Development of a human lymphoblastoid cell line constitutively expressing human CYP1B1 cDNA: substrate specificity with model substrates and promutagens

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An AHH-1 TK+/− cell derivative was developed that stably expresses human cytochrome P4501B1 (CYP1B1) cDNA in an extrachromosomal vector which confers resistance to l-histidinol and co-expresses NADPH cytochrome P450 oxidoreductase (OR). The CYP1B1-expressing cell line was designated hlBl/OR. Microsomes prepared from CYP1B1 cDNA expressing cells exhibit elevated levels of 7-ethoxyresorufin deethylase (EROD), 7-ethoxy-4-trifluoromethylcumarin deethylase (EFCD), benzo(a)pyrene hydroxylase (BPH), bufuralol 1′-hydroxylase, testosterone hydroxylase activities and spectrally quantifiable cytochrome P450. CYP1B1-containing microsomes did not contain detectable coumarin 7-hydroxylase, p-nitrophenol hydroxylase, lauric acid hydroxylase, (S)-mephenytoin 4′-hydroxylase or diclofenac 4′-hydroxylase activities. Kinetic parameters for selected substrates were compared among CYP1B1 and the two additional members of the CYP1 family, CYP1A1 and CYP1A2. For BPH and EFCD, the rank order of rates of substrate metabolism were CYP1A1>CYP1B1>CYP1A2. For EROD, the rank order of substrate metabolism was CYP1A1>CYP1A2>CYP1B1. For both EROD and EFCD the apparent Kₘ values for CYP1B1 were more similar to CYP1A1 than to CYP1A2. In order to begin to characterize the promutagen activating ability of CYP1B1, the mutagenicity of selected chemicals was examined in hlBl/OR cells; there was increased sensitivity (CYP1B1-expressing relative to control cells) to the mutagenicity of benzo(a)pyrene, cyclopenta[c,d]pyrene, 4-methylnitrosamino-1-(3-pyridyl)-1-butanol and aflatoxin B₁ (AFB). CYP1B1, expressed in this system, appears to be particularly efficient at activating AFB.

Introduction

Cytochromes P450 (CYP) are a family of haemoproteins which are the principal enzymes involved in the activation of many promutagens and procarcinogens (Guengerich, 1988; Gonzalez, 1989; Gonzalez and Gelboin, 1994). These enzymes are expressed in many tissues in vivo with the highest levels of this class of enzymes found in the liver of mammals (Gonzalez and Gelboin, 1994). Individual cytochrome P450 forms have unique, yet often overlapping substrate specificity. For example, at least five different hepatic cytochrome P450 forms have been reported to activate aflatoxin B₁ (AFB) (Aoyama et al., 1990) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNK) (Crespi et al., 1991b; Smith et al., 1992). Aromatic amines are usually activated by a single form (CYP1A2) (Butler et al., 1989).

One of the more recently identified human cytochrome P450 forms, CYP1B1, was first recognized as being 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-inducible in human keratinocytes (Sutter et al., 1994). The CYP1B1 protein is also expressed in MCF-7 cells upon treatment with TCDD (Spinke et al., 1994). Northern analysis of RNA isolated from different human tissues reveals that CYP1B1 is expressed in the kidney, prostate, uterus and mammary tissues, but at much lower levels in the liver (Sutter et al., 1994). Mouse CYP1B1 (P450EF) is expressed in C3H 10T1/2 cells (Shen et al., 1994; Savas et al., 1994). Rat CYP1B1 is expressed at high levels in the adrenal gland of untreated rats (Otto et al., 1991; Walker et al., 1995) and in many additional tissues upon TCDD treatment. The significance of CYP1B1 in xenobiotic metabolism, in general, and procarcinogen metabolism, in particular, is not well understood.

In addition to CYP1B1, the CYP1 family contains two additional members, CYP1A1 and CYP1A2. CYP1A1 has been recognized as having a principal role in the metabolic activation of many polycyclic aromatic hydrocarbons (PAH) (Shimada et al., 1992) and CYP1A2 has been recognized a principal role in the activation of many aromatic and heterocyclic amines (Butler et al., 1989; Eaton et al., 1995). CYP1A2 is primarily a hepatic enzyme, whereas, CYP1A1 and CYP1B1 appear to be principally extrahepatic and most readily detected following administration of inducers (Sesarid et al., 1990; Sutter et al., 1994).

Here we report the development of a human cell line stably expressing CYP1B1 cDNA. This cell line, designated hlBl/OR, metabolizes prototypic substrates for CYP1 and contains spectrophotometrically quantifiable and immunologically detectable CYP1B1 protein. The AHH-1 TK+/− human B-lymphoblastoid cell line, which was used in these studies, is a convenient vehicle to measure the induction of gene mutations at the hypoxanthine phosphoribosyl transferase (hprt) and thymidine kinase loci and also the induction of chromosomal aberrations, aneuploidy and micronuclei (Crespi et al., 1987; Crespi et al., 1991a; Crofton-Sleigh et al., 1993). The CYP1B1 cDNA-expressing cell lines were characterized and used to study the P450 form-specific activation of mutagens at the hprt locus. Finally, we have compared properties of cDNA-expressed CYP1B1 with cDNA-expressed CYP1A1 and CYP1A2.

Material and methods

Cells, tissue culture, DNA introduction

AHH-1 TK+/− human B lymphoblastoid cells were maintained in Roswell Park Memorial Institute (RPMI) medium 1640 supplemented to 9% v/v with horse serum. Plasmid vector was introduced into AHH-1 TK+/− cells by electroporation using a BTX model 600 Electro cell Manipulator (San Diego, CA, USA). Cells containing vectors were initially selected by resistance to hygromycin B, then shifted l-histidinol-selection to accomplish vector

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amplification. Cells bearing recombinant plasmids were maintained in medium either with 100-400 µg/ml hygromycin B or without histidine and containing 2 mM L-histidinol (pEBVHisk-based vectors) (Crespi et al., 1990a).

Enzymes and reagents
Restriction endonucleases, Klenow fragment of DNA polymerase I and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA, USA). 7-Ethoxy-4-trifluoromethylcoumarin and 7-hydroxy-4-trifluoromethylcoumarin were purchased from Molecular Probes, Eugene, OR, USA. NNK and 3'-hydroxybenz(a)pyrene were obtained from the NCI Chemical Carcinogen Repository (Research Triangle Park, NC, USA). Radiolabelled lauric acid and (S)-mephenytoin were obtained from Amersham, Arlington Heights, IL, USA.

All other chemicals were purchased from Sigma Chemical Company, St. Louis, MO, USA.

Cytotoxicity and mutagenicity assays
Cytotoxicity was estimated by measuring cell growth after treatment. After cultures have resumed exponential growth, the cumulative growth of the mutagen-treated cultures was divided by the cumulative growth of the negative control cultures to yield relative survival. Induction of mutation at the hprt locus was measured by previously published protocols (Crespi et al., 1987) with minor modifications. Human lymphoblasts were exposed to the mutagens for 24 h. cDNA-derived NADD1 cytochrome P450 oxdoreductase (OR) is expressed from the glucocorticoid-inducible mouse mammary tumour virus long terminal repeat. OR expression was increased by the addition of 1 µM dexamethasone. Each replicate culture contained 3 × 10^6 cells. After a 7 day phenotypic expression period, the mutant fraction was measured by plating 7.5 × 10^4 cells in three 96-well microtitre plates in the presence of 6-thioguanine (0.6 µg/ml) and 50 µg/ml 3′-methyladenine. Duplicate cultures were grown in 96-well plates with selection. NNK was dissolved in dimethyl sulphoxide (DMSO) and dissolved in water for delivery to the cell cultures or other chemicals were dissolved in dimethyl sulphoxide (DMSO).

DNA manipulations, vectors
Bacterial transformations, plasmid DNA isolations and construction of the expression vectors were performed according to standard protocols. For CYP1B1 expression, a derivative of the pRedHyHo vector (Crespi et al., 1995), designated pBlueHyHo was used. pBlueHyHo was produced from pRedHyHo by introduction of the LacZ β complementation from pUC19 into the HindIII site between the HisD expression unit and the OR expression unit. This construct was performed as follows: the polylinker in pUC19 was cleaved with XbaI and ligated to the XbaI site of pRedHyHo, then ligated into XbaI digested pBluescriptII and XbaI ligated. This fragment was blunt ended by treatment with the Klenow fragment of DNA polymerase. This content is slightly lower than that previously reported for CYP1B1 (range ~2-fold) and one population was chosen for further characterization and designated h1B1/OR cells.

The stability of cDNA-derived enzymatic activity in h1B1/OR cells was monitored as a function of time of growth in cell culture. Cells were propagated and monitored periodically for sensitivity to BP cytotoxicity. The cell line was stable for at least 2 months of continuous exponential growth. This level of stability is sufficient for most applications in toxicology and drug metabolism (Crespi et al., 1991a).

Characterization of the enzyme
Microsomes prepared from h1B1/OR cells contained spectrophotometrically and immunologically detectable CYP1B1 protein. This protein was not detectable in control cells (without cDNA-bearing vector) (Figure 2). The level of expression (spectral P450 content) under culture conditions similar to those used in the mutagenicity assay was 14 pmol P450/mg microsomal protein. This content is slightly lower than that previously reported for CYP1A1 (h1A1v2 cells, 25 pmol P450/mg) and CYP1A2 (h1A2v2 cells, 38 pmol P450/mg). No spectrally active CYP was detectable in control microsomes CYPIA1 or CYP1A2 were not detectable by Western blot at loading up to 150 µg/lane (data not shown).

The level of OR activity, as measured by cytochrome c reductase, was elevated ~1.5-fold relative to microsomes prepared from control cells. We have found that when cytochrome P450 and OR cDNAs are co-expressed from a single vector, the levels of OR present can vary substantially for different P450 cDNAs. The results for CYP1B1 are at the low end of the range. For example, when CYP2C9/OR or CYP2E1/OR are co-expressed, the level of cytochrome c reductase activity is higher [250 or 80 nmol/(mg min)] respect-
Human lymphoblastoid cells expressing CYPIBI cDNA

Fig. 1. Schematic map of the CYPIBI cDNA expression vector pBlueHyHoB1 cDNA expression units are transcribed in the direction of the arrow. The identity of the different DNA elements are as follows: 5'tk, herpes simplex virus thymidine kinase gene promoter; 3'tk, herpes simplex virus thymidine kinase gene polyadenylation signal; HaMSV MMTV-LTR, the Harvey murine sarcoma virus enhancer linked to the mouse mammary tumour virus long terminal repeat promoter; OriP, the origin of DNA replication from Epstein-Barr virus; hOR, the human NADPH-cytochrome P450 oxidoreductase; Hyg, the hygromycin B resistance gene; HisD, the E.coli histidinol dehydrogenase gene. The arrows indicate the direction of transcription. Selected restriction sites (EcoRI, NarI, HindIII, XhoI and Sall) are also shown.

Fig. 2. Western immunoblot of microsomes prepared from human lymphoblastoid cells. Microsomal samples, 30 mg per lane, were analysed by SDS-PAGE and Western immunoblot. Immunoreactive proteins were detected by enhanced chemiluminescence procedure. Lane 1, control microsomes, P450 not detected, lane 2, hIB1/OR microsomes, 1 pmol P450. The positions of the 60 and 50 kDa protein markers are indicated at the right of the blot.

Analysis of metabolism of a panel of cytochrome P450 substrates which microsomes prepared from CYPIBI-expressing cells revealed elevated EROD, EFCD, BPH (Table I), bufuralol 1'-hydroxylase and testosterone hydroxylase (Table II). No detectable coumarin 7-hydroxylase, p-nitrophenol hydroxylase, lauric acid hydroxylase, (S)-mephenytoin 4'-hydroxylase or diclofenac 4'-hydroxylase activities were present.

The lack of coumarin 7-hydroxylase, a reaction catalysed by CYP2A6 (Yamano et al., 1990), p-nitrophenol hydroxylase, a reaction catalysed by CYP2A6 and CYP2E1, lauric acid hydroxylase, a reaction catalysed by CYP4A and CYP2E1 (Clarke et al., 1994), (S)-mephenytoin 4'-hydroxylase, a reaction catalysed by CYP2C19 (Wrighton et al., 1993) and diclofenac 4'-hydroxylase, a reaction catalysed by CYP2C9 (Leeman et al., 1993) were consistent with the enzyme-selective nature of these substrates and supports the prediction of activities based on comparisons of CYPIB1 primary amino acid sequence to those of CYP1A1 and CYP1A2 (Sutter et al., 1994).

The detection of bufuralol 1'-hydroxylase and testosterone hydroxylase activities were surprising. The rates of metabolism per unit enzyme were relatively low (-300-fold lower than CYP2D6/bufuralol or CYP3A4/testosterone) but the metabolite signal was substantially (10 fold) above machine background and/or incubations with control microsomes. CYPIB1-catalysed bufuralol 1'-hydroxylase activity was not inhibited by the addition of 1 mM quinidine, a CYP2D6-selective inhibitor (data not shown) indicating that the metabolism was not due to presence of CYP2D6. Concurrent incubations with cDNA-expressed CYP2D6 with and without 1 mM quinidine showed 95% inhibition. With CYPIB1, two hydroxylated testosterone metabolites were observed. These metabolites co-chromato-

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present in CYPIBI microsomes should not have affected this rank order. The apparent K_m for CYPIBI-catalyzed EROD was 580 nM, which is similar to the apparent K_m for CYP1A1 (1100 nM) and much higher than the K_m for CYP1A2 (4.8 nM). The turnover number for CYPIBI (0.74/min) was lower than for either CYP1A1 (1.1/min) or CYP1A2 (0.9/min). The slightly higher OR levels present in CYPIBI microsomes again should not have affected this rank order. BPH activity was measured at a single substrate concentration (25 μM). The turnover number at this concentration for CYPIBI (0.27/min) was intermediate relative to CYP1A1 (0.0/min) or CYP1A2 (0.07/min). The slightly higher OR levels present in CYPIBI microsomes should not have affected this rank order.

**Mutagenicity assays**

The ability of cDNA-derived CYPIB1 to activate promutagens was examined using hB1/OR cells. Four promutagens, benzo(a)pyrene (BP), cyclopenta(c,d)pyrene (CPP), AFB and NNK were examined for the ability to induce gene mutations at the hprt locus. We, and others, have previously demonstrated that AFB and NNK are activated by multiple CYP forms (Shimada and Guengerich, 1989; Forrester et al., 1990; Aoyama et al., 1990; Crespi et al., 1991b,c; Smith et al., 1992). The polycyclic aromatic hydrocarbons (PAH), BP and CPP are prototypical CYP1A1 substrates (Penman et al., 1994). BP was clearly mutagenic to hB1/OR cells (Figure 3). BP induced a monotonic increase in mutant fraction in the concentration range 10–1000 ng/ml. Over this same concentration range BP is non-mutagenic to control cells (Penman et al., 1994). BP was relatively toxic to hB1/OR cells. Comparison of this dose–response data with that previously published for CYP1A1 (Penman et al., 1994) and taking into consideration that the hA1 and hB1/OR cell lines have roughly comparable cytochrome P450 contents, indicates that CYPIB1 and CYP1A1 have roughly comparable abilities to activate BP to a gene mutagen. The slightly higher OR levels present in hB1/OR cells should not affect this conclusion. The activation of BP by CYPIB1-expressing human lymphoblasts stands in contrast to the lack of a response induced by BP with CYPIB1 present in the Salmonella typhimurium umu gene expression system (Shimada et al., 1996). The bacterial system does indicate that both CYP1A1 and CYP1A2 are capable of activating BP; therefore, the difference cannot be completely attributed to a lack of sensitivity of the system to this compound. The activation of BP by CYPIB1 in human lymphoblasts is consistent with the detection of BP metabolites by fluorometric assay (Table I), although the phenols detected by this assay are not expected to be directly mutagenic. Metabolism of BP by CYPIB1 and CYP1A2 expressed in human lymphoblasts was also detectable (Table I). The discordance between the systems cannot be attributed to the presence of microsomal epoxide hydrolase in human lymphoblasts (and...
its absence in the bacterial system) since the lymphoblastoid cell line does not have detectable microsomal epoxide hydrolase activity (Crespi et al., 1985). In contrast to BP, several BP diols produced a detectable response with CYP1B1 in the *S. typhimurium umu* gene expression system (Shimada et al., 1996). Therefore, different responses of BP in the two systems could be due to differences in sensitivity of the different host cells to the range of electrophiles produced by BP metabolism. Additional experiments are required to clarify this point.

CYP was also clearly mutagenic to h1B1/OR cells (Figure 3). A monotonic increase in mutant fraction was observed over the concentration range 5–60 ng/ml. CPP was non-mutagenic to control cells over the concentration range tested (Penman et al., 1994). Like BP, CPP is relatively toxic to h1B1/OR cells. Comparison of this dose–response data with that previously published for CYP1A1 (Penman et al., 1991a), CYP1A2 (Crespi et al., 1991c), CYP2A6 (Crespi et al., 1990a), CYP3A4 (Crespi et al., 1991c) indicate that CYP1B1 is among the most efficient enzymes for activating AFB to a gene mutagen. Relevant parameters are summarized in Table III. We have listed the minimum effective concentration of AFB (A in Table III) which was calculated as the point where the concentration-response curve crosses the 99% upper confidence limit from the historical negative control data base (a mutant fraction $7 \times 10^{-6}$, all cell lines appear to have similar negative control mutant frequencies). Comparison of these values indicates the relative sensitivities of the different cell lines to AFB mutagenicity. However, the cell lines have different cytochrome P450 contents, indicates that as with BP, CYP1B1 and CYP1A1 have roughly comparable abilities to activate CPP to a gene mutagen. The slightly higher OR levels present in h1B1/OR cells should not affect this conclusion.

The efficient activation of AFB by CYP1B1-expressing human lymphoblasts stands in sharp contrast to the complete lack of a response induced in the *S. typhimurium umu* gene expression system (Shimada et al., 1996). This bacterial system does give positive responses for AFB when either CYP1A1 and CYP1A2 are present. However, the difference in response between CYP1A1 and CYP1A2 is less pronounced in the *S. typhimurium* system relative to the human lymphoblast system (three-fold versus 40-fold; Penman et al., 1994) and substantially higher AFB concentrations were needed to generate a relatively weak response in *S. typhimurium*. It is possible that CYP1B1 is a relatively low capacity enzyme for AFB activation and insufficient electrophilic metabolite is formed to generate a detectable response in *S. typhimurium*. Further experiments are needed to clarify this point.

AFB is a known human (and rat) hepatocarcinogen and it has been established that among the mixture of enzymes present in human liver, CYP1A2 appears to be the most important enzyme at low AFB concentrations (Eaton et al., 1995), although CYP3A4 may be involved at high AFB exposures. Given the extrahepatic nature of CYP1B1, one would expect little role of this enzyme in hepatic AFB activation. However, it is possible that CYP1B1 may play a role in extrahepatic AFB activation, particularly for individuals exposed to inducers.

NNK induced a monotonic increase in mutant fraction over the concentration range 10–100 ng/ml (Figure 3). NNK was also toxic to h1B1/OR cells. NNK is non-toxic and non-mutagenic to control cells at exposure concentrations up to 300 ng/ml (Crespi et al., 1991b). We had previously demonstrated that multiple human P450 forms can activate NNK (Crespi et al., 1991b; Penman et al., 1994). After allowing for differences in cellular P450 content (using the same approach used for AFB above), the ability to metabolically activate NNK is approximately CYP1A1 = CYP1A2 = CYP1B1 = CYP2A6 > CYP2E1 = CYP2D6. It is interesting to note that, per unit induced mutant fraction, NNK is much more toxic.
... to CYP1B1-expressing cells than to cells expressing other cytochrome P450 enzymes (Figure 4). NNK is an asymmetric nitrosamine and this observation may be indicative of a different pathway of metabolism for CYP1B1 than for CYP1A1, CYP1A2, CYP2A6, CYP2D6 or CYP2E1. In order to test this hypothesis, NNK metabolism was analyzed in microsomes prepared from h1B1/OR cells. Unfortunately, when sodium bisulphite (5 mM) was added to the incubation mixture to trap the keto aldehyde, thereby allowing quantification of the two α-hydroxylation pathways (Peterson et al., 1991), the metabolism of NNK was inhibited by 50%. Therefore, sodium bisulphite was not added to the assay mixtures, and the metabolite peak was quantified as a mixture of keto and alcohol. The apparent $K_m$ and $V_{max}$ values were 1.2 mM and 1.8/min respectively. The apparent $K_m$ value for CYP1B1 was 3-10-fold higher than that reported for CYP2D6 (Penman et al., 1993), CYP1A2 (Smith et al., 1992) or CYP2A6 (Patten et al., 1996). Since humans are exposed to low levels of NNK, and CYP1B1 exhibited a high $K_m$ for this cytochrome P450, such as CYP2A6, may play a more important role in the activation of NNK in vivo. With regard to activation of other nitrosamines, N-nitrosodimethylamine was negative for CYP1B1 activation using the S.typhimurium umu gene expression system (Shimada et al., 1996). Therefore, it is unclear how broadly CYP1B1 activates nitrosamines.

The development of the h1B1/OR cell line complements the other isogenic AHH-1 TK$^{+}$ human B-lymphoblastoid cell lines expressing human cytochromes P450 (Crespi et al., 1990a,b, 1991c; Penman et al., 1993, 1994). For all of these expressed human P450s, spectrophotometric determination of P450 content is possible, allowing comparisons of metabolism and protoxin activation on both a per cell and per pmole P450 basis. This enables a quantitative analysis of the relative efficiency of different P450 enzymes to carry out specific biotransformations in an intact cellular system.

The present study, in combination with the study by Shimada et al., 1996 indicate that CYP1B1 activates a broad range of structurally diverse procarcinogens including, polycyclic aromatic hydrocarbon (and dihydriodiol derivatives), aromatic amines and amides, heterocyclic amines, nitro polycyclic aromatic hydrocarbons, AFB and NNK. This broad substrate specificity, coupled with the expression of CYP1B1 in multiple human tissues (Sutter et al., 1994; Shimada et al., 1996), suggests that CYP1B1 could have a significant role in cancer induction in humans. The striking differences in response for the activation of BP and AFB in the S.typhimurium umu gene expression system and human lymphoblasts serves to underscore the need to test procarcinogens in multiple systems.

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References


Table III. Comparison of the abilities cDNA expressed cytochromes P450 to activate aflatoxin B$_1$ to a gene mutagen. The minimum detectable concentration was calculated as the AFB concentration where the dose-response curve crossed the 99% upper confidence limit for the historical negative control data base ($7 \times 10^{-6}$) (Penman and Crespi, 1987)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cell line</th>
<th>A. Minimum detectable concentration (ng/ml)</th>
<th>B. P450 content (pmol/ng microsomes)</th>
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<td>1.7</td>
<td>150</td>
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</table>

Fig. 4. Plot of relative survival versus induced mutant fraction for NNK activated by CYP1B1 (diamonds) or all other cytochrome P450s (squares). The data for all other cytochrome P450s is taken from (Crespi et al., 1990a;b, 1991c; Penman et al., 1993, 1994).


