Developmental Changes in Human Liver CYP2D6 Expression

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ABSTRACT:

Within the human cytochrome P450 family, specific forms show developmental expression patterns that can affect drug clearance, efficacy, and safety. The objective of this study was to use dextromethorphan O-demethylase activity and quantitative Western blotting to identify CYP2D6 developmental expression patterns in a large (n = 222) and developmentally diverse set of pediatric liver samples. Immunodetectable levels of CYP2D6 protein determined for selected samples across all age categories showed a significant correlation with the corresponding dextromethorphan O-demethylase activity. Of gender, ethnicity, postmortem interval, and genotype, only increasing gestational age was associated with CYP2D6 activity and protein content in prenatal samples. In contrast, both age and genotype were associated with CYP2D6 expression in postnatal samples. CYP2D6 expression in liver samples from neonates less than 7 days of age was higher than that observed in first and second trimester samples, but not significantly higher than third trimester fetal samples. In contrast, expression in postnatal samples greater than 7 days of age was substantially higher than that for any earlier age category. Higher CYP2D6 expression also was observed in liver samples from Caucasians versus African Americans. Finally, using phenotype categories inferred from genotype, CYP2D6 activity was higher in postnatal samples predicted to be extensive or intermediate metabolizers versus poor metabolizers. These results suggest that age and genetic determinants of CYP2D6 expression constitute significant determinants of interindividual variability in CYP2D6-dependent metabolism during ontogeny.

The cytochromes P450 enzyme superfamily is the predominant system responsible for xenobiotic metabolic clearance in animals, including humans. These enzymes are characterized by tremendous diversity in substrate specificity, regulation, and expression that results in significant interindividual variability in metabolism and clinical pharmacological response (Wrighton and Stevens, 1992; Guengerich and Parikh, 1997). In adults, differences in cytochrome P450 expression and attributable metabolism are determined by genetic variability and, to a lesser extent, by enzyme induction due to drug therapy, xenobiotic exposure, or dietary factors (Guengerich et al., 1997; Ozdemir et al., 2000; Lamba et al., 2002). Understanding factors influencing metabolism in a pediatric population presents additional challenges as ontogenesis, or the change in enzyme expression status as a function of age, is superimposed upon any genetic and/or environmental factors. Where metabolic clearance is defined largely by a single cytochrome P450, this phenomenon can result in pediatric patients who present as phenotypically similar but actually have disparate genotypes that have yet to be realized.

Although CYP2D6 accounts for 2% or less of the hepatic cytochrome P450 content (Shimada et al., 1994), this enzyme is responsible for the oxidative metabolism of approximately 12% of clinically relevant drugs (Williams et al., 2004). Furthermore, the CYP2D6 locus exhibits a high degree of genetic polymorphism that clearly has been linked to the variable pharmacological response to a variety of analgesic, cardiovascular, and antidepressant drugs (Heim and Meyer, 1992; Eichelbaum et al., 1997; Flores and Mogil, 2001). For these reasons, this enzyme has been the target of extensive study. Initial phenotyping studies showed that CYP2D6 activity is bimodally distributed, with poor metabolizer status attributable to approximately 7% of the Caucasian population. As the number of identified CYP2D6 allelic variants has increased to more than 80 and the ethnic diversity of subjects studied has expanded, the difficulty in accurately predicting the metabolic capacity from genotype has become apparent (Griese et al., 1998; Zanger et al., 2001). These discrepancies, in combination with the delay in the expression of phenotype during prenatal and postnatal development, may lead to errors in clinical practice unless the application of genetic medicine is balanced with a complete understanding of CYP2D6 ontogeny. This concern is particularly relevant, as pharmaceutical companies have recently increased the scope of pediatric clinical trials in response to the recognition of the unmet medical need (Milne, 1999) and the objectives outlined in the Pediatric Exclusivity Act (Food and Drug Administration, 1998) and Best Pharmaceuticals for Children Act (2002).

Because of ethical and logistical concerns related to performing in vivo studies in pediatric patients, relatively few studies have been conducted on changes in CYP2D6 expression during early development. However, the use of in vitro techniques to define human drug metabolism enzymology and associated interindividual variability is well established scientifically and endorsed from a regulatory per-

ABBREVIATIONS: PCR, polymerase chain reaction; PMI, postmortem interval.
TABLE 1
Liver donor demographics

<table>
<thead>
<tr>
<th>Age at death (yr)</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Postnatal (n = 160, months)</td>
<td>3.7 (0–215)</td>
</tr>
<tr>
<td>Fetal (n = 62, weeks gestational age)</td>
<td>20.0 (11.7–41)</td>
</tr>
<tr>
<td>Time between death and tissue processing (PMI) (hr)</td>
<td>17.0 (1–41)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>133 (60)</td>
</tr>
<tr>
<td>Female</td>
<td>78 (35)</td>
</tr>
<tr>
<td>Unknown</td>
<td>11 (5)</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
</tr>
<tr>
<td>Northern European</td>
<td>93 (42)</td>
</tr>
<tr>
<td>African</td>
<td>83 (37)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>19 (9)</td>
</tr>
<tr>
<td>Other</td>
<td>4 (2)</td>
</tr>
<tr>
<td>Unknown</td>
<td>23 (10)</td>
</tr>
</tbody>
</table>

Values are presented as median (range) or n (%).

The objective of this study was to further define CYP2D6 ontogeny by the characterization of marker dextromethorphan O-demethylase activity, CYP2D6 protein levels, and CYP2D6 genotype in a large liver tissue set that included both fetal and pediatric liver samples (Koukouritaki et al., 2002).

Materials and Methods

Materials. Human liver samples were obtained from the Brain and Tissue Bank for Developmental Disorders, University of Miami (National Institute of Child Health and Human Development, NOI-HD-8-3283 and NOI-HD-8-3284, respectively) and the Central Laboratory for Embryology Research, University of Washington (National Institute of Child Health and Human Development, HD-00836) as described previously (Koukouritaki et al., 2002) and in accordance with ethical guidelines. All samples were stored at ~80°C. A total of 222 liver samples representing ages from 8 weeks’ gestation to 18 years’ postbirth were analyzed. Donor estimated gestational age, postnatal age, and cause of death were available for all samples (Table 1). Samples from individuals succumbing to disease processes that might have involved liver damage or disease were excluded from the tissue bank. Information on sex and ethnicity were provided for some samples. Adult pooled human liver microsomes were purchased from Xenotech, LLC (Kansas City, KS). Human cDNA-expressed CYP2D6 Supersomes and peptide-specific monoclonal antibody against CYP2D6 were purchased from BD Gentest (Woburn, MA), and horseradish peroxidase-conjugated sheep antirabbit IgG, nitrocellulose membrane, and enhanced chemiluminescence Western blotting kits were obtained from GE Healthcare (Princeton, NJ). Ten percent precast polyacrylamide gels were from Bio-Rad (Hercules CA). Dextromethorphan and dextrorphan were purchased from Sigma/RBI (St. Louis, MO). DNAzol reagent and QIAamp DNA Mini kits were obtained from Invitrogen (Carlsbad, CA) and SQIAGEN (Valencia, CA), respectively. Oligonucleotide primers for both DNA amplification and single-base extension were from Integrated DNA Technologies, Inc. (Coralville, IA). The ExoSAP-IT mix, containing both exonuclease I and shrimp alkaline phosphatase, and shrimp alkaline phosphatase alone were purchased from United States Biochemical Corp. (Cleveland, OH). CEQ SNP-Primer Extension kits were from Beckman Coulter, Inc. (Fullerton, CA). All other reagents and materials were obtained from common commercial sources.

Preparation of Hepatic Microsomal Suspensions. Microsomal suspensions from individual liver samples were prepared, and protein concentrations were determined as described previously (Koukouritaki et al., 2002).

Dextromethorphan O-Demethylase Activity Determination. CYP2D6-dependent dextromethorphan O-demethylation was measured using 25 μg of human liver microsomal protein, 20 μM dextromethorphan (saturating based on reported kinetic values), and 50 mM phosphate buffer (pH 7.4) in a total volume of 0.2 ml. Incubations were performed in a 96-well cluster tube format. Chilled samples were preincubated for 3 min at 37°C before the addition of NADPH to a final concentration of 1 mM. After 30 min of incubation, reactions were terminated with 50 μl of cold acetonitrile. All activity assays were performed on the same day and in duplicate, and assays on each sample were performed a minimum of two times. Each 96-well format also contained samples with varying amounts of the metabolite standard, dextrorphan, in addition to incubations of dextromethorphan with pooled human liver microsomal samples to assess variability across the 96-well format.

Dextromethorphan was quantitated by high-pressure liquid chromatography using Shimadzu LC-10AD pumps (Shimadzu, Kyoto, Japan), an Htc PAL autosampler (Leap Technologies, Carrboro, NC) equipped with a narrow-bore reverse-phase column (Aquasil C-18 50 × 2.1 mm, 34 μm) and Thermo Electron Corporation, West Palm Beach, FL.) and using a step gradient (5% B for 0.5 min to 95% B in 1.0 min and held for 24.5 min) at a flow rate of 0.5 ml/min. The mobile phase consisted of solvent A, water with 0.1% (v/v) formic acid, and solvent B, acetonitrile (10:90) with 0.1% (v/v) formic acid. The effluent from the high-pressure liquid chromatography was introduced into a Sciex API 4000 triple quadrupole mass spectrometer (MDS Sciex, Thornhill, Ontario, Canada) via a Turbo IonSpray source. Mass spectrophotometric analysis was performed in the positive ion mode using multiple reaction monitoring at unit resolution for the transition 258 → 157 m/z. Instrument parameters were optimized for the product ion at m/z 157. Needle voltage was 4500 and the collision energy was set to 53 eV with 5 mtor nitrogen as the collision gas. The dextromethorphan limit of detection was 0.007 pmol. Unknown dextromethorphan concentration amounts were read from a linear regression of known standards (0.12 pmol–1.00 nmol) using 1/x weighting. All unknown samples fell within the range of concentrations used to prepare the standard curve, the lower limit of which gave a signal 10-fold higher than background. All replicates were averaged and calculations of activity (nanomoles per minute per milligram) were performed in Excel (Microsoft, Seattle, WA).

CYP2D6 Immunooquantitation. Fetal, pediatric, and adult human liver microsome samples were analyzed for immunodetectable CYP2D6 essentially as described by Koukouritaki et al. (2002). Briefly, microsomal protein suspensions (1 μg of protein) along with known amounts of cDNA-expressed CYP2D6 Supersomes (2.5-fmol of CYP2D6 protein) were fractionated by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and transferred to nitrocellulose membranes. After blocking and washing, the membranes were incubated with the primary MAB-2D6 antibody at 1:5000 dilution and secondary horseradish peroxidase-conjugated sheep anti-mouse IgG at 1:2000 dilution at room temperature for 1 h. The antibody-bound CYP2D6 protein was detected by chemiluminescence using ECL kits (GE Healthcare). After film development, the optical densities of the immunoreactive CYP2D6 bands were read from a Kodak DC120 digital camera and digitized using Digital Science 1D v3 software (Kodak IBI, New Haven, CT). CYP2D6 concentrations in liver samples were determined by linear regression using the known amounts of CYP2D6 Supersome samples as standards (SigmaPlot V 9.01 and SigmaStat V 3.11; SysStat Software, San Jose, CA). If the amount of CYP2D6 in 1 μg of sample liver microsomes was outside of the standard curve range, the immunooquantitation was repeated using 10 μg of microsomal protein.

DNA Isolation and Genotype Analysis. Genomic DNA was isolated from...
Dextromethorphan O-demethylation has been well documented as a sensitive and selective CYP2D6 probe (Schmid et al., 1985) and, as such, was used along with mass spectrometry detection to determine CYP2D6 activity in 222 fetal and pediatric liver microsome samples. As shown in Fig. 1A, activity for first and second trimester fetal samples was either undetectable (41 of 46 samples) or very low (< 0.01 nmol/min/mg of microsomal protein). However, 10 of 14 samples (71%) from the third trimester fetal group showed measurable dextromethorphan O-demethylase activity. After birth, a substantial increase in enzyme activity was observed compared with that for the prenatal age group (Fig. 1B) that did not appear to change further as a function of postnatal age (Fig. 1C).

Given potential issues with donor sample quality attributable to the degradation of enzymes as a function of postmortem interval (PMI), immunodetectable levels of CYP2D6 protein were determined to assess whether measurements of enzyme activity reflected hepatic CYP2D6 protein levels. No correlation was observed between either dextromethorphan O-demethylase activity or CYP2D6 protein content and the PMI (data not shown), consistent with other studies using these same tissue samples (Stevens et al., 2003; Koukouritaki et al., 2004). In contrast, there was good correlation between dextromethorphan O-demethylase activity and CYP2D6 protein in individual tissue samples (Fig. 2) (r² = 0.686, P < 0.001).

In addition to PMI, the relative contributions of age, gender, ethnicity, and genotype on dextromethorphan O-demethylase activity and
CYP2D6 protein levels initially were evaluated. For the fetal samples, increasing gestational age was the only factor significantly associated with increasing dextromethorphan O-demethylase activity and CYP2D6 protein \( (P < 0.001 \text{ and } P = 0.008, \text{ respectively}) \) (Table 3). Among the tissue samples from individuals born prematurely, increasing postnatal age was associated with increasing CYP2D6 protein content \( (P = 0.005) \) but only marginally associated with increasing dextromethorphan O-demethylase activity \( (P = 0.086). \) In contrast, for the postnatal samples, both age and genotype were significantly associated with increasing dextromethorphan O-demethylase activity \( (P = 0.018 \text{ and } P = 0.003, \text{ respectively}) \), whereas age \( (P < 0.001) \), genotype \( (P = 0.009) \), and ethnicity \( (P = 0.007) \) were associated with increasing CYP2D6 protein (Table 3).

Based on this preliminary analysis and visual inspection of the scatter plots (Fig. 1), both dextromethorphan O-demethylase activity and CYP2D6 protein content were grouped into one of four age groups: gestational age <26 weeks (first and second trimester), gestational age 26 to 40 weeks (third trimester), postnatal ages 0 to 7 days, and postnatal ages >7 days to 18 years (Fig. 3, A and B). Because genotype was an important covariate in determining expression in the postnatal sample set, predicted poor metabolizers (Table 2) were excluded from the analysis of postnatal samples. Attempts were made to subdivide the >7 days to 18 years group into more defined age brackets, e.g., >7 days to 28 days, >28 days to 6 months, and >6 months to 18 years. However, no statistical differences were observed among these latter categories (data not shown). Although there is a trend for increased activity and CYP2D6 protein content between the grouped first and second trimester and third trimester liver samples, there was no statistical difference between these age groups. There also was no statistical difference in CYP2D6 protein content or dextromethorphan O-demethylase activity between third trimester liver samples and tissues from neonates less than 7 days of age. The difference between dextromethorphan O-demethylase activity and CYP2D6 protein in neonates less than 7 days of age and the activity observed in first and second trimester fetal samples (10–26 weeks gestational age) were significant, but not compared with third trimester fetal samples. In contrast, there was a highly significant difference between both CYP2D6 activity and protein in the postnatal samples >7 days to 18 years of age compared with either fetal age group or the neonatal 0- to 7-day age group. The role of ethnicity in expression of hepatic CYP2D6 also was examined for the postnatal age group. Because of the relatively small number of Hispanic samples in the data set, the analysis of the postnatal samples was limited to Caucasians and African Americans. Among these two postnatal groups, the observed activity in African Americans was less than that observed in Caucasians (Fig. 4). Among all postnatal pediatric samples, the observed median activity of 0.032 nmol/min/mg of microsomal protein was considerably less than that determined in a pooled sample of adult liver microsomes (0.168 nmol/min/mg of microsomal protein).

Based on the stepwise linear regression, genotype also contributes significantly to the differences in dextromethorphan O-demethylase activity in the postnatal age groups. When the postnatal samples were grouped based on predicted phenotype (Table 2), the observed dextromethorphan O-demethylase activity of the poor metabolizer phenotype group was significantly less than that for either the intermediate or extensive metabolizer phenotype groups (Fig. 5). However, the difference between the intermediate and extensive metabolizer phenotype groups was not statistically significant.

**Discussion**

Low levels of CYP2D6 protein and activity were observed in a small percentage of first or second trimester fetal liver samples. Although the percentage of samples exhibiting detectable protein and activity increased in third trimester fetal liver samples, the values remained no greater than 3 to 5% of the levels observed in adult liver. CYP2D6 protein and activity remained similar to third trimester fetal levels during the 1st week of life, but then increased significantly thereafter. Furthermore, there were no significant differences in either protein or activity with increasing age after 1 week postnatal age. These data are consistent with the in vivo longitudinal data reported by Blake et al. (2007) who suggested that the phenotype reflects the CYP2D6 genotype by 2 weeks of age. The low fetal levels of CYP2D6-dependent activity and protein also are consistent with the results of Treluyer et al. (1991), as was the lack of difference between CYP2D6 activity and protein in the 1st week of life versus the third trimester. Because CYP2D6 protein was independent of gestational age in their sample set, Treluyer et al. (1991) concluded that there was an important contribution from a birth-dependent control process. Similar to the conclusion of these authors, of the factors examined (estimated gestational age, postnatal age, postmortem interval, gender, and ethnicity), only postnatal age was significantly associated with increasing CYP2D6 activity, consistent with a major contribution from an unidentified birth-dependent process.

Johnson et al. (2008) have argued that adjustment of the in vivo data reported by Blake et al. (2007) for maturation of renal function would suggest a gradual increase of hepatic CYP2D6 activity such that adult enzyme levels would not be achieved until approximately 6 months of age. However, the initial assessment of the data reported herein would be more consistent with a rapid rise in hepatic CYP2D6 activity because there was no discernible increase in activity in liver samples beyond 1 week after birth. Yet, the median activity for the >7 days to 18 years age group of 0.033 nmol/min/mg of protein is at the low range of reported values for adult human liver microsomes (Pearce et al., 1996; Gorski et al., 1999) and is considerably lower than the activity determined for a pooled adult human liver microsomal sample (0.168 nmol/min/mg). This observation is probably due to the purposeful weighting of this sample set with earlier ages. The median age of all postnatal samples was only 3.7 months (Table 1) and that for the >7 days to 18 years age group was only 5.4 months.
TABLE 3
Factors associated with increasing CYP2D6 activity or protein in fetal and postnatal human liver microsomal samples

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Dependent Variable</th>
<th>Factor</th>
<th>Standardized β</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal</td>
<td>Activity</td>
<td>Increasing gestational age</td>
<td>0.601</td>
<td>4.452</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Protein</td>
<td>Increasing gestational age</td>
<td>0.640</td>
<td>3.116</td>
<td>0.008</td>
</tr>
<tr>
<td>Premature</td>
<td>Activity</td>
<td>Increasing gestational age</td>
<td>0.365</td>
<td>1.799</td>
<td>0.086</td>
</tr>
<tr>
<td></td>
<td>Protein</td>
<td>Increasing postnatal age</td>
<td>0.688</td>
<td>3.422</td>
<td>0.005</td>
</tr>
<tr>
<td>Postnatal</td>
<td>Activity</td>
<td>Genotype</td>
<td>0.245</td>
<td>3.025</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>Protein</td>
<td>Increasing postnatal age</td>
<td>0.194</td>
<td>2.393</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ethnicity</td>
<td>-0.268</td>
<td>2.756</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Genotype</td>
<td>0.257</td>
<td>2.677</td>
<td>0.009</td>
</tr>
</tbody>
</table>

Thus, these data also would be consistent with a longer period of maturation as suggested by Johnson et al. (2008) but have insufficient power to detect an intermediate level of CYP2D6 activity in tissue samples from older neonates and infants less than 6 months of age. Clearly, the ultimate goal of being able to reliably predict the disposition of and response to the administration of compounds to children of different ages that depend on CYP2D6 for elimination remains to be achieved.

Another possible explanation for the observed low activity in the >7 days to 18 years age group would be sample quality. However, the lack of correlation between either CYP2D6 activity or protein levels and PMI argues against this explanation. Furthermore, for enzymes that show little change with ontogeny, reported protein and activity levels with these same tissue samples were consistent with adult values (Stevens et al., 2003; Koukouritaki et al., 2004), again arguing against sample quality being a significant issue.

Among postnatal liver samples, median dextromethorphan O-demethylase activity was higher in Caucasians (0.043 nmol/min/mg of protein) than in African Americans (0.029 nmol/min/mg of protein). This observation is consistent with the report of lower CYP2D6 functional capacity in black Americans, as measured by the urinary dextromethorphan/dextrorphan metabolic ratio (Gaedigk et al., 2002). Several investigators have reported a population shift toward lower CYP2D6 activity for populations of African descent, probably because of a higher incidence of the reduced function CYP2D6*17 and CYP2D6*29 alleles compared with the alleles in the Caucasian population. Both the CYP2D6*17 and CYP2D6*29 alleles are prevalent in African Americans, with frequencies of 21 and 7%, respectively (Bradford, 2002), with even higher frequencies in African populations in which there is less admixture with other ethnic groups (Masimi-rembwa et al., 1996; Bradford et al., 1998). These genetic differences probably explain the differences observed in the current study in that the allelic frequencies of the CYP2D6*17 and CYP2D6*29 alleles were 20.9 and 7.2%, respectively, in the liver samples from African-American donors in contrast to 1.3 and 0%, respectively, in the liver samples from Caucasian donors. However, extrapolation of these genotypes to phenotypes is complicated by evidence of altered substrate specificity for CYP2D6*17 and CYP2D6*29, such that the concordance between phenotype is dependent upon the probe substrate used and the genotypic composition of the ethnic population under investigation (Wenerholm et al., 1999, 2001). CYP2D6 probe-dependent analysis was not feasible for the current study because of constraints with tissue sample amounts.

CYP2D6 is highly polymorphic with more than 80 known alleles, several of which have a significant functional impact. Although a large number of alleles have been identified, many of these are relatively rare. Using the long-range PCR DNA amplification approach described by Gaedigk et al. (2002) to detect gene duplication and deletion together with multiplexed single-base extension to detect the presence or absence of 11 single nucleotide polymorphisms, it was possible to identify 14 CYP2D6 alleles that account for greater than 90% of the variability observed in the population groups included in this study (Bradford, 2002; Gaedigk et al., 2002, 2007). Consistent
with the concept that during early life stages, individuals must mature into a phenotype consistent with genotype, genetic variability was not associated with CYP2D6 protein or dextromethorphan O-demethylase activity in fetal samples. However, both age and genotype were strongly associated with CYP2D6 protein content and activity in the postnatal liver samples with significantly higher activity observed in samples predicted to be extensive or intermediate metabolizers versus those predicted to be poor metabolizers. Also consistent with this concept, the percentage of samples with nondetectable dextromethorphan O-demethylase activity decreased from 87% in first or second trimester samples to 29 to 38% in third trimester or 1-week postnatal samples, respectively, to only 13% in tissue samples from individuals older than 7 days of age. The latter percentage is similar to the expected percentage of poor metabolizers in the study population. Combined with the inability to discern significant increases in either CYP2D6 protein or activity in postnatal samples from individuals older than 7 days of age, these data suggest that CYP2D6 variability in individuals older than 1 to 2 weeks of age largely is due to genetic variability.

References


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