FEBS Letters 374 (1995) 270–272 FEBS 16197

Expression of cytochrome P450 CYP1B1 in breast cancer

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Received 18 September 1995

Abstract The expression of CYP1B1 has been identified in breast cancer using the reverse transcriptase-polymerase chain reaction and immunoblotting. CYP1B1 mRNA was expressed in the majority of breast tumours and immunoblotting of breast tumours identified a single protein band of molecular weight 60 kDa corresponding to the predicted molecular weight of human CYP1B1. This is the first study to identify CYP1B1 expression in a tumour where it may represent a previously unknown pathway for the metabolism of oestradiol and chemotherapeutic drugs.

Key words: Breast; Cancer; Cytochrome P450; Neoplasm

1. Introduction

The P450s are a multigene superfamily of constitutive and inducible enzymes which play a central role in the oxidative metabolism of a diverse range of xenobiotics, including carcinogens, therapeutic drugs and several groups of biologically active endogenous compounds, such as steroid hormones and fatty acids [1–5]. The major families of P450 involved in xenobiotic metabolism each consist of several individual forms with specific regulatory mechanisms and substrate specificities [6]. Until recently, the CYP1 gene family which is the main P450 family involved in the metabolism of polycyclic aromatic compounds and arylamines and is regulated by the Ah receptor complex, was considered to be composed of only two distinct but closely homologous members, CYP1A1 and CYP1A2 [7–10]. In man, CYP1A1 is an inducible P450 found primarily in extrahepatic tissues while CYP1A2 is constitutively expressed in liver [11]. Recently, a new dioxin-inducible CYP1 P450 subfamily has been identified, containing one form to date, CYP1B1 [12]. Using immunohistochemistry, we have previously identified a CYP1-immunoreactive protein in breast cancer [13] and in this study we have investigated the expression of individual forms of the two known CYP1 gene subfamilies in breast cancer.

2. Materials and methods

2.1. Tumour samples

Samples of breast tissue were obtained from patients undergoing surgery for primary breast cancer. In accordance with current pathology guidelines requiring the majority of each breast biopsy to be submitted for histopathological examination, only small samples were available for this study and were, therefore, used either for RT-PCR or immunoblotting. RT-PCR was performed on breast cancers from 42 patients (age range 33–87). Immunoblotting was performed on breast cancers obtained from 6 patients (age range 45–67). The tissue samples were frozen in liquid nitrogen and stored at −80°C prior to analysis.

2.2. RT-PCR

Total RNA was extracted using essentially the method of Chomczynski and Sacchi [14]. The recovered RNA pellet was washed twice with 95% ethanol before being air-dried and resuspended in 20 μl diethyl pyrocarbonate (Sigma, Poole, UK)-treated distilled water. cDNA was synthesized from the isolated RNA using oligo (dT) with the following reaction conditions: RNA (20 μl) in 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM dithiothreitol, 40 U human placental ribonuclease inhibitor (Promega, Southampton, UK), 0.125 mM each of dATP, dCTP, dGTP and dTTP (Promega), 18 U avian myeloblastosis virus reverse transcriptase (Promega) and 0.5 mg oligo $(dT)_{15}$ primer (Promega). cDNA was then synthesized at 42°C for 60 min and the reaction was stopped by heating to 80°C for 5 min.

The CYP1B1 primers recognize sequences in the 3′ non-coding region of the CYP1B1 gene and give a product of 489 bp [12]. The CYP1A primers were designed to recognize sequences common to both CYP1A1 and CYP1A2 and give PCR products of 649 bp for CYP1A1 and 643 bp for CYP1A2, respectively. Each product contained an *Rsa*I restriction site (corresponding to nt 1102 for CYP1A1 and nt 1002 for CYP1A2, respectively [7,9]) and gave fragments of 535 and 114 bp for CYP1A1 and 507 and 136 bp for CYP1A2, respectively. The primers were synthesized and purified using high-performance liquid chromatography by Oswel DNA Service (Edinburgh, UK) with the following sequences:

CYP1B1

Forward `5′-AAC TGT CCA TCA GGT GAG GT-3′ (nt 2104-2123)`

Reverse `5′-TAA GGA AGT ATA CCA GAA GGC-3′ (nt 2573-2593)`

CYP1A

Forward `5′-GAG CAT GTG AGC AAG GAG G-3′ (CYP1A1, nt 567-585; CYP1A2, nt 495-513)`

Reverse `5′-CGG AAG GTC TCC AGG ATG AA-3′ (CYP1A1, nt 1197-1216; CYP1A2, nt 1119-1138)`

β-Actin primers were bought from Stratagene (Cambridge, UK) and had the following sequences:

Forward `5′-TGA CGG GGT CAC CCA CAC TGT-GCC CAT CTA-3′ (nt 1038-1067)`

Reverse `5′-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3′ (nt 1876-1905)`

PCR using the hot-start technique [15] was performed in a 100-μl reaction volume consisting of 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, either 1.5 mM $MgCl_2$ (for CYP1B1) or 2.5 mM

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Abbreviations: P450, cytochrome P450; RT-PCR, reverse transcriptase-polymerase chain reaction.

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SSDI 0014-5793(95)01126-9

$MgCl_2$ (for CYP1A), 0.2 mM (CYP1B1) or 0.4 mM (CYP1A) each of dATP, dCTP, dGTP and dTTP, 0.5 U Taq DNA polymerase (Promega), 2 μl cDNA and either 100 pmol of each primer for both CYP1B1 and β-actin or 85 pmol of each CYP1A primer. 35 cycles of amplification were performed using the following conditions: for CYP1B1: 94°C for 30 s (denaturation), 60°C for 30 s (annealing), 72°C for 1 min (extension); and a final 2-min extension at 72°C; for CYP1A: 94°C for 1.3 min (denaturation), 55°C for 36 s (annealing), 72°C for 1.3 min (extension) and a final 2-min extension at 72°C. For β-actin, the conditions were (as recommended by the manufacturer's data sheet): denaturation at 94°C for 5 min, annealing at 60°C for 5 min followed by 35 cycles of amplification consisting of 94°C for 48 s (denaturation), 60°C for 48 s (annealing), 72°C for 1.5 min (extension) and a final extension at 72°C for 10 min. Both positive and negative controls were included in the PCR. The positive control for CYP1B1 was a 2.78-kb CYP1B1 cDNA which had been cloned into pcDNAII. Positive controls used for CYP1A were cloned full-length human CYP1A1 and CYP1A2 cDNA sequences which were kindly provided by Professor D.W. Nebert (University of Cincinnati, USA). The negative control was sterile distilled water in place of template cDNA. Both the β-actin primers and CYP1A primers span intron sequences and the absence of any band at a higher molecular weight than expected from the cDNA confirms absence of contaminating DNA in the RNA preparations.

After PCR, 10 μl of the PCR product was electrophoresed on a 1.5% agarose gel which incorporated 0.007% w/v ethidium bromide. The PCR product was purified with a Wizard miniprep DNA purification system (Promega) used according to the manufacturer's instructions and further analysed by direct sequencing (CYP1B1 and CYP1A) and restriction enzyme digestion with *Rsa*I (CYP1A) as follows. The CYP1B1 and CYP1A PCR products were sequenced by the direct dideoxy sequencing technique of Sanger [16] with a T7 sequencing kit (Pharmacia, Milton Keynes, UK) used according to the manufacturer's protocol. The sequencing primers were the same primers used for PCR. Purified CYP1A PCR product was also digested with *Rsa*I for 2 h at 37°C with the following conditions: 25 μl DNA in 10 mM Tris-HCl pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 20 mg bovine serum albumin (Sigma) and 0.02 U *Rsa*I (Promega), then heated to 95°C for 2 min to stop the reaction. Restriction digests were analysed by electrophoresis on an ethidium bromide-containing agarose gel as above.

2.3. Immunoblotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis and immunoblotting of breast tumours using an enhanced chemiluminescence technique to identify immunoreactive proteins were performed as described previously [17,18].

3. Results

3.1. RT-PCR

Of the 42 tumours studied by RT-PCR, 40 of the tumours amplified for β-actin and the 2 tumours which did not amplify for β-actin are not considered further. 29 of the 40 tumours (73%) amplified for CYP1B1 (Fig. 1) and sequencing of the purified PCR product showed identity with CYP1B1. RT-PCR for CYP1A showed that 10 of the 40 tumours (25%) amplified for CYP1A (Fig. 2) and direct sequencing of the undigested CYP1A PCR product showed identity with CYP1A1. Following digestion of the PCR product with *Rsa*I, two bands of 535 and 114 bp, respectively, were identified, consistent with the digestion products of CYP1A1 (Fig. 3). None of the tumours, however, showed evidence of expression of CYP1A2 mRNA (Fig. 3). There was only 1 tumour which expressed CYP1A1 without co-expressing CYP1B1 mRNA while 10 of the tumours showed no amplification for either CYP1B1 or CYP1A1.

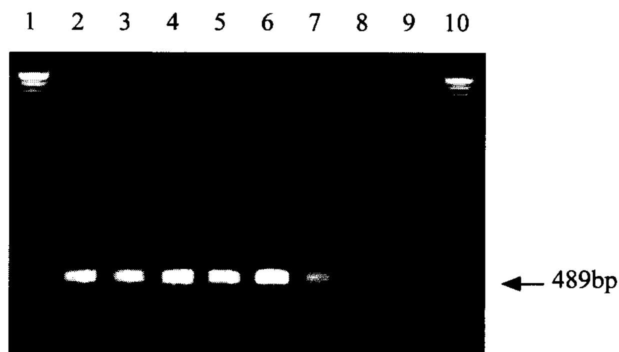

Fig. 1. Ethidium bromide stained agarose gel of CYP1B1 breast tumour PCR product. Lanes 1 and 10, molecular weight markers (λ*Hin*dIII fragments); lanes 2–7, breast tumours; lane 8, negative control (water); lane 9, positive control (CYP1B1 cDNA).

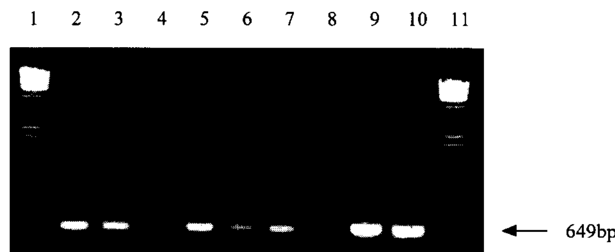

Fig. 2. Ethidium bromide stained agarose gel of breast tumours following PCR with CYP1A primers. Lanes 1 and 11, molecular weight markers (λ*Hin*dIII fragments); lanes 2–7, breast tumours; lane 8, negative control (water); lanes 9 and 10, positive controls (CYP1A1 and CYP1A2 plasmids, respectively).

3.2. Immunoblotting

Although CYP1B1 mRNA has previously been identified by Northern blotting in several normal human tissues [12] to date the presence of CYP1B1 protein has not been demonstrated. Immunoblotting of breast tumours was performed using an anti-CYP1 antibody which we have shown to recognize human CYP1B1 expressed in *E. coli*. All of the breast tumours examined showed a single immunoreactive band at 60 kDa (Fig. 4) corresponding to the predicted molecular weight of human CYP1B1.

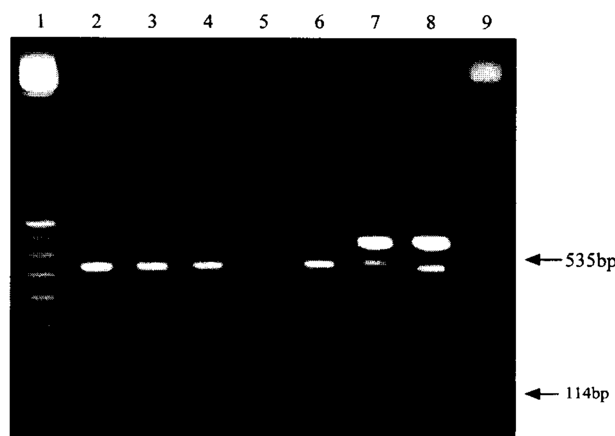

Fig. 3. Ethidium bromide stained agarose gel of CYP1A PCR products following *Rsa*I digestion. Lanes 1 and 9, molecular weight markers (100-bp ladder); lanes 2–6, breast tumours; lanes 7 and 8, positive controls (CYP1A1 and CYP1A2 plasmids, respectively).

Fig. 4. Immunoblot of breast tumours showing an immunoreactive band at 60 kDa. Lanes 1–6, breast tumours.

4. Discussion

The expression of individual forms of the CYP1 gene family in breast cancer has been investigated. It had been widely thought that the CYP1 gene family consisted of only one subfamily, containing two highly homologous members, CYP1A1 and CYP1A2. Recently, however, a new member of the CYP1 family, CYP1B1, belonging to a new subfamily, was identified in dioxin-induced human keratinocytes and showed ~40% identity with both CYP1A1 and CYP1A2 [12]. In the current study, both CYP1A1 and CYP1B1 mRNA were shown to be expressed in breast tumours, with CYP1B1 mRNA being the most frequently expressed. CYP1A2 mRNA was not identified in any of the tumours.

The strategy we used for identifying individual members of the CYP1A subfamily was to use primers which recognize sequences common to both CYP1A1 and CYP1A2, followed by *Rsa*I restriction enzyme digestion to give different pairs of fragments for CYP1A1 and CYP1A2, respectively, with sizes that allowed their resolution on agarose gel electrophoresis. These results were confirmed by direct dideoxy sequencing of the undigested PCR product.

Both CYP1A1 and CYP1B1 have been shown to be regulated by compounds which act at the Ah receptor [8,12]. However, this study clearly shows differential expression of CYP1A1 and CYP1B1, with CYP1B1 being the most frequently expressed member of the CYP1 gene family in breast cancer.

The expression of CYP1B1 in breast cancer is likely to have important metabolic consequences for the tumour cells, e.g. with respect to oestrogen metabolism as breast cancer is an oestrogen-dependent tumour. Dioxin induction of MCF-7 breast cancer cells resulted in marked NADPH-dependent 4-hydroxylation of 17β-oestradiol which was not inhibited by an anti-CYP1A1 antibody [18] and was inhibited by an antibody raised against mouse P450EF [19], now identified as murine CYP1B1 [20], suggesting that CYP1B1 may contribute to the 4-hydroxylation of oestradiol.

Acknowledgements: This study has been supported by grants from The Scottish Office Home and Health Department (G.I. Murray, W.T. Melvin, S.W.B. Ewen and M.D. Burke), Aberdeen Royal Hospitals NHS Trust (G.I. Murray, W.T. Melvin, S.W.B. Ewen and M.D. Burke), American Forest and Paper Association (W.F. Greenlee and C.B. Marcus), General Electric Corporation (W.F. Greenlee) and NIH Grants ES-07009 (W.F. Greenlee) and ES-05311 (C.B. Marcus). We thank Professor D.W. Nebert (University of Cincinnati) for the generous gift of the CYP1A1 and CYP1A2 plasmids.

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