HMF, secondly, the lobular stromal HMFs showed very effective TCDD-induced AhR down-regulation. Although ligand binding is required for activation of AhR, subsequent nuclear translocation and DNA binding, a substantial proportion of the receptor was detected in HMFs tightly bound to the nuclear fractions in the absence of ligand treatment. This finding may parallel other data from human cells in culture. Significant levels of AhR in the nuclear fraction of untreated HeLa cells were detected by immunofluorescence localization (49). We also distinguished two cytosolic AhR species (106 and 104 kDa) of which only the 104 kDa protein translocated preferentially to the nucleus. This is consistent with a report of two functionally distinct forms of AhR in human cell lines (50). We have seen a similar preferential nuclear translocation of allelic variants of mouse AhR in mouse BMS2 bone stromal fibroblasts (S.Heidel and C.R.Jefcoate, unpublished data).

A previous study has reported an absence of TCDD-induced CYP1A1 in human dermal fibroblasts, which was attributed to inhibition of AhR activity by a putative repressor (29). Our previous reports (20,21,27,28,30) and the data presented here indicate that TCDD induction in fibroblasts cannot be readily evaluated by CYP1A1 expression, since this form is not typically expressed in these cells. We observed induction of CYP1B1 in all human fibroblasts examined. However, we found that the extent of inducibility is linked to the human genotype rather than to the tissue source of the fibroblasts. The lack of correlation with AhR or ARNT is certainly compatible with the presence of an AhR-suppression mechanism. The apparent heterogeneity in HMFs with respect to AhR transfer to the nucleus indicates the presence of other modulatory mechanisms, although in this case, slow nuclear translocation was not correlated with a deficiency in CYP1B1 induction.

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References

dioxin-genesis for plasmogen activator inhibitor-2 and interleukin-1β.
urokinase-plasminogen activator mRNA by 2,3,7,8-tetrachlorodibenzo-p-
dioxin in human keratinocyte cell line. Toxicol. Appl. Pharmacol., 133,
34–42.
(1994) Mouse cytochrome p450EF: representative of a new 1B subfamily of
cytochrome P450s. Cloning, sequence determination and tissue
expression via the aryl hydrocarbon receptor and protein stabilization in
tumor-induced protein expression in some hormone-independent breast cancer
regulation of nuclear aryl hydrocarbon receptor DNA-binding and
14, 5653–5660.
activated form of the aryl hydrocarbon receptor in the nucleus of HeLa
cells in the absence of exogenous ligand. Arch. Biochem. Biophys., 329,
47–55.
distinct forms of the human Ah receptor. J. Biochem Toxicol., 10,
95–102.
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28, 1998