FUNCTIONAL CHARACTERIZATION OF CYTOCHROME P450 2B6 ALLElic VARIANTS

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ABSTRACT:

Cytochrome P450 (P450) 2B6 is a hepatic enzyme of potential importance for the metabolism of clinically used drugs and environmental or abused toxicants. Genetic polymorphisms of CYP2B6 (CYP2B6*2, CYP2B6*3, CYP2B6*4, CYP2B6*5, CYP2B6*6, CYP2B6*7; wild-type, CYP2B6*1) were found previously in white and Japanese populations. In the present study, the goal was to investigate the effects of amino acid substitutions on CYP2B6 function. Wild-type (CYP2B6.1) and all of the known variants of CYP2B6 (CYP2B6.2, CYP2B6.3, CYP2B6.4, CYP2B6.5, CYP2B6.6, and CYP2B6.7) were transiently expressed in COS-1 cells, and their 7-ethoxy-4-trifluoromethylcoumarin O-deethylation activities were determined. The levels of the variant CYP2B6 proteins were relatively low compared with that of CYP2B6.1, although the differences were not significant. The activities of 7-ethoxy-4-trifluoromethylcoumarin O-deethylation on the basis of the CYP2B6 protein level at low (0.5 μM) and high (50 μM) substrate concentrations varied among wild-type and variant CYP2B6 proteins. All CYP2B6 enzymes showed typical Michaelis-Menten kinetics. The $V_{max}$ value of CYP2B6.6 was significantly higher than that of CYP2B6.1. Those CYP2B6 variants having a Lys262Arg substitution (CYP2B6.4, CYP2B6.6, and CYP2B6.7) showed increased values for $V_{max}$ and $K_m$, whereas the kinetic parameters of CYP2B6.2 and CYP2B6.3 were not affected by the corresponding amino acid substitution. These results may mean that Lys262 in combination with other amino acid residues such as Gln172 and Arg487 is associated with the CYP2B6 function and that the genetic polymorphism of CYP2B6 leads to interindividual differences in xenobiotic metabolism.

Members of the cytochrome P450 (P450) superfamily of heme-proteins catalyze the oxidative metabolism of xenobiotic chemicals such as drugs, carcinogens, and toxins, as well as endogenous substances such as steroids, fatty acids, and vitamins (Nelson et al., 1996). The CYP2B6 gene has been mapped to chromosome 19 between 19q12 and 19q13.2, and is composed of nine exons (Guengerich, 1995; Nelson et al., 1996). This P450 isoform is expressed in liver and many extrahepatic tissues including kidney, intestine, and lung (Gonzalez et al., 1992; Ekins and Wrighton, 1999; Gervot et al., 1999). CYP2B6 plays important roles in the metabolism of a number of therapeutic drugs such as the antineoplastic agent cyclophosphamide, antiestrogen tamoxifen, anticonvulsant 5-mephénytoin, and benzodiazepine diazepam (Chang et al., 1993; White et al., 1995; Heyn et al., 1996; Ono et al., 1996). In addition, CYP2B6 has been implicated in the metabolism of procarcinogens including aflatoxin B1, 6-aminochrysene, and 7,12-dimethylbenz[a]anthracene (Aoyama et al., 1990; Mimura et al., 1993; Shou et al., 1996).

Most P450 isoforms involved in the metabolism of xenobiotic chemicals, particularly the CYP2 family, are known to be affected by common genetic polymorphisms, which can have profound effects on drug metabolism and the efficacy of drug therapy (Meyer and Zanger, 1997; Daly et al., 1998; Ingelman-Sundberg et al., 1999). Substantial interindividual differences have been found in the contribution of CYP2B6 to cyclophosphamide 4-hydroxylation in a panel of human liver microsomes, and this may be related to the large interindividual variability in hepatic levels of CYP2B6 mRNA and protein (Yamano et al., 1989; Chang et al., 1993; Code et al., 1997; Gervot et al., 1999). Large interpatient differences in the clinical pharmacokinetics and biotransformation of this drug have also been reported (Boddy et al., 1992; Chen et al., 1995). A part of such variation can be expected to be caused by the genetic polymorphism of CYP2B6, although the reason for this is not clear.

Recently, Lang et al. (2001) identified nine point mutations of the CYP2B6 gene in a population of white persons. Five of these cause amino acid substitutions in exons 1, 4, 5, and 9. They found six different alleles termed CYP2B6*2, CYP2B6*3, CYP2B6*4, CYP2B6*5, CYP2B6*6, and CYP2B6*7 (wild-type, CYP2B6*1) by haplotype analysis (Table 1). More recently, Hiratsuka et al. (2002) have found that the allelic variants of CYP2B6 exist in the Japanese population with frequencies comparable with those in the white population, albeit with some differences. The 516G>T mutation was reported to be the only one of the five nonsynonymous mutations seen exclusively in combination with other amino acid mutations in both populations. However, very little has been reported about the effects of genetic polymorphisms of CYP2B6 on the detailed enzymatic properties or about comparisons among identified allelic variants. The data should provide valuable infor-
mation for predicting the biological effects of xenobiotics metabolized mainly by CYP2B6.

The purpose of this study was to investigate whether the amino acid substitutions in CYP2B6 affect enzyme function. To achieve this, cDNAs of wild-type and variant CYP2B6 were constructed, and the corresponding CYP2B6 proteins (CYP2B6.1, CYP2B6.2, CYP2B6.3, CYP2B6.4, CYP2B6.5, CYP2B6.6, and CYP2B6.7) were transiently expressed in COS-1 cells. The enzymatic properties of CYP2B6 proteins were characterized using the marker substrate 7-ethoxy-4-trifluoromethylcoumarin.

Materials and Methods

Materials. Human adult normal liver mRNA was purchased from BioChain Institute (Hayward, CA). The SuperScript first-strand synthesis system, Platinum Pfx DNA polymerase, pENTR/t-TOPO vector, pcDNA-DEST40 mammalian expression vector, Lipofectamine 2000 and OPTI-MEM medium were purchased from Invitrogen (Carlsbad, CA). QuikChange Multi site-directed mutagenesis kit was obtained from Stratagene (La Jolla, CA). COS-1 cells were obtained from the Japanese Collection of Research Biosource (Osaka, Japan). Dulbecco’s modified Eagle’s medium and fetal bovine serum were purchased from Sigma-Aldrich (St. Louis, MO). 7-Ethoxy-4-trifluoromethyl-coumarin and 7-hydroxy-4-trifluoromethyl-coumarin were purchased from Ultrafine Chemicals (Manchester, UK). NADPH was obtained from Oriental Yeast (Tokyo, Japan). ABI BigDye terminator cycle sequencing reaction kit v2.0 was from Applied Biosystems (Foster City, CA). Rabbit anti-human CYP2B6 antibody (synthetic peptide corresponding to amino acids 265-276) was from Affiniti Research Products (Exeter, UK). Horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin and enhanced chemiluminescence-plus reagents were from Amersham Biosciences (Piscataway, NJ). All other chemicals and reagents were of the highest quality commercially available.

Construction of CYP2B6 Plasmids. The mRNA from normal human liver was reverse-transcribed to cDNA using the SuperScript first-strand synthesis system. The cDNA of CYP2B6*1 was amplified by polymerase chain reaction (PCR) from 50 ng of single stranded cDNA template using the forward primer, 5’-CACCATTGAACCTACGGGTCTCCTCCTC-3’ and the reverse primer, 5’-TCACGGGGCCAGGAAGCGG-3’. The underlined sequence was introduced to perform directional TOPO cloning. The amplification mixture (50 μl) contained 1 U of Platinum Pfx DNA polymerase, 1 x Pfx amplification buffer, 1 mM MgSO4, 0.3 mM dNTPs, and 0.3 μM each of forward and reverse primers. The cycling parameters were as follows: denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 15 s and 72°C for 2 min, and termination by a 7-min extension at 72°C. The PCR products were cloned into the pENTR/t-TOPO vector and sequenced in both the forward and reverse directions using the ABI BigDye terminator cycle sequencing reaction kit v2.0 on the DNA analyzer ABI Prism 3700 (Applied Biosystems). The plasmid carrying the CYP2B6*1 cDNA was used as a template to generate point mutations in the target regions. Four single variant CYP2B6s (CYP2B6*2, CYP2B6*3, CYP2B6*4, and CYP2B6*5), one double variant CYP2B6 (CYP2B6*6), and one triple variant CYP2B6 (CYP2B6*7) were constructed with the QuikChange Multi site-directed mutagenesis kit according to the manufacturer’s instructions using the 5’- phosphorylated primers. The primers for the respective mutations were MP-C64T for CYP2B6*2, MP-C777A for CYP2B6*3, MP-A785G for CYP2B6*4, MP-C1459T for CYP2B6*5, MP-G516T/MP-A785G for CYP2B6*6, and MP-G516T/MP-A785G/MP-C1459T for CYP2B6*7 (Table 2). All CYP2B6 plasmids were sequenced to confirm successful mutagenesis. The wild-type and variant CYP2B6 cDNAs were subsequently subcloned into the pcDNA-DEST40 mammalian expression vector.

Expression of CYP2B6 Enzymes. COS-1 cells, an African green monkey kidney cell line, were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum under 5% CO2 at 37°C. Cells (5.5 x 10^6/cm^2) were seeded in a 10-cm culture dish one day before transfection. On the following day, the culture medium was replaced with OPTI-MEM medium, and the CYP2B6 expression plasmids (15 μg DNA) were transfected using Lipofectamine 2000 according to the manufacturer’s instructions. After 48 h, the cells were washed twice with ice-cold phosphate-buffered saline and harvested in 100 mM potassium phosphate buffer (pH 7.4) containing 250 mM sucrose. For comparison, cells transfected with pcDNA3.1 as a null vector were used as a negative control. Microsomes from COS-1 cells were prepared by sequential centrifugation according to a standard procedure described by Ekins et al. (1999). After centrifugation, the microsomes were suspended in 100 mM potassium phosphate buffer (pH 7.4) containing 10% glycerol and stored at −80°C until use. The protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Quantification of CYP2B6 Protein Levels. Microsomal proteins (20 μg) from COS-1 cells were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli, 1970) and electrotransferred to a polyvinylidene fluoride sheet (Atto, Tokyo, Japan) as described by Towbin et al. (1979). The sheet was incubated with rabbit anti-human CYP2B6 antibody as the primary antibody and then with horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin as the secondary antibody. Immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (enhanced chemiluminescence-plus reagents), and the band densities were determined with the bioimaging analyzer Typhoon 9400 (Amersham Biosciences).

Assay for CYP2B6-Dependent Metabolism. 7-Ethoxy-4-trifluoromethyl-coumarin O-deethylation activity was determined by measurement of the formation of 7-hydroxy-4-trifluoromethyl-coumarin, according to the method of Morse and Lu (1998) with some modifications. The incubation mixture contained 7-ethoxy-4-trifluoromethyl-coumarin as a substrate (0.5, 1.0, 2.0, 5.0, 10, 20, and 50 μM), microsomes from COS-1 cells (500 μg of protein/ml) and 1 mM NADPH in 50 mM potassium phosphate buffer (pH 7.4) in a final volume of 250 μl. 7-Ethoxy-4-trifluoromethyl-coumarin was dissolved in methanol (final concentration in the reaction medium, 0.5% (v/v)). After preincubation at 37°C for 2 min, the reaction was initiated by the addition of NADPH. The mixture was incubated at 37°C for 15 min and terminated with 10 μl of 2.0 M phosphoric acid. The samples were centrifuged at 6000g for 20 min. The supernatant was filtered and analyzed by high-performance liquid chromatography (HPLC). HPLC was performed using an LC-10AVP system (Shimadzu, Kyoto, Japan) equipped with an Inertsil ODS-80A column (150 x 4.6 mm i.d.; GL Sciences, Tokyo, Japan). The column was kept at 40°C. The elution was performed isocratically with 20 mM sodium perchlorate (pH 2.5)-acetoniitrite (47:53, v/v) at a flow rate of 1.0 ml/min. The detection was based on fluorescence intensity with excitation at 342 nm and emission at 495 nm. The sample standards were prepared in the same manner as the incubation samples. Under these conditions, the retention times of 7-hydroxy-4-trifluoromethyl-coumarin and 7-ethoxy-4-trifluoromethyl-coumarin were 4.2 and 13.5 min, respectively. The detection limit for 7-hydroxy-4-

### Table 1

<table>
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<tr>
<th>Allele</th>
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<th>Nucleic Acid Changes</th>
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* Wild-type.
of 7-ethoxy-4-trifluoromethylcoumarin O-deethylation were performed. The calculated kinetic parameters are summarized in Table 3. The $K_m$ values of wild-type and variant CYP2B6s ranged from 5.5 to 7.7 $\mu$M, and a significant increase in CYP2B6.6 was observed. The values of $V_{\text{max}}$ and $V_{\text{max}}/K_m$ of wild-type and variant CYP2B6s on the basis of the microsomal protein level were 8.9 to 18 pmol/min/mg of protein and 1.4 to 2.3 $\mu$mol/min/mg of protein, respectively. However, there was no significant difference in the values of $V_{\text{max}}$ and $V_{\text{max}}/K_m$ in any variant CYP2B6. When the enzymatic activities were normalized to CYP2B6 protein levels, the values of $V_{\text{max}}$ and $V_{\text{max}}/K_m$ of CYP2B6.1 were 3.9 pmol/min/pmol CYP2B6 and 0.7 $\mu$mol/min/pmol CYP2B6, respectively. The $V_{\text{max}}$ values of CYP2B6.4, CYP2B6.6, and CYP2B6.7 were significantly increased 2.0 to 2.6-fold compared with that of CYP2B6.1 (Fig. 3). With respect to $V_{\text{max}}/K_m$ the values of CYP2B6.4, CYP2B6.5, CYP2B6.6, and CYP2B6.7 were significantly higher than that of CYP2B6.1 (1.6–1.9-fold).

**Discussion**

Although CYP2B6 is generally regarded as a minor component of the P450 genes in the liver, it has been shown to catalyze the oxidation of a number of structurally diverse xenobiotics in heterologous cell expression systems (Code et al., 1997; Rendic and Di Carlo 1997; Ekins and Wrighton, 1999; Gervot et al., 1999). The metabolic activ-
Experimental conditions are described under Materials and Methods. Each point represents the mean of four separate experiments derived from independent preparations. Open bar, 0.5 μM substrate; hatched bar, 50 μM substrate. * p < 0.05, ** p < 0.01 versus CYP2B6.1.

It has been suggested that the oxidative metabolism of 7-ethoxy-coumarin and testosterone can be used as classical substrates for CYP2B6; however, other P450 isoforms such as CYP1A2, CYP2A6, CYP2E1, and CYP3A4 are also involved in these reactions (Imaoka et al., 1996; Shimada et al., 1999). On the other hand, the levels of the CYP2B6 protein in a panel of human liver microsomes have been reported to correlate strongly with the activities of 5-mephenytoin N-demethylation and 7-ethoxy-4-trifluoromethylcoumarin O-deethylation (Heyn et al., 1996; Code et al., 1997; Ekins et al., 1998). We also confirmed that 7-ethoxy-4-trifluoromethylcoumarin is selectively O-deethylated by CYP2B6 at a substrate concentration of 5 μM using microsomes from insect cells expressing specific human P450s, although it was impossible to determine the activity of 5-mephenytoin N-demethylation for reasons of analytical sensitivity (data not shown).

Therefore, we used 7-ethoxy-4-trifluoromethylcoumarin O-deethylation as a probe to characterize the enzymatic function of wild-type and variant CYP2B6s. All of the variant CYP2B6s were capable of O-deethylating 7-ethoxy-4-trifluoromethylcoumarin as well as wild-type CYP2B6 at low and high substrate concentrations; however, the activity on the basis of the CYP2B6 protein level varied among wild-type and variant CYP2B6s. Furthermore, from the ratio of ac-
tivity at low and high substrate concentrations, the proteins could be roughly classified into two groups: 1, wild-type and single variant CYP2B6s, and 2, double and triple variant CYP2B6s, suggesting that the affinity toward 7-ethoxy-4-trifluoromethycoumarin differs among CYP2B6 variants. Further studies are required to identify the metabolic ability of variant CYP2B6s toward other substrates.

Previous studies have estimated that the $K_m$ value for 7-ethoxy-4-trifluoromethycoumarin O-deethylation in microsomes from human B-lymphoblastoid cells expressing wild-type CYP2B6 is 1.7 to 2.9 μM (Ekins et al., 1996; Code et al., 1997). Code et al. (1997) have reported that the kinetics for 7-ethoxy-4-trifluoromethycoumarin O-deethylation with the recombinant CYP2B6, as well as in human liver microsomes, display a distinctive “hook,” suggesting an allosteric activation mechanism in the Eadie-Hofstee plots, and that data best fit the Hill equation. Additionally, the $O$-deethylation of 7-ethoxy coumarin of the Gln172His-substituted mutant protein (516G/H9262) has been successfully crystallized by modification of the hook, suggesting an allosteric mechanism.

The total frequencies of allelic variants of CYP2B6 in populations have been reported to be high (20–33%) (Ariyoshi et al., 2001; Lang et al., 2001; Hiratsuka et al., 2002). These amino acid substitutions have been seen alone or combination with other point mutations, meaning that the coexistence of multiple CYP2B6 variants may also complicate the interindividual differences in xenobiotic metabolism.

In conclusion, we expressed amino acid-substituted enzymes of six CYP2B6 variants as well as wild-type CYP2B6 in COS-1 cells, and examined their enzymatic properties using 7-ethoxy-4-trifluoromethycoumarin O-deethylation. The protein levels of the variant CYP2B6s were slightly lower probably due to the decreased stability of the proteins. The values of $V_{max}$ and $V_{max}/K_m$ of CYP2B6.4, CYP2B6.6, and CYP2B6.7 having a Lys262Arg substitution on the basis of the CYP2B6 protein level were significantly higher than those for CYP2B6.1. These findings may mean that the amino acid at position 262 is a significant residue in the CYP2B6 function and that polymorphic alleles of CYP2B6 cause the variations in the metabolic ability toward xenobiotics.

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References


