Characterization of the Novel Defective CYP2C9*24 Allele

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ABSTRACT:

CYP2C9 is involved in the metabolism of more than 100 currently used drugs, corresponding to about 10 to 20% of commonly prescribed drugs (Kircheiner and Brockmoller, 2005). Some of them (e.g., anticoagulant warfarin) have a narrow therapeutic index (Miners and Birkett, 1998). Clinically available warfarin is a racemic mixture, and CYP2C9 is responsible for the metabolism of more potent S-enantiomer (Kaminsky and Zhang, 1997). The human CYP2C9 gene is highly polymorphic (http://www.imm.ki.se/CYPalleles/). The two most common variant alleles in Caucasian populations are CYP2C9*2, with a point mutation in exon 3 (430C>T), and CYP2C9*3, with a point mutation in exon 7 (1075A>C). Both are associated with a decrease in the catalytic activity of the enzyme (Rettie et al., 1994; Sullivan-Klose et al., 1996). It was shown in many studies that the patients with polymorphic alleles require significantly lower doses of warfarin and are more susceptible to bleeding complications than the carriers of two wild-type alleles (Kircheiner and Brockmoller, 2005).

We have previously reported a novel 1060G>A sequence variant in exon 7 of CYP2C9 gene, leading to substitution of acidic amino acid glutamate to basic lysine (E354K). The new allele, designated CYP2C9*24, was present in the heterozygous state in one warfarin-treated patient. To characterize the CYP2C9*24 allele, we expressed the wild-type and CYP2C9.24 protein in a recombinant yeast expression system and a human embryonic kidney (HEK)-293 cell system. Carbon monoxide difference spectra were recorded on dithionite-reduced microsomes, and protein was determined by Western blotting. Transformation with CYP2C9.1 cDNA resulted in detectable CYP2C9 protein in yeast or HEK-293 cells, whereas only small amounts of the protein were detected in yeast transfected with CYP2C9.24 cDNA. A strong differential absorption peak at 450 nm was observed with microsomes of yeast transfected with CYP2C9.1 cDNA, whereas no peak was detected with microsomes of yeast transfected with CYP2C9.24 cDNA or empty pYeDP60 plasmid. These results suggest that CYP2C9.24 may be improperly folded, both in yeast and mammalian cells, resulting in improper heme incorporation and rapid intracellular degradation. The data obtained in the expression systems are consistent with our findings in vivo. In conclusion, we have identified a novel defective CYP2C9 variant allele of potential importance for drug metabolism in vivo.

Materials and Methods

Preparation of CYP2C9 Expression Constructs. The yeast expression vector pYeDP60, which contained the wild-type CYP2C9 cDNA (AstraZeneca, R&D, Umeå, Sweden), was used as a template to generate mutant CYP2C9*24 cDNA. QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to introduce 1060G>A mutation with mutagenic oligonucleotides mut2C9_F (5'-CAGATGCTGTGGTGACAGTCCA-GAGATACATTG-3') and mut2C9_R (5'-CAATGTATCTCTGGACCTTGT-GCACCACAGCATCTG-3'). The CYP2C9*1 and CYP2C9*24 alleles were amplified from pYeDP60 vector using primers that introduced KpnI restriction site before the start codon (5'-ATAAGTTACCAGTGGATCTCCTGTTG-3') and EcoRI site after the stop codon (5'-ATAAGTTACCAGTGGATCTCCTGTTG-3') and EcoRI site after the stop codon (5'-ATAAGTTACCAGTGGATCTCCTGTTG-3'). Fragments were purified by QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA). All the constructs were verified by sequencing.

Expression in Yeast. pYeDP60 containing CYP2C9*1 or CYP2C9*24 cDNA were transfected by lithium acetate procedure in Saccharomyces cerevisiae strain W(R) that overexpresses yeast reductase (Pompon et al., 1996). Yeast cells transfected with empty pYeDP60 were used as a negative control. After 2 to 4 days of incubation at 28°C, the colonies were transferred to a new SGI (Mountain View, CA) plate (1 g/l casamino acids, 7 g/l yeast nitrogen base, 20 g/l glucose, and 20 mg/l tryptophan) making 2- to 3-cm lines and incubated for an additional 2 to 4 days at 28°C until the yeast had grown thick. Then the yeasts were transferred into 20 ml of SGI medium and grown overnight at 28°C until OD_{600 nm} was around 10. The culture was poured into 230 ml of yeast peptone glycerol ethanol medium (10 g/l yeast extract, 10 g/l bacto-peptone, 5 g/l glucose, and 2% ethanol) and grown at 28°C to OD_{600 nm} around 10 to 13. Induction was started by the addition of 28 ml of 20% galactose, and the growth was continued until OD_{600 nm} reached 30 (Pompon et al., 1996).

The yeast cells were recovered by centrifugation, washed once with distilled water, and resuspended in Tris EDTA KCl buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.1 M KCl). Cells were spun down again and resuspended in 2.5 ml of TES buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.6 M sorbitol), with added Complete protease inhibitor mixture (Roche Applied Science, Pentzberg, Germany). The yeast cells were mechanically disrupted using glass beads. Five milliliters of cold TES buffer was added to the crude extract three times, and the supernatant was withdrawn and centrifuged at 15,000g for 10 min at 4°C. The supernatant was then transferred to a new tube and centrifuged at 34,000g for 1 h at 4°C. The pellet, consisting of microsomes, was dissolved in 2.5 ml of cold Tris EDTA glycerol medium (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 20% glycerol) (Pompon et al., 1996). Tris-HCl, pH 7.4, 1 mM EDTA, and 0.6 M sorbitol), with added Complete protease inhibitor mixture (Roche Applied Science, Pentzberg, Germany). The yeast cells were mechanically disrupted using glass beads. Five milliliters of cold TES buffer was added to the crude extract three times, and the supernatant was withdrawn and centrifuged at 15,000g for 10 min at 4°C. The supernatant was then transferred to a new tube and centrifuged at 34,000g for 1 h at 4°C. The pellet, consisting of microsomes, was dissolved in 2.5 ml of cold Tris EDTA glycerol medium (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 20% glycerol) (Pompon et al., 1996).

Results and Discussion

Most of the genes encoding enzymes involved in drug metabolism are genetically diverse. CYP genes, in particular, have higher density of polymorphic sites and higher genetic diversity than any other human genes examined. Among subfamilies involved in phase I drug metabolism, CYP2 displays the highest level of genetic diversity. It is particularly important to predict the function of nonsynonymous sequence variation in CYP genes and to correlate that function to phenotypic observation because of their role in drug metabolism and adverse drug reactions (Solus et al., 2004).

In the present study, we have characterized a rare variant CYP2C9*24 allele that was previously found in one warfarin-
radiation rate were recently reported in variant CYP1B1 allele with N453S substitution (Bandiera et al., 2005).

To further test this hypothesis, we heterologously expressed wild-type and mutated CYP2C9 proteins in mammalian HEK-293 cells. Carbon monoxide difference spectral analysis of the microsomal fraction obtained from transfected HEK-293 cells showed that only wild-type CYP2C9.1 protein was correctly folded (60 pmol/mg microsomal protein) (Fig. 2A), whereas no cytochrome P450 carbon monoxide spectrum was observed for the cells transfected with CYP2C9*24 cDNA or with empty pcDNA3.1 plasmid. Western blot analysis revealed that only cells transfected with wild-type construct produced detectable levels of CYP2C9.1 in all independent replications of transfection (Fig. 2B).

In conclusion, our data suggest that the substitution of acidic amino acid glutamate to basic lysine may lead to incorrectly folded CYP2C9.24 protein that is rapidly degraded in both yeast and mammalian cells. Furthermore, our data support our observations of lower warfarin dose requirements and lower (S)-warfarin clearance in a patient with the CYP2C9*24 allele (Herman et al., 2006).

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