ABSTRACT:
Genotyping of the highly polymorphic cytochrome P450 2D6 (CYP2D6) permits a gross classification of individual phenotype (viz. ultra-rapid, extensive, and poor metabolizers). It does not, however, provide a precise prediction of CYP2D6 activity, particularly in an individual possessing at least one functional CYP2D6 allele. It has been suggested that the level of mRNA expression or enzyme activity in lymphocytes, isolated from blood, could potentially provide a better quantitative estimate of in vivo hepatic enzymatic activity in human subjects. Although short sequences of CYP2D6 mRNA have been detected in human lymphocytes by reverse transcriptase-polymerase chain reaction (RT-PCR) that suggests the potential use of lymphocyte RNA as a readily accessible biomarker, it is not known whether a functional enzyme is expressed in human lymphocytes. In this study, human lymphocyte activity was assessed with a CYP2D6-specific, high-turnover probe substrate that is severalfold more sensitive than traditional markers of CYP2D6 (e.g., dextromethorphan). CYP2D6 catalytic activity could not be detected in homogenates of human lymphocytes, even at high protein concentrations and with supplementation of enzyme cofactors. Further RT-PCR analysis of lymphocytes collected from eight human donors revealed the presence of only a fragment, but not the complete transcript, of CYP2D6 mRNA. Northern blot RNA transcript analysis also failed to indicate the presence of the full-length transcript in lymphocytes. Collectively, these data indicate that human lymphocytes express neither the full-length CYP2D6 mRNA transcript nor functional enzyme activity. Therefore, the utility of lymphocytes as a functional biomarker for CYP2D6 enzyme activity is not clear at present.
Experiments to detect in vitro CYP2D6 activity

In Table 1, the substrate concentration, lymphocyte protein concentration, supplemented incubation time, amount of metabolite formed, and detection method are presented. The data represent the mean of duplicate measures.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration</th>
<th>Lymphocyte Protein Concentration</th>
<th>Supplemented</th>
<th>Incubation Time</th>
<th>Amount of Metabolite Formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextromethorphan</td>
<td>12 µM</td>
<td>0–5.0 mg/ml</td>
<td>None</td>
<td>0–45 min</td>
<td>N.D.</td>
</tr>
<tr>
<td>R-568</td>
<td>2 µM</td>
<td>0–5.0 mg/ml</td>
<td>bs and reductase</td>
<td>30 min</td>
<td>N.D.</td>
</tr>
<tr>
<td>R-568</td>
<td>2 µM</td>
<td>1.2 mg/ml</td>
<td>bs and reductase</td>
<td>30 min</td>
<td>N.D.</td>
</tr>
<tr>
<td>R-568</td>
<td>2 µM</td>
<td>2.3 mg/ml</td>
<td>0.4 pmol CYP2D6</td>
<td>5 min</td>
<td>6.12</td>
</tr>
<tr>
<td>R-568</td>
<td>2 µM</td>
<td>1.2 mg/ml</td>
<td>0.4 pmol CYP2D6</td>
<td>5 min</td>
<td>4.64</td>
</tr>
<tr>
<td>R-568</td>
<td>2 µM</td>
<td>0 mg/ml</td>
<td>0.4 pmol CYP2D6</td>
<td>5 min</td>
<td>9.230</td>
</tr>
<tr>
<td>N.D., not detected; indicates that metabolite formation was below the detection limit which is 15 fg of M1 (R-568 metabolite) and 50 fg of dextrorphan.</td>
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</tr>
</tbody>
</table>

Chromatography was performed with a YMC Basic C-8 column (100 × 2.1 mm; YMC Inc. USA, Milford, MA) as described above. The flow rate of mobile phase was 0.2 ml/min for 5.5 min and 0.3 ml/min for the next 4.5 min, for a total run time of 10 min per sample. The mass spectrometer was operated in the positive ion electrospray mode. The ions monitored and their associated retention times were as follows: R-568 metabolite M1, m/z 290, retention time 2.9 min; R-568 (parent), m/z 304, retention time 4.6 min; capsaicin (internal standard), m/z 306, retention time 8.7 min. On-column limits of quantitation of both R-568 and M1 (parent and metabolite compounds, respectively) were 15 fg per injection.

Northern Blot Analysis to Detect the CYP2D6 RNA Transcript. A full-length CYP2D6 cDNA transcript was used as the probe to detect CYP2D6 mRNA on the blot. The cDNA transcript was prepared from a plasmid containing the human full-length CYP2D6 cDNA kindly provided to us by Dr. Frank Gonzalez (National Institutes of Health, Bethesda, MD). This probe was biotinylated with the use of the BioNick labeling system (Invitrogen, Carlsbad CA) according to the manufacturer’s instructions. In brief, 1 μg of CYP2D6 cDNA was mixed with the deoxyxynucleoside-5'-triphosphate mix (containing biotin-14-DATP) and enzyme (Escherichia coli DNA polymerase I) and incubated at 16°C for 1 h. The resulting biotinylated probe was purified by precipitation from the reaction mixture with ethanol.

Total RNA was extracted from human donor lymphocytes obtained from the Puget Sound Blood Center and a liver obtained from the University of Washington Human Liver Bank. Both donors were homozygous wild-type for CYP2D6 genotype. RNA extraction from these tissues was accomplished with the use of the Ambion Totally RNA kit (Ambion, Austin, TX).

Diluted aliquots of total RNA (4–24 µg of lymphocyte RNA and 5–20 µg of liver RNA) were electrophoresed in a 1.0% agarose gel containing 2.2 M formaldehyde at 4 V/cm (Sambrook et al., 1989). We also included a lane containing the full-length CYP2D6 mRNA transcript as a positive control. NorthernMax running buffer (Ambion) was used for gel preparation and electrophoresis.

Following electrophoresis, RNAs were transferred to a Biodyne A membrane (Invitrogen) overnight. The nucleic acids were subsequently crosslinked to the membrane by baking at 80°C for 2 h.

To hybridize the biotinylated CYP2D6 probe to the immobilized RNA, we first conditioned the membrane with UltraHyb hybridization buffer (Ambion) for 30 min at 42°C in a hybridization bag. Two hundred picograms of the double-stranded biotinylated probe were denatured and added to the hybridization bag. This was left to hybridize overnight at 42°C with moderate shaking. Unbound probe was then removed with successive washes with standard saline citrate buffer containing SDS.

The blot was detected with the use of the Photostar-Stop detection kit (New England Biolabs, Beverly MA), a chemiluminescence-based detection system, according to the manufacturer’s instructions. The blot was visualized following exposure to X-ray film.

Detection of the Full-Length or Complete CYP2D6 mRNA Transcript with RT-PCR. To detect CYP2D6 mRNA, we relied on a RT-PCR assay with a very high degree of sensitivity. The detection limit of this assay is approximately 500 copies of the full-length CYP2D6 transcript. This assay is similar to previously published methods from our laboratory (Wang et al., 2000; Yang et al., 2002). Total lymphocyte RNA was extracted from human donors provided by the Puget Sound Blood Center. RNA extraction was accomplished
with the use of the Ambion Totally RNA kit (Ambion) and quantitated spectrophotometrically. In every experiment, both positive and negative controls were included to validate results from the RT-PCR assay. Negative controls contained no RNA template, whereas positive controls contained human liver total RNA from a CYP2D6-positive liver.

RT-PCR was performed using the Titan One-Tube RT-PCR System (Roche Diagnostics, Indianapolis IN). In brief, 0 to 1 μg of total RNA from lymphocytes was mixed with a 200 μM concentration of each deoxynucleoside-5'-triphosphate, 2.5 μl of dithiothreitol, 1 μl of the enzyme mix (avian myeloblastosis virus reverse transcriptase, Taq and Tgo DNA polymerases), 0.4 μM concentration of each primer, 6 units of RNase inhibitor, 10 μl of 5× RT-PCR buffer, and sterile nuclease-free H2O to a final volume of 50 μl. The primers, of novel design, were as follows: forward 5'-AGT GGC CAT CTT GCT CC, and reverse 5'-CGG GGC ACA GCA CAA AGC. These primers amplified the entire CYP2D6 mRNA transcript and resulted in a 1.5-kb product.

Reactions were carried out in the PerkinElmer model 2400 Thermocycler (Applied Biosystems, Foster City CA) using standard thin-walled reaction tubes. The first step, reverse transcription, was performed for 45 min at 47°C. This was immediately followed by denaturation at 94°C for 2 min, followed by 10 cycles at 94°C for 30 s, 59°C for 30 s, and 68°C for 1.5 min, and 25 additional cycles at a 62°C (instead of 59°C) annealing temperature and an incremental increase of 5-s extension time per cycle. The final extension was performed at 68°C for 7 min.

Following separation of products by electrophoresis on a 2.0% agarose gel, formation of the CYP2D6 product was quantified based on the digitized band intensity of the fluorescence image of the agarose gel stained with ethidium bromide.

Detection of a Truncated CYP2D6 mRNA Transcript and β-Actin with RT-PCR. To detect a truncated CYP2D6 mRNA transcript, we again utilized the Titan One-Tube RT-PCR system as described above. The primers for this reaction were as follows: forward 5'-TGT TCC TGG CGC GCT AT, and reverse 5'-CTC CTC GGT CAC CCA. These primers amplified a 112-bp fragment 356 bp downstream from the start codon and specific for CYP2D6. The target sequence is located internal to the full-length CYP2D6 RNA transcript. Primer design was based on the CYP2D6 sequence deposited in GenBank (accession number M33388.1).

A fragment of β-actin (450 bp) was detected with the use of the following primers: forward 5'-CGT ACC ACT GCC ATC GTG AT, and reverse 5'-GTG TTG GCC TAC AGG TCT TTG, and amplified with conditions identical to those of the short CYP2D6 mRNA fragment. Reaction mixtures and equipment used were the same as those described above for the full-length CYP2D6.
transcript. Again, the same set of positive and negative controls described above was included for both the β-actin and short-fragment reactions.

RT-PCR was carried out as described above with the following exceptions: primer annealing was at 60°C throughout, elongation was for 45 s, and the second cycling step was 35 cycles, not 25. Products of short-fragment CYP2D6 RNA were separated on 2.0% agarose gel stained with ethidium bromide and analyzed as described above based on digitized band intensity of the fluorescence images.

Results

To determine whether lymphocytes express functional CYP2D6 protein, we incubated homogenates of human lymphocytes with the well established CYP2D6-specific substrate, dextromethorphan. We tried to maximize product formation by varying 1) lymphocyte lysate concentrations up to 5.0 mg/ml total protein, 2) dextromethorphan concentrations to 12 µM, and 3) incubation times from 5 to 45 min. None of these attempts led to a detectable formation of metabolite.

To evaluate whether very low CYP2D6 enzyme activity in lymphocytes was not detected due to limited sensitivity of dextromethorphan assay, we turned to a much more sensitive CYP2D6-selective probe, R-568. R-568 is a calcimimetic (Coburn and Maung 2000; Goodman et al., 2000) that has been reported to be a highly sensitive and specific catalytic probe for CYP2D6 enzyme. We have shown recently that R-568 is about 20-fold more sensitive than dextromethorphan for assessing CYP2D6-mediated metabolism (L. McConnachie, K. Kowdley, A. Levy, B. Tung, K. Thummel, B. Phillips, M. Baijai, V. Chi, J. Esmay, D. Shen, and R. Ho, manuscript submitted). However, even with 5 mg/ml of lymphocyte protein and incubation times up to 30 min, no enzymatic oxidation of R-568 was detected (Table 1 and Fig. 1). Because this LC/MS assay for R-568 has a detection limit of 15 fg on-column, we estimated that as little as 2.5 fmol of product would have been detected under our experimental conditions.

We then investigated whether the inability to detect CYP2D6 activity was due to inadequate or absent cofactors and coenzymes necessary for product formation. We systematically supplemented the incubate with cytochrome b5 and NADPH-P450 reductase, which are necessary for P450-mediated catalysis. These cofactors were added in a 1:3:20 molar ratio (CYP2D6/b5/reductase). The amount of coenzymes employed was based on an estimate of a 100-fold lower CYP2D6 protein expression in lymphocytes relative to liver (7 pmol/mg). This ratio is consistent with reports by other investigators (Yamazaki et al., 1999; Venkatakrishnan et al., 2000). The supplementation also included an excess of cofactors. However, cofactor and coenzyme supplementation did not result in detectable metabolite formation (Table 1; Fig. 1). In contrast, when we added a small amount of recombinant CYP2D6 enzyme (0.4 pmol; equivalent to about 1/20th of this enzyme reported to be present in each milligram of liver protein) into the lymphocyte homogenate, the R-568 product (metabolite) M1 was readily detected (Table 1; Fig. 1).

The lack of CYP2D6 catalytic activity is consistent with the results obtained with Northern blot analysis for CYP2D6 RNA. In this experiment, we were only able to detect the CYP2D6 RNA transcript in the human liver. No hybridization of the probe occurred in lanes containing increasing amounts of human lymphocyte RNA (Fig. 2).

We confirmed the negative data obtained with Northern blot analysis with an additional analysis by RT-PCR. Typical results from the RT-PCR experiments are depicted in Fig. 3 (A–C). In lymphocyte sample collected from eight different donors, no full-length (1.5-kb) CYP2D6 mRNA transcript was detectable, even using up to 1 µg of total lymphocyte RNA as a template (Fig. 3C). Based on detection limits of this assay, we estimated that the full-length transcript, if expressed in lymphocytes, must be expressed at fewer than 2500 copies per µg of total RNA. On the other hand, a truncated CYP2D6 RNA fragment (112 bp) found within full-length transcript was readily detectable in the same set of eight donors (Fig. 3B). It should be noted that β-actin was expressed at similar levels and was readily detectable from the same lymphocyte donors (Fig. 3A). Given that RT-PCR is an exquisitely sensitive method of detecting mRNA transcripts, and we have employed up to 1 µg of total RNA in each reaction, it is unlikely that the full-length CYP2D6 transcript is expressed in lymphocytes.

Discussion

Based on immunoblot data, CYP2D6 content in human liver is estimated to be approximately 7 pmol/mg of total liver protein (Shimada et al., 1994). Assuming lymphocyte protein expression is 2 orders of magnitude lower, about 70 fmol of CYP2D6 per mg of
protein could be present in lymphocytes. Under this assumption, 42 to 81 fmol of CYP2D6 would have been present in our lymphocyte homogenates (1.2 and 2.3 mg/ml protein in 0.5-ml incubation volume). These estimates of CYP2D6 protein concentrations are low, but well within the detection limit of our activity assay using R-568. In a separate set of experiments, we verified that the R-568 assay is capable of detecting NPS-1378 [the O-desmethyl metabolite (M1) of R-568] formation from as low as 50 fmol of recombinant CYP2D6 (data not shown). Our failure to detect CYP2D6 activity in human lymphocyte incubations means that either functional CYP2D6 is completely absent, or the amount of CYP2D6 expressed in lymphocytes must be more than 100-fold lower than that in the liver. Even if the latter scenario were to be true, such a low level of enzyme expression in human lymphocytes is unlikely to be practical for use as a routine surrogate marker for liver CYP2D6 activity.

The inability to detect turnover of R-568 in human lymphocyte homogenates could have been due to high protein concentrations masking CYP2D6 activity (e.g., through nonspecific binding of R-568). This interference was ruled out by our ready ability to detect metabolite formation with the addition of 0.4 pmol of recombinant CYP2D6 to lymphocyte homogenates (Table 1). The lack of CYP2D6 enzyme activity in lymphocytes is explained by the absence of full-length RNA transcripts as demonstrated by Northern blot detection and RT-PCR analysis (Fig. 3). Instead, truncated mRNA transcripts were readily detectable (Figs. 2 and 3). Our data agree with previous reports of the presence of short fragments of CYP2D6 mRNA in human lymphocytes (Carcillo et al., 1996; Krovat et al., 2000). However, the detection of a CYP2D6 mRNA fragment does not indicate the presence of a full transcript that is needed for producing a functional enzyme but, rather, the production of truncated transcripts. The present result calls into question the potential utility of lymphocytes as a biomarker. It is very unlikely that the truncated CYP2D6 mRNA fragment, although present in abundance, could be translated into functional protein and reflect regulation of hepatic CYP2D6.

Transcription of CYP2D6 in the liver has been reported to be, at least partially, under control of HNF4 (Zanger et al., 2001; Hara and Adachi, 2002); the associated transcription factors are not present in lymphocytes. There are several reports indicating that although HNF4 plays a role in the regulation of numerous P450s (e.g., Jover et al., 2001), its expression is limited to the kidney, intestine, pancreas, and liver (Peiler et al., 2000). Thus, the expression of CYP2D6 is tissue-specific; lymphocytes, lacking this transcription factor, do not appear to express full-length mRNA transcripts or functional enzyme. It is not known whether there is an immunoreactive CYP2D6 protein expressed in lymphocytes (Krovat et al., 2000).

One previous study has suggested that expression of short-sequence CYP2D6 mRNA in lymphocytes is correlated with in vivo CYP2D6 activity (Carcillo et al., 1996) and that the presence of truncated CYP2D6 mRNA in lymphocytes might be a viable biomarker of hepatic activity (Krovat et al., 2000). However, the present data do not provide a physiological basis for the putative linkage. We have shown that only short fragments of CYP2D6 message are expressed in lymphocytes, and that this expression is independent of total message transcribed. Figure 3, A to C, shows that the abundance of short-fragment product is not related to full-length CYP2D6 product because the bands intensities of liver and lymphocyte short-fragment products are very similar; yet, there are no full-length products for lymphocytes. Therefore, the presence of fragments of CYP2D6 mRNA transcripts in lymphocytes is not necessarily related to transcription to a full-length product or to translation of functional protein. Interestingly, our observation with CYP2D6 transcription in lymphocytes is probably an exception among P450 genes, since several reports have established that there would be measurable expression of CYP1B1, CYP2E1, and CYP1A1 mRNA, and functional CYP1A1 protein (Vanden Heuvel et al., 1993; Raucky et al., 1997; Baron et al., 1998; Dey et al., 2001). It appears that CYP2D6 does not codifferentiate or distribute with the above-mentioned P450 isoenzymes and that lymphocyte expression of P450s is isof orm-specific. The regulatory mechanisms leading to differential tissue expression of cytochrome P450 isoenzymes, particularly their expression in lymphocytes, warrant further exploration.

In summary, we have conducted a thorough investigation of CYP2D6 enzymatic activity and mRNA expression in human lymphocytes. With a highly sensitive CYP2D6 probe substrate, R-568, no enzyme activity was detectable in lymphocytes. These data are supported by the absence of full-length CYP2D6 mRNA transcripts as assessed by Northern blot and RT-PCR analyses. Only truncated transcripts of CYP2D6 mRNA were present in lymphocytes. Together, these data suggest that it is not feasible to use human lymphocyte expression of CYP2D6 as a quantitative biomarker of hepatic CYP2D6 activity.

References


