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ARTICLE

Immunohistochemical Localization of Cytochrome P450 CYP1B1 in Breast Cancer with Monoclonal Antibodies Specific for CYP1B1

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SUMMARY Cytochrome P450 CYP1B1 is a recently identified member of the CYP1 P450 family. We have shown that this P450 displays increased expression in several types of human cancer, indicating that CYP1B1 is a potential tumor biomarker. In this study we developed monoclonal antibodies (MAbs) to CYP1B1 that are effective on formalin-fixed, paraffin-embedded tissue sections and investigated the presence of CYP1B1 in a series of primary breast cancers. The MAbs were generated using a synthetic peptide coupled to carrier protein as the immunogen. The MAbs specifically recognized CYP1B1 and did not recognize either CYP1A1 or CYP1A2, related CYP1 forms. The MAbs were tested by immunohistochemistry and were found to be effective on formalin-fixed, paraffin-embedded tissue sections. The majority of breast cancers showed positive immunoreactivity for CYP1B1, and in each case CYP1B1 was specifically localized to tumor cells. The presence of CYP1B1 in breast cancer cells is likely to contribute to their metabolism of estradiol because CYP1B1 is a specific estradiol hydroxylase. **(J Histochem Cytochem 47:1457–1464, 1999)**

KEY WORDS
breast cancer
CYP1B1
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Cytochrome P450 (P450) CYP1B1 is the only known member of a recently identified subfamily of the CYP1 gene family. Human CYP1B1 was originally isolated from a dioxin-treated keratinocyte cell line (Sutter et al. 1994) as part of a series of investigations to identify differential expression of genes caused by exposure to dioxin. The human CYP1B1 gene is located on chromosome 2p22–21 spanning 12 kb and is composed of three exons and two introns (Tang et al. 1996). The mRNA is 5.2 kb and encodes for a protein of 543 amino acids (Sutter et al. 1994). This is the largest known human P450 gene, in terms of both mRNA size and number of amino acids, and is also the simplest structurally. Both nucleic acid and amino acid sequence analyses show that CYP1B1 displays only approximately 40% homology with CYP1A1 and CYP1A2. The low degree of similarity with existing members of the CYP1 family resulted in this P450 being assigned to a new CYP1 subfamily, CYP1B, which to date only contains the single member CYP1B1. Indeed, hybridization studies of human DNA suggest that there is only one member of the CYP1B gene family (Sutter et al. 1994). The CYP1B1 gene is transcriptionally activated by ligands of the Ah receptor including planar aromatic hydrocarbons (Sutter et al. 1994; Hakkola et al. 1997), and the most potent of these Ah receptor agonists for inducing transcription of the CYP1B1 gene appears to be dioxin (Hakkola et al. 1997).

Orthologous forms of this P450 have also recently been isolated from a benzanthracene-induced cell line derived from mouse embryo fibroblasts (Savas et al. 1994; Shen et al. 1994) and adult rat adrenal cortex (Bhattacharyya et al. 1995; Walker et al. 1995). Although there is a high degree (greater than 80%) of both nucleic acid and amino acid sequence homology between the human, mouse, and rat forms of CYP1B1, there also appear to be considerable species differences regarding tissue-specific expression, regulation, and met-

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abolic specificity of CYP1B1 (Savas et al. 1994,1997; Sutter et al. 1994; Bhattacharyya et al. 1995).

Breast cancer is the most common cancer to affect women and is usually an estrogen-dependent tumor. Human CYP1B1 expressed in yeast (*S. cerevisiae*) shows high specific activity towards the 4-hydroxylation of 17β-estradiol (Hayes et al. 1996), converting it to 4-hydroxyestradiol, and human CYP1B1 is considered to be the most efficient 4-hydroxylase of 17β-estradiol. In contrast, mouse CYP1B1 does not appear to act as an estradiol hydroxylase (Savas et al. 1997), indicating species differences in the metabolic capability of CYP1B1. Liehr and Ricci (1996) showed that there is a significant level of 4-hydroxylation of 17β-estradiol in breast cancer microsomes. They showed considerably higher 4-hydroxylation of 17β-estradiol in microsomes prepared from breast cancer compared with only a very low level of 4-hydroxylation in normal breast tissue. Both immunoreactive CYP1B1 protein (Murray et al. 1997) and CYP1B1 mRNA (McKay et al. 1995) have been identified in breast cancer, indicating that CYP1B1 is a major form of cytochrome P450 expressed in breast cancer.

Our initial immunohistochemical studies of CYP1B1 (Murray et al. 1997), which showed enhanced CYP1B1 expression in several types of human cancer including breast cancer, were performed using a polyclonal antibody to CYP1B1. In this study we have developed monoclonal antibodies (MAbs) specific for human CYP1B1 using a synthetic peptide as the immunogen, characterized the antibodies for use in immunohistochemistry, and used these antibodies to investigate the expression of CYP1B1 by immunohistochemistry in a series of primary human breast cancers.

Materials and Methods

P450 Sequence Alignment, Peptide Selection, and Immunization

Based on a combination of structural homology modeling and sequence alignment of the human CYP1B1 amino acid sequence with the human CYP1A1 and CYP1A2 amino acid sequences, a 148-amino-acid segment located in the C-terminal third of the CYP1B1 protein was predicted to contain regions of amino acids that would be located on the external aspect of the CYP1B1 protein. Peptides of either 14 or 15 amino acid residues corresponding to this segment of the CYP1B1 protein were synthesized in the University of Aberdeen Protein Facility. The individual peptide sequences and amino acid location on the CYP1B1 protein are listed in Table 1. Individual peptides were then conjugated to ovalbumin using glutaraldehyde, as previously described (Duncan et al. 1992), and each peptide conjugate was used as an immunogen. Individual peptide conjugates mixed with Freund's incomplete adjuvant were injected ip into BALB/c mice and the mice were re-immunized with the same peptide conjugate 2–4 weeks after the initial immunization. The presence of an immune response to each peptide conjugate was determined by testing serum (obtained 10 days after the second immunization) from each mouse for recognition of expressed CYP1B1 by immunoblotting. The mice whose sera gave the best recognition of CYP1B1 were then given a final immunization with the appropriate peptide conjugate and were used for production of MAbs.

Table 1 Peptide sequences and amino acid location on CYP1B1 protein of peptides used for immunization

Peptide	Peptide sequence	Location of peptide in CYP1B1 protein
A	NLPYVLAFLYEAMRF	377–391
B	SSFVPVTIPHATTAN	392–406
C	TSVLGYHIPKDTVVF	407–421
D	VNQWSVNHDPVKWPN	422–436
E	PENFDPARFLDKDGL	437–451
F	INKDLTSRVMIFSVG	452–466
G	KRRCIGEELSKMQLF	467–481
H	LFISILAHQCDFRAN	482–496
I	PNEPAKMNFSYGLT	497–510
J	IKPKSFKVNVTLRE	511–524

Monoclonal Antibodies to CYP1B1

Four days after the final immunization with peptide conjugate, the mice were sacrificed, their spleens isolated, and splenic cells fused with mouse myeloma cells (Ag8.653). The resultant hybridoma clones were then screened for antibody production by enzyme-linked immunoassay (ELISA), using the relevant peptide conjugated with bovine serum albumin (BSA). The BSA conjugates were bound to an ELISA plate by incubation overnight at 4C in 50 mM sodium carbonate/bicarbonate buffer, pH 9.6, and the ELISA was performed as described previously (Duncan et al. 1992; Murray et al. 1998a). Hybridoma clones that were strongly positive by ELISA were subcloned twice and further tested by immunoblotting, using expressed CYP1B1, expressed CYP1A1, control microsomes, and human liver microsomes. The MAbs were isotyped using an Isostrip kit (Roche Diagnostics; Lewes, Sussex, UK) according to the manufacturer's instructions.

Tissues

Samples of normal adult human tissues (liver, kidney, lung, pancreas, adrenal cortex, brain, stomach, jejunum, colon, breast, ovary, and endometrium) were obtained from fresh, unfixed tissue samples submitted to the Department of Pathology, University of Aberdeen for diagnosis. Samples of tissue were frozen in liquid nitrogen and stored at −75C before preparation of microsomes. Samples of primary breast cancer ($n = 60$) were obtained from samples of breast tissue submitted to the Department of Pathology, University of Aberdeen for diagnosis. All the samples of breast tissue were from needle core biopsies of palpable breast lumps performed as part of the diagnostic protocol before definitive treatment. The core biopsies were fixed in 10% neutral buffered formalin at room temperature (RT) for 18–24 hr and then routinely embedded in paraffin.

The diagnosis of breast cancer was performed with hematoxylin and eosin-stained sections using standard histopathological criteria. All tumors were graded according to the Nottingham grading system (Elston and Ellis 1991), and the estrogen receptor status of the breast cancers was assessed by immunohistochemistry for estrogen receptor protein, as previously described (King et al. 1997). The lymph node status for presence or absence of lymph node metastasis was assessed from subsequent axillary lymph node samples (when performed) submitted for histopathological examination. The clinicopathological characteristics of the breast cancers are described in Table 2.

Expressed CYP1B1 and CYP1A1

Microsomes prepared from human lymphoblastoid cells containing expressed human CYP1B1, expressed human CYP1A1, or control lymphoblastoid cells that contained only vector were obtained from Gentest (Woburn, MA). The microsomes containing individual expressed P450s are supplied by Gentest with a specific P450 content per milligram of microsomal protein.

Preparation of Microsomes

Frozen samples of tissues were thawed on ice in 0.01 M Tris-HCl, pH 7.4, containing 1.15% KCl. The thawed samples of tissue were dissected free of connective tissue and fat, finely chopped with a scalpel, and then homogenized in 0.01 M Tris-HCl containing 0.25 M sucrose and 15% glycerol using a Polytron PT3000 homogenizer (Kinematica; Lucerne, Switzerland). The homogenates were then centrifuged at 15,000 × g for 20 min at 4C using a Centrikon T-124 centrifuge (Kontron Instruments; Cumbernauld, UK). The resultant supernatants were then centrifuged at 180,000 × g (44,000 rpm) for 1 hr at 4C using a Centrikon T-1160 centrifuge (Kontron Instruments). The pellet obtained after centrifugation was resuspended in 0.1 M Tris-HCl containing 15% glycerol and 1 mM EDTA (Sigma–Aldrich; Poole, Dorset, UK) and centrifuged again at 180,000 × g (44,000 rpm) for 1 hr at 4C. The final microsomal pellet was resuspended in 0.1 M Tris-HCl containing 15% glycerol and 1 mM EDTA, and the microsomal samples were then stored at −75C before use. The protein concentration of each sample of microsomes was determined using Bradford's method (Bradford 1976). Bovine serum albumin (Sigma–Aldrich) was used as the protein standard.

Table 2 Clinicopathological characteristics of breast cancer

Age (mean and range)	52.3 years (34–76)
Histological type	
Invasive ductal carcinoma	52 (86.7%)
Invasive lobular cardcinoma	8 (13.3%)
Grade of breast cancer	
Grade 1	9 (15%)
Grade 2	27 (45%)
Grade 3	24 (40%)
Lymph node status	
Negative (no metastasis)	33 (55%)
Positive (presence of metastasis)	22 (36.7%)
Not determined	5 (8.4%)
Estrogen receptor status	
Negative	23 (38.3%)
Positive	35 (58.3%)
Not determined	2 (3.4%)

Immunoblotting

Samples of microsomal proteins were electrophoretically separated at constant current in a 10% polyacrylamide gel using a Hoefer SE600 vertical gel electrophoresis apparatus (Amersham Pharmacia Biotech; Little Chalfont, Bucks, UK) and then transferred at constant current for 18 hr to nitrocellulose (Hybond ECL; Amersham Pharmacia Biotech) by electroblotting using a Hoefer TE42 blotting system (Amersham Pharmacia Biotech). After electrophoretic transfer, nonspecific protein binding sites were blocked by incubation of the nitrocellulose membrane for 60 min at RT in wash buffer consisting of 2% nonfat milk (Marvel; Premier Beverages, Stafford, UK) in 10 mM PBS containing 0.05% Tween-20 (Sigma). The nitrocellulose was then sequentially incubated with immune mouse serum (1:250) or CYP1B1 MAb (1:100) and goat anti-mouse immunoglobulin conjugated to horseradish peroxidase (1:2000; Bio-Rad, Hemel Hempstead, UK). After incubation with each antibody, the membrane was washed for five 10-min periods with wash buffer, and after removal of unbound secondary antibody the membrane was further washed in 10 mM PBS for five 10-min periods. Horseradish peroxidase was then demonstrated using an enhanced chemiluminescent technique (ECL plus; Amersham Pharmacia Biotech), which was performed as previously described (McKay et al. 1995; Murray et al. 1997). Briefly, the nitrocellulose membrane was incubated in a solution consisting of 4 ml of Detection Reagent 1 (lumigen PS-3; Amersham Pharmacia Biotech) and 100 μl of Detection Solution 2 (luminol PS-3; Amersham Pharmacia Biotech) for 5 min at RT, blotted dry, wrapped in clear plastic film, and then exposed to X-ray film (Amersham Pharmacia Biotech). The exposed X-ray film was then developed in an automatic film processor (X-omat; Kodak, Rochester, NY).

Immunohistochemistry

Immunohistochemical detection of CYP1B1 was performed with a catalyzed signal amplification method (King et al. 1997). In this study, we used fluorescein tyramide rather than biotinylated tyramide, which we had used in our previous study (King et al. 1997), to avoid any possible interference from endogenous biotin. Sections (4 μm thick) were cut onto aminoethyl propoxy silane-coated slides, then dewaxed in xylene, rehydrated in 100% ethanol, 95% ethanol, and washed in 0.05 M Tris-HCl, pH 7.6, containing 150 mM NaCl (TBS). Endogenous peroxidase was inhibited with a solution consisting of 90 ml methanol and 3 ml hydrogen peroxide (30% w/v). Antigen retrieval was performed by microwaving the sections in 0.01 M citrate buffer, pH 6.0, for 20 min in a microwave (Proline; Proline, Hull, UK) operated at full power (800 W). After the antigen retrieval step, sections were allowed to cool to RT and then the primary anti-CYP1B1 MAb was applied. In one experiment during the initial immunohistochemical characterization of the CYP1B1 MAbs, antigen retrieval was not performed.

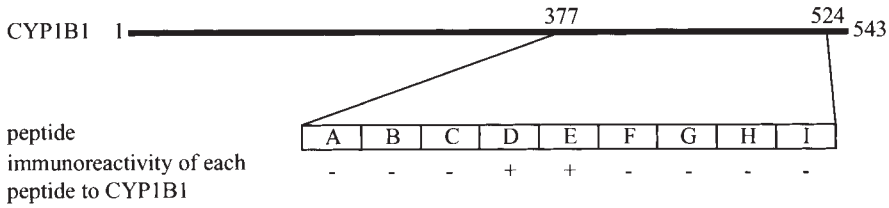

Figure 1 Region of CYP1B1 protein to which individual peptides were synthesized and used for immunization of mice. The immunoreactivity of individual mouse sera towards CYP1B1 after immunization with each peptide is indicated.

The primary antibody was applied as a tissue culture supernatant at various dilutions (undiluted to 1:160) for 60 min at RT. After incubation in primary antibody, the sections were washed in TBS for three successive 5-min periods, and then peroxidase-conjugated rabbit anti-mouse immunoglobulin (1:100 in TBS containing 4% normal human serum; Dako, High Wycombe, UK) was applied for 30 min at RT. The sections were then washed in TBS and in TBS containing 0.05% Tween-20 (TNT buffer). The sections were then further washed in TNT buffer and fluorescein tyramide (NEN; Hounslow, Middlesex, UK) applied for 10 min at RT. The sections were then further washed in TNT buffer, followed by application of monoclonal mouse anti-fluorescein (1:20; Dako) for 30 min at RT. After further washing in TNT buffer, peroxidase-conjugated rabbit anti-mouse immunoglobulin (1:100 in TBS containing 4% normal human serum) was applied for 30 min at RT. After washing in TBS, sites of bound peroxidase were then demonstrated colorimetrically using a solution containing diaminobenzidine and hydrogen peroxide (Liquid DAB plus; Dako). After incubating the sections for 10 min at RT in the peroxidase substrate solution, the reaction was stopped by washing the slides in cold tapwater and the enzyme reaction product was intensified using 0.5% copper sulfate. The slides were then washed in cold tapwater, counterstained with hematoxylin, dehydrated in alcohol, cleared in xylene, and mounted in a synthetic mounting medium (DPX; BDH, Poole, Dorset, UK).

The sections were examined with brightfield light microscopy by two independent observers (MCEM, GIM) to establish the presence or absence of immunostaining, and its distribution, cellular localization, and intensity. The intensity of immunostaining in each tumor was assessed as strong (3), moderate (2), weak (1), or negative (0). The proportion of cells showing positive immunoreactivity was also assessed semiquantitatively using the following scale: less than 5% of cells (0), 5–25% (1), 26–75% (2), and greater than 75% (3) of cells. A tumor was regarded as positive if more than 5% of tumor cells showed immunostaining. A tumor was classified as negative if there was complete absence of immunostaining in tumor cells or if less than 5% of tumor cells showed positive immunoreactivity. An overall CYP1B1 immunohistochemical score was obtained by assigning a numerical value from 0–3 for the results for the intensity of CYP1B1 immunoreactivity and the proportion of cells showing CYP1B1 immunoreactivity, respectively, and then combining these values. This resulted in an overall CYP1B1 immunohistochemical score ranging from 0 to 6. The overall CYP1B1 immunohistochemical score was grouped into four categories; 0–2 (score for weak immunoreactivity and low number of positive cells), 3–4 and 5–6 for comparison of CYP1B1 immunohistochemistry with histological grade, tumor type, presence of lymph node metastasis, and estrogen receptor status.

The positive control tissue used consisted of sections of a breast cancer that we had previously shown to contain CYP1B1 by both immunoblotting and immunohistochemistry with a polyclonal antibody to CYP1B1 (Murray et al. 1997). Negative controls used in place of the primary CYP1B1 MAb were TBS and tissue culture media. In one experiment, MAb was liquid-phase preabsorbed with the peptide (peptide E, 10 nmol of peptide/ml antibody) that had

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human CYP1B1      PENFDPARFLDKDGL   (peptide E)
rat CYP1B1        PEDFDPARFLDKDGF
mouse CYP1B1      PEDFDPARFLDKDGF
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Figure 2 Sequence alignment of the peptide (Peptide E) used to generate the MAbs to CYP1B1, with the corresponding sequences of mouse and rat CYP1B1. Amino acid residues common to all three P450s are indicated by an asterisk.

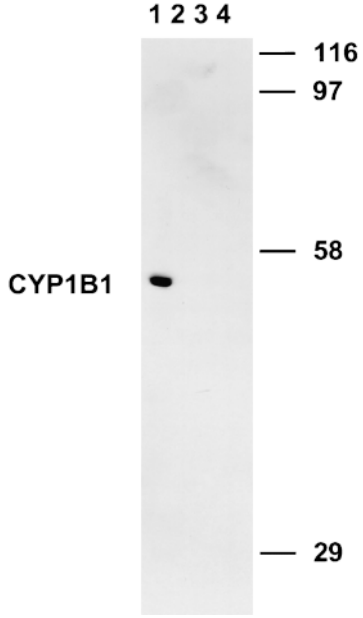

Figure 3 Immunoblot of expressed CYP1B1 with MAb 5D3, demonstrating specificity for CYP1B1. Lane 1, expressed CYP1B1 (10 μg of microsomal protein corresponding to 0.86 pmol of CYP1B1); Lane 2, expressed CYP1A1 (10 μg of microsomal protein, corresponding to 0.53 pmol of CYP1A1); Lane 3, control lymphoblastoid microsomes containing only vector (10 μg of microsomal protein); Lane 4, normal human liver microsomes (10 μg of microsomal protein). Molecular weight markers are shown at right in kD.

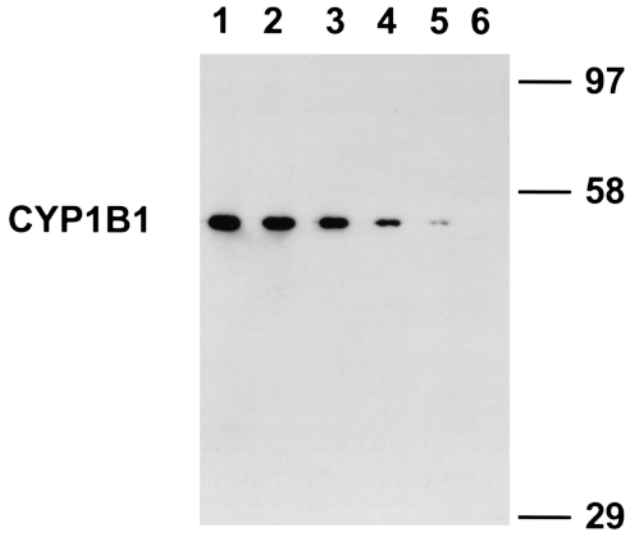

Figure 4 Immunoblot demonstrating the minimal detectable amount of CYP1B1 with MAb 5D3. Lane 1–Lane 6, decreasing amounts of expressed CYP1B1. Lane 1, 0.86 pmol of CYP1B1; Lane 2, 0.43 pmol of CYP1B1; Lane 3, 0.21 pmol of CYP1B1; Lane 4, 0.1 pmol CYP1B1; Lane 5, 0.05 pmol of CYP1B1; Lane 6, 0.025pmol of CYP1B1. Molecular weight markers are shown at right in kD.

been used as the immunogen for the MAbs before performance of immunohistochemistry.

Results

Development of MAbs to CYP1B1

The immune response of sera from mice injected with each of the peptide conjugates was assessed by SDS-PAGE and immunoblotting, using expressed CYP1B1 as the antigen. The sera of mice injected with different peptides showed a variable immune response (Figure 1). Sera of mice that had been injected with Peptides D and E both showed recognition of CYP1B1 and were judged to have produced a positive immune response. Sera from mice injected with Peptide E gave a marginally stronger recognition of expressed human CYP1B1 than Peptide D. However, sera from mice injected with Peptides A and B and Peptides F–J showed no apparent recognition of CYP1B1 and were considered to have produced no significant immune response. Mice that had been immunized with Peptide E were therefore chosen to develop MAbs to CYP1B1. The peptide sequence used to develop the MAbs shows a high degree of similarity with corresponding rat CYP1B1 and mouse CYP1B1 sequences, with 13 of the 15 amino acid residues being identical (Figure 2).

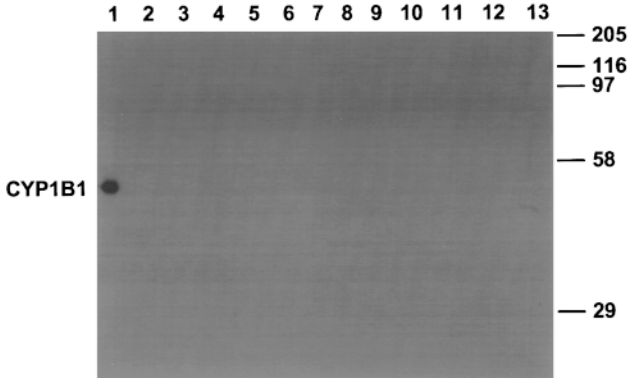

Figure 5 Immunoblot of CYP1B1 of microsomes prepared from various normal human tissues. Lane 1, expressed CYP1B1 (5 μg of microsomal protein corresponding to 0.43 pmol of CYP1B1); Lane 2, liver; Lane 3, kidney; Lane 4, lung; Lane 5, pancreas; Lane 6, adrenal cortex; Lane 7, brain (medulla); Lane 8, stomach; Lane 9, jejunum; Lane 10, colon; Lane 11, breast; Lane 12, ovary; Lane 13, endometrium (30 μg of microsomal protein loaded per lane of normal human tissue). Molecular weight markers are shown at right in kD.

Five MAbs were developed from mice immunized with Peptide E. The individual antibodies were designated 5C4, 5D3, 5D9, 5E2, and 5G7, and isotyping showed that all the antibodies are $IgG_{1\kappa}$ subtype. All the MAbs recognized a single immunoreactive band of molecular size 52 kD, corresponding to the expected molecular size of expressed human CYP1B1 by immunoblotting, and did not recognize expressed human CYP1A1 or any protein present in vector-only control microsomes or human liver microsomes (Figure 3). Serial dilutions of expressed CYP1B1 indicated that the minimal detectable amount of CYP1B1 by immunoblotting was 0.05 pmol of expressed CYP1B1 (Figure 4). CYP1B1 was not identified by immunoblotting of microsomes prepared from a range of normal adult human tissues, including kidney, stomach, small intestine, colon, lung, and endometrium (Figure 5).

Immunohistochemistry on formalin-fixed, paraffin-embedded sections of breast cancer used as the positive control showed that three of the MAbs (5D3, 5E2 and 5G7) demonstrated strong staining, whereas two of the MAbs (5C4, 5D9) showed no immunoreactivity. All MAbs that showed positive immunoreactivity required an antigen-retrieval step for optimal immunohistochemical results, and all three MAbs showed an identical pattern of localization and distribution of immunohistochemical staining. Therefore, only one of the MAbs (5D3) was used for subsequent immunohistochemical studies of breast cancer. Liquid-phase preincubation of anti-CYP1B1 antibody with Peptide E before performance of immunohistochemistry almost completely abolished immunoreactivity.

CYP1B1 immunoreactivity was identified in 46 (76.7%) of cases of breast cancer, whereas there was no detectable CYP1B1 immunoreactivity in 14 cases (23.3%). In each case in which CYP1B1 immunoreactivity was observed, the immunoreactivity was localized to the cytoplasm of tumor cells (Figure 6). The intensity of the CYP1B1 immunoreactivity ranged from

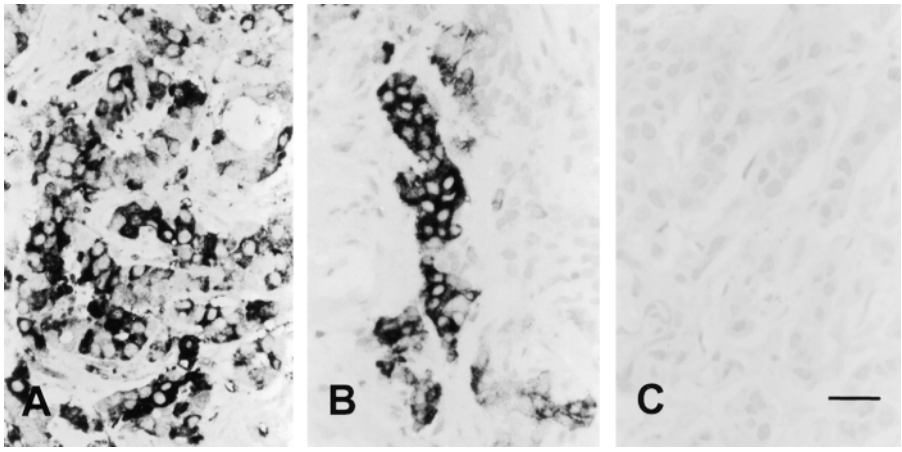

Figure 6 Immunohistochemical demonstration of CYP1B1 in breast cancer with MAb 5D3. (**A**) Localization of CYP1B1 in a Grade 3 invasive ductal carcinoma. There is strong CYP1B1 immunoreactivity in a high proportion of tumor cells. (**B**) Localization of CYP1B1 in a Grade 3 invasive lobular carcinoma, demonstrating strong immunoreactivity in breast cancer cells. (**C**) There is no immunoreactivity in a Grade 3 invasive ductal carcinoma when the primary CYP1B1 MAb is replaced by TBS in the immunohistochemical procedure. Bar = 60 μm.

strong in 10 (16.7%) cases to moderate in 12 (20%) cases, and weak immunoreactivity was observed in 24 (40%) cases. The proportion of cells showing CYP1B1 immunoreactivity ranged from high in 10 (16.7%) cases to moderate in 27 (45%) cases, to low in nine (15%) cases. The relationship between the intensity of CYP1B1 immunoreactivity and the proportion of cells showing CYP1B1 immunoreactivity is summarized in Table 3. Twelve (20%) cases had an overall CYP1B1 immunohistochemical score of 5 or 6, 26 (43.4%) cases had a score of 3 or 4, 8 (13.3%) cases had a score of 2, and 14 (23.3.%) cases had a score of 0. The presence of CYP1B1 in different grades, histological types of breast cancer, lymph node status, and estrogen receptor status is summarized in Tables 4–7. There was no statistical relationship between the presence of CYP1B1 and the histological type of the tumor, tumor grade, the presence or absence of lymph node metastasis, or estrogen receptor status. There was no CYP1B1 immunoreactivity in stromal cells or connective tissue in the following cell types, including lymphocytes and plasma cells, when they were present in individual biopsy specimens.

Discussion

CYP1B1 shows increased expression in a variety of tumors, including breast cancer (Murray et al. 1997), and there is elevated CYP1B1-associated 4-estradiol hydroxylase mono-oxygenase activity in breast cancer (Liehr and Ricci 1996). CYP1B1 is also capable of metabolizing a variety of putative human carcinogens, including polycyclic aromatic hydrocarbons and heterocyclic amines (Shimada et al. 1996; Crespi et al. 1997). Therefore, CYP1B1 appears to have potentially important roles in tumor development and progression, as a potential target for anti-cancer drugs, and as a tumor biomarker. Our initial studies of the presence of CYP1B1 in individual types of tumors were performed with a polyclonal antibody to CYP1B1, and for each tumor type only a small number of tumor samples were investigated (Murray et al. 1997). To evaluate more fully the role of CYP1B1 in tumor development and progression and its potential as a tumor marker requires MAbs that specifically recognize CYP1B1 in formalin-fixed, paraffin-embedded tissue sections, and also requires a larger number of tumors to be studied. In this study we developed MAbs to CYP1B1, demonstrated that they sensitively and specifically detect CYP1B1 by immunoblotting and immunohistochemistry, and then used these MAbs to investigate the presence of CYP1B1 in a series of primary breast cancers.

The strategy we used to develop MAbs to CYP1B1 was a combination of structural molecular modeling and sequence alignment to identify regions of CYP1B1 that were likely to be located on the external aspect of

Table 3 Comparison of the intensity of CYP1B1 immunoreactivity with the proportion of cells displaying CYP1B1 immunoreactivity

	Proportion of cells displaying CYP1B1 immunoreactivity			
Intensity of CYP1B1 immunoreactivity	Less than 5%	5–25%	26–75%	Greater than 75%
Negative (n = 14)	14	0	0	0
Weak (n = 24)	0	8	15	1
Moderate (n = 12)	0	1	9	2
Strong (n = 10)	0	0	3	7

Table 4 Presence of CYP1B1 in different histological types of breast cancer

	Histological type of breast cancer	
Overall CYP1B1 immunoreactivity	Invasive ductal (n = 52)	Invasive lobular (n = 8)
Negative (n = 14)	12	2
2 (n = 8)	6	2
3–4 (n = 26)	23	3
5–6 (n = 12)	11	1

Table 5 Comparison of CYP1B1 with different grades of breast cancer

Overall CYP1B1 immunoreactivity	Grade of breast cancer		
	Grade 1 (n = 9)	Grade 2 (n = 27)	Grade 3 (n = 24)
Negative (n = 14)	2	6	6
2 (n = 8)	1	5	2
3–4 (n = 26)	5	11	10
5–6 (n = 12)	1	5	6

the CYP1B1 protein and therefore were likely to be immunogenic. We have recently used a similar approach to develop MAbs to matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases (Murray et al. 1998a,b). A region located in the C-terminal third of the CYP1B1 protein, encompassing the heme binding region, was identified and peptides consisting of either 14 or 15 amino-acid residues were synthesized and conjugated to carrier protein. Each peptide was injected into mice, and the immunoreactivity of each peptide for CYP1B1 was assessed by immunoblotting using microsomes prepared from human lymphoblastoid cells containing expressed CYP1B1 and microsomes containing vector only. Only sera from mice immunized with Peptides D and E showed recognition of CYP1B1, whereas none of the other peptides showed significant immunoreactivity for CYP1B1. Mice that had been immunized with Peptide E were selected for the development of MAbs to CYP1B1. The peptide sequence used for generation of the MAbs shows a high degree of similarity (13 of 15 amino acids identical) with rat CYP1B1 and mouse CYP1B1, and it is anticipated that these MAbs would also recognize rat CYP1B1 and mouse CYP1B1. All the MAbs were specific for CYP1B1 and did not recognize either CYP1A1 or CYP1A2, the other known members of the CYP1 gene family. Furthermore, the MAbs did not recognize any other protein in human liver microsomes. Human liver microsomes were used as a source for CYP1A2 because CYP1A2 is one of the major forms of P450 constitutively expressed in liver, and liver microsomes also act as a source for several other forms of P450, thus providing a broad screen to confirm the specificity of the MAbs for CYP1B1.

Table 6 Comparison of CYP1B1 in lymph node-positive and lymph node-negative breast cancer cases (lymph node status was not determined in 5 cases)

Overall CYP1B1 immunoreactivity	Lymph node status	
	Lymph node negative (n = 33)	Lymph node positive (n = 22)
Negative (n = 14)	6	8
2 (n = 8)	7	1
3–4 (n = 24)	12	12
5–6 (n = 9)	8	1

Table 7 Comparison of presence of CYP1B1 immunoreactivity in breast cancer with presence of estrogen receptor (estrogen receptor status not determined in 2 cases)

Overall CYP1B1 immunoreactivity	Estrogen receptor status	
	Negative (n = 23)	Positive (n = 35)
Negative (n = 14)	3	11
2 (n = 7)	5	2
3–4 (n = 26)	12	14
5–6 (n = 11)	3	8

In this study, we were not able to detect CYP1B1 by immunoblotting in a range of normal human tissues. The absence of CYP1B1 protein in both normal human liver and a range of extrahepatic tissues is consistent with our previous immunohistochemical studies (Murray et al. 1997), in that we were unable to detect CYP1B1 protein. In this study, we loaded a relatively high amount of microsomal protein (30 μg) per lane and used a highly sensitive chemiluminescent detection system. It therefore appears likely that even a very low level of CYP1B1 protein in the normal tissues studied would have been detected with this system. However, Shimada et al. (1996) identified CYP1B1 mRNA in a wide range of normal tissues with a sensitive Northern hybridization procedure, suggesting that CYP1B1 expression may be regulated at the posttranscriptional level.

A further major aim of this study was to develop antibodies to CYP1B1 that could be used in immunohistochemistry and that were effective on formalin-fixed, paraffin-embedded sections of tissue. All the MAbs were evaluated by immunohistochemistry on formalin-fixed, paraffin-embedded sections of breast cancer that we have previously shown by immunoblotting to contain a relatively high level CYP1B1 (Murray et al. 1997), and we found that three of the MAbs effectively detected CYP1B1 by immunohistochemistry. Because the three antibodies that gave positive immunoreactivity all produced an identical pattern and intensity of immunoreactivity, we used only one of the antibodies to investigate CYP1B1 expression.

In this study, we found that 77% of breast cancers contained CYP1B1, and in each tumor CYP1B1 was specifically localized to tumor cells. The high frequency of expression of CYP1B1 in breast cancer is very similar to the findings of our previous studies of a small number of breast cancers and support the concept that CYP1B1 is a major form of cytochrome P450 present in breast cancer (McKay et al. 1995; Murray et al. 1997). CYP1B1 was found in all histological types of breast cancer, and the presence of CYP1B1 was not associated with any particular histological type of breast cancer, nor with the presence or

absence of lymph node metastases nor with the presence or absence of estrogen receptor protein. Recently, an association between a CYP1B1 polymorphism (valine→leucine) at codon 432 and estrogen receptor status in breast cancer has been described (Bailey et al. 1998). There is also the potential for crosstalk between the estrogen receptor complex and the aryl hydrocarbon receptor complex (Wang et al. 1998) that is involved in the transcriptional regulation of CYP1B1 (Schmidt and Bradfield 1996).

Because CYP1B1 is involved in estrogen metabolism, acting as a specific C4 hydroxylase of 17β-estradiol (Hayes et al. 1996), the presence of CYP1B1 in breast cancer cells is likely to make a significant contribution to the intratumoral metabolism of estradiol. The presence of CYP1B1 in breast cancer also provides a molecular target for drugs specifically activated by CYP1B1 (Murray et al. 1997).

The development of MAbs to CYP1B1 that are effective in formalin-fixed, paraffin-embedded sections should make them particularly useful for investigating CYP1B1 expression in different tumor types and related preneoplastic lesions.

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