Biochemical Pharmacology, Vol. 56, pp. 243–251, 1998.

ELSEVIER

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(98)00133-6

Comparative Studies on the Catalytic Roles of Cytochrome P450 2C9 and Its Cys- and Leu-Variants in the Oxidation of Warfarin, Flurbiprofen, and Diclofenac by Human Liver Microsomes

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ABSTRACT. *S*-Warfarin 7-hydroxylation, *S*-flurbiprofen 4′-hydroxylation, and diclofenac 4′-hydroxylation activities were determined in liver microsomes of 30 humans of which 19 were wild-type (Arg144 · Ile359), 8 were heterozygous Cys (Cys144 · Ile359), and 3 were heterozygous Leu (Arg144 · Leu359) allelic variants of the cytochrome P450 2C9 (*CYP2C9*) gene. All of the human samples examined contained P450 protein(s) immunoreactive with anti-CYP2C9 antibodies in liver microsomes. Individuals with the Cys144 allele of *CYP2C9* had similar, but slightly lower, activities for the oxidations of these substrates than those of wild-type *CYP2C9*. One of the three human samples heterozygous for the Leu359 allele had very low V_{max} and high K_m values for the oxidation of three substrates examined, while the other two individuals gave kinetic parameters comparable to those seen in the wild-type and Cys144 *CYP2C9*. Reverse transcriptase-polymerase chain reaction analysis, however, showed that all of the three human samples with the heterozygous Leu359 variant were found to express both Ile359 and Leu359 variants at relatively similar extents in liver RNA of three humans. These results suggest that the Cys144 variant of CYP2C9 catalyzes the CYP2C9 substrates at rates comparative to, but slightly lower than, those of wild-type CYP2C9, while the Leu359-allelic variant has slower rates for the oxidation of these drug substrates. Activities for the oxidation of these CYP2C9 substrates in humans with heterozygous Leu359 allele is likely to be dependent on the levels of expression of each of the wild- and Leu-variants in the livers. However, one of the humans with a heterozygous Leu allele was found to have very low activities towards the oxidation of CYP2C9 substrates. The basis of this defect in catalytic functions towards CYP2C9 substrates is unknown. BIOCHEM PHARMACOL **56**;2:243–251, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. CYP2C9; polymorphism; warfarin; flurbiprofen; diclofenac; RT–PCR

P450¶ comprises a superfamily of enzymes that catalyze oxidation of a great number of xenobiotic chemicals such as drugs, toxic chemicals, and carcinogens and a variety of endobiotic chemicals including steroids, fatty acids, prostaglandins, and vitamins [1, 2]. CYP2C subfamily members have been shown to be major P450 forms in mammalian livers and play important roles in the oxidation of clinically used drugs and other xenobiotic chemicals [3–5]. CYP2C9 has been reported to be present at levels of about 20% of total P450 in human liver microsomes and to catalyze the oxidation of diverse xenobiotic chemicals such as tolbutamide, warfarin, flurbiprofen, phenytoin, hexobarbital, and diclofenac [4, 5].

At least three genotypes in the *CYP2C9* gene, namely Arg144 · Ile359 (wild-type), Cys144 · Ile359 (Cys allele), and Arg144 · Leu359 (Leu allele), have been found in humans by analyzing genomic DNA isolated from human blood and tissue samples [6–9]. Ethnic-related differences exist in the occurrence of the *CYP2C9* genetic polymorphism where the Cys allele of *CYP2C9* is detected only in Caucasians with a frequency of ~20%, while the Leu allele is determined in Caucasians and Orientals with frequencies of 3–10% [6, 8–10]. Most of the human samples containing variant forms of *CYP2C9* are heterozygous for Cys and Leu alleles [9, 10], while there exist, as very rare cases, the homozygous Leu359/Leu359 variant and the heterozygous Arg144Cys · Ile359Leu variant; in the latter two cases, defective catalytic activities have been suggested in these

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¶ *Abbreviations:* P450 or CYP, cytochrome P450; and RT-PCR, reverse transcriptase–polymerase chain reaction.

Received 13 October 1997; accepted 13 March 1998.

human samples [6]. However, it remains unclear whether or not humans with heterozygous Cys and Leu alleles of *CYP2C9* have slower rates for the oxidation of several CYP2C9 substrates in liver microsomes.

The aim of the present study was to characterize in different samples the catalytic properties of humans who express heterozygous Cys and Leu allele variants of *CYP2C9*. We determined the oxidation activities of several CYP2C9 substrates including *S*-warfarin, *R*,*S*-, *R*- and *S*-flurbiprofen, and diclofenac in liver microsomes of 30 Caucasians genotyped for the *CYP2C9* gene, of which 19 were wild-type (Arg144 · Ile359), 8 were heterozygous Cys allele (Cys144 · Ile359), and 3 were heterozygous Leu allele (Arg144 · Leu359) [9]. Catalytic activities of these CYP2C9 substrates by cDNA-expressed CYP2C9 and its mutant proteins in yeast microsomes were also determined and compared with those catalyzed by human liver microsomes. Results of RT–PCR analysis of the expression of Ile359 and Leu359 in humans heterozygous for the Leu allele are reported.

MATERIALS AND METHODS

Chemicals

R,*S*-Flurbiprofen, *R*,*S*-warfarin, and sodium diclofenac were purchased from the Sigma Chemical Co. *S*-Mephenytoin, *R*- and *S*-warfarin, and 7-hydroxywarfarin were obtained from the Ultrafine Chemicals Co. and 4′-hydroxydiclofenac was from the Gentest Co. The *S*- and *R*-flurbiprofen enantiomers and 4′-hydroxyflurbiprofen were gifts from the Upjohn Co. Other chemicals and reagents used in this study were obtained from sources described previously or were of the highest qualities commercially available [4, 11].

Enzyme Preparation

Human liver samples were obtained from organ donors or patients undergoing liver resection as described previously [9, 11]. In this study, we used only Caucasian samples, since there are no cases of Cys-allele of *CYP2C9* in Japanese samples [9]. Liver microsomes were suspended in 10 mM of Tris–Cl buffer (pH 7.4) containing 1.0 mM of EDTA and 20% glycerol (v/v) [12].

CYP2C9 was purified to electrophoretic homogeneity from human liver microsomes as described [13]. Yeast microsomes containing cDNA-expressed CYP2C9 and its Cys- and Leu-variants were prepared as described [6]. NADPH-P450 reductase was purified from liver microsomes of phenobarbital-treated rabbits by the method of Yasukochi and Masters [14] as modified by Taniguchi *et al.* [15]. Rabbit anti-human CYP2C9 antibodies were prepared as described [13, 16].

Enzyme Assays

Standard reaction mixtures in a final volume of 0.20 mL for the determination of substrate oxidation by P450 enzymes included human liver microsomes (0.1 mg of protein/mL) in 50 mM of potassium phosphate buffer (pH 7.4) containing an NADPH-generating system consisting of 0.5 mM of $NADP^+$, 5 mM of glucose-6-phosphate, and 0.5 U of glucose-6-phosphate dehydrogenase/mL, and various concentrations of substrates [4, 11]. In cases of recombinant CYP2C9 and its variants (5 pmol) in yeast microsomes, rabbit NADPH-P450 reductase (15 pmol) was added to the incubation mixture and the buffer was replaced by 50 mM of potassium HEPES buffer (pH 7.4) containing 0.1 mM of EDTA and 1.5 mM of $MgCl_2$ [6]. 7-Hydroxylation of warfarin enantiomers, 4′-hydroxylation of flurbiprofen enantiomers, and 4′-hydroxylation of diclofenac were determined by the original methods with slight modifications [17–19]. Briefly, the reaction was conducted at 37° for 15 min and terminated by the addition of 10 μL of 60% $HClO_4$ (w/v). Warfarin, flurbiprofen, diclofenac, and their hydroxylated metabolites were separated on HPLC with a C_{18} 5-μm analytical column (4.6 × 150 mm). The elution was conducted with a mixture of 36% CH_3CN (v/v) containing 0.04% H_3PO_4 for the warfarin assay and 45% CH_3CN (v/v) containing 20 mM of $NaClO_4$ (pH 2.5) for the flurbiprofen and diclofenac assays.

S-Mephenytoin 4′-hydroxylation and testosterone 6β-hydroxylation were determined as described previously [9, 20].

P450 contents were estimated spectrally by the method of Omura and Sato [21]. Human P450 proteins in liver microsomes were estimated by coupled sodium dodecyl sulfate-polyacrylamide gel electrophoresis/immunochemical development ("Western-blotting") [22]. Protein concentrations were estimated by the method of Lowry *et al.* [23].

Analysis of Genetic Polymorphism in CYP2C9 *Genes*

The genotyping for the detection of *CYP2C9* genetic polymorphisms was performed according to a method described elsewhere [8, 9]. Briefly, PCR amplification was performed in mixtures (final volume of 25 μL) containing 1× Ex *Taq* buffer, the 4 dNTPs, primers, genomic DNA (25 ng) isolated from human livers, and 0.5 U of Ex *Taq* polymerase (Takara) with a Perkin–Elmer 2400 thermocycler. The DNA fragments thus obtained were digested with *Ava*II (for the detection of Arg144/Cys144) and *Kpn*I (for the detection of Ile359/Leu359), separated by electrophoresis using 10% polyacrylamide gels (Bio-Rad), and visualized after treatment with SYBR™ Green I nucleic acid gel stain (FMC Bioproducts Inc.).

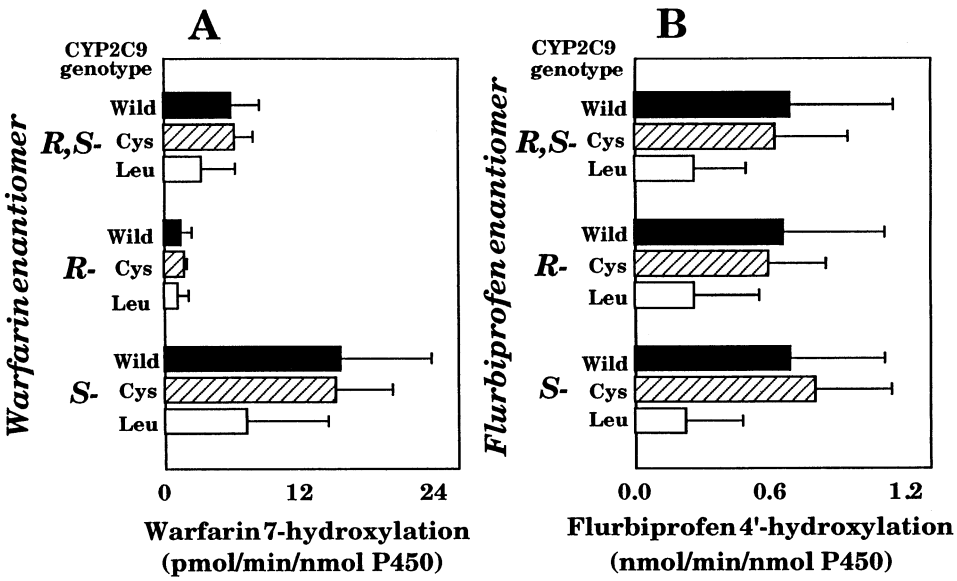

FIG. 1. Oxidation of *R,S-*, *R-*, and *S*-enantiomers of warfarin (A) and flurbiprofen (B) by liver microsomes of 30 human samples genotyped for *CYP2C9* gene into wild-type, heterozygous Cys allele, and heterozygous Leu allele. Substrate concentrations used were 100 μM for warfarin and 10 μM for flurbiprofen. Data are means ± SD.

Expression of Ile359 *and* Leu359 *Variants of* CYP2C9 *Gene in Human Liver RNA*

Total RNA was isolated from livers of different human samples using the guanidinium thiocyanate extraction procedure as described previously [24, 25].

Two mismatched PCR primers, *g* (5′-TGCACGAG GTCCAGAGATGC-3′) and *i* (5′-TGCACGAGGTC CAGAGGTAC-3′), in exon 7 of the *CYP2C9* gene were synthesized for the detection of expression of Ile359 and Leu359 variants, respectively, in liver RNA of different human samples as described [6, 8]. The reverse primer C6*R* (5′-GTAGCACAGAAGTCAGGGAA-3′) in exon 8 was newly synthesized to amplify a 131-bp DNA fragment from liver RNA of different human samples. Primers *g* and C6*R* were used for the detection of an Ile359 variant, and the primers *i* and C6*R* were for a Leu359 variant; in both cases, the RT–PCR amplification product was expected to generate a 131 bp fragment. RT–PCR amplification was carried out in a system (total volume of 50 μL) consisting of a 0.2-μM concentration of each of the primers, 1x reaction buffer, 0.3 mM of dNTPs, 2.5 mM of $Mn(OAc)_2$, 20 U of RNase inhibitor, 5 U of r*Tth* DNA polymerase (RT–PCR high-*Plus*-kit, Toyobo Co.), and 2 μg of RNA. RT–PCR amplification was an initial reaction at 60° for 30 min, at 94° for 2 min, at 94° for 1 min, at 66° for 1.5 min (25, 30, or 40 cycles), and a final extension at 66° for 7 min. The PCR products were digested with *Nsi*I and *Kpn*I for the detection of Ile359 and Leu359 polymorphism, respectively, in different human samples. After gel electrophoresis of the digests, the DNA fragments were visualized with SYBR™ Green I nucleic acid gel stain.

Statistical Analysis

Kinetic parameters for *S*-warfarin 7-hydroxylation, *S*-flurbiprofen 4′-hydroxylation, and diclofenac 4′-hydroxylation by recombinant CYP2C9 and by human liver microsomes were estimated using a computer program (KaleidaGraph program from Synergy Software) designed for nonlinear regression analysis. The correlations between activities of P450-dependent drug oxidation activities in different human liver microsomal preparations were analyzed using a linear regression analysis program (InStat program from GraphPad Software).

Statistical analysis was performed using Student's *t*-test.

RESULTS

Oxidation of Warfarin and Flurbiprofen Enantiomers by Liver Microsomes of 30 *Human Samples*

In this study, we used 30 human samples in which 19 were wild-type (Arg144 · Ile359), 8 were heterozygous Cys (Cys144 · Ile359), and 3 were heterozygous Leu (Arg144 · Leu359) allelic variants of *CYP2C9*. None of the human samples examined were homozygous Cys144/Cys144 or Leu359/Leu359 variants or the heterozygous Arg144Cys · Ile359Leu variant.

We first determined the abilities to oxide *R,S-*, *R-*, and *S*-enantiomers of warfarin and flurbiprofen by liver microsomes of these human samples (Fig. 1). Formation of 7-hydroxywarfarin by human liver microsomes was the highest when *S*-warfarin was used as a substrate, followed by *R,S-* and *R*-warfarin (Fig. 1A). In contrast, 4′-hydroxyflurbiprofen formation was almost the same when *R,S-*, *R-*, and *S*-flurbiprofen enantiomers were used as substrates (Fig. 1B). There were no statistically significant differences in catalytic activities for the oxidations of warfarin and flurbiprofen in liver microsomes of wild-type and Cys- and Leu-variants of CYP2C9. Good correlations ($r > 0.7$) between activities of *S*-warfarin 7-hydroxylation, *R,S-*, *R-* and *S*-flurbiprofen 4′-hydroxylations, and diclofenac 4′-hydroxylation were found in liver microsomes of these 30

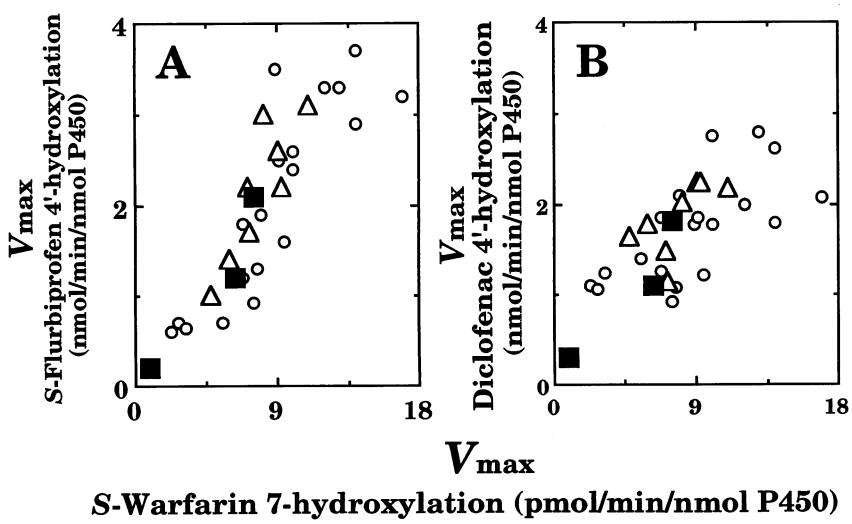

FIG. 2. Relationships of V_{max} values between *S*-warfarin 7-hydroxylation and *S*-flurbiprofen 4′-hydroxylation (A) and diclofenac 4′-hydroxylation (B) activities in liver microsomes of 30 different humans. Genotypes for the *CYP2C9* gene are wild-type (○), heterozygous Cys allele (△), or heterozygous Leu allele (■). Oxidations of *S*-warfarin, *S*-flurbiprofen, and diclofenac were determined in substrate concentration ranges between 1 and 100 μM with 0.05 μM of P450 in the incubation mixture.

human samples (data not shown). Based on these results, we used *S*-warfarin, *S*-flurbiprofen, and diclofenac as substrates for further analysis to characterize CYP2C9 and its variants in human liver microsomes.

Kinetic Analysis of S-Warfarin 7-Hydroxylation, S-Flurbiprofen 4′-Hydroxylation, and Diclofenac 4′-Hydroxylation by Human Liver Microsomes

Kinetic analysis of the oxidations of *S*-warfarin, *S*-flurbiprofen, and diclofenac were examined in liver microsomes of 30 human samples (Figs. 2 and 3). There were correlations in V_{max} values for the oxidation of *S*-warfarin 7-hydroxylation and *S*-flurbiprofen 4′-hydroxylation ($r = 0.85$, Fig. 2A) or diclofenac 4′-hydroxylation ($r = 0.71$, Fig. 2B) by liver microsomes of 30 humans. One human sample (HL-C6) genotyped for the *CYP2C9* gene as heterozygous for the Leu allele had very low activities for these three reactions.

Analysis of K_m values for *S*-warfarin 7-hydroxylation, *S*-flurbiprofen 4′-hydroxylation, and diclofenac 4′-hydroxylation by liver microsomes suggested that only one human sample, HL-C6, had high K_m values as compared with those of humans with wild-type and Cys alleles (Fig. 3). Two other humans heterozygous for the Leu allele had K_m values compatible with those of the other two groups.

Comparison of Liver Microsomal P450 Levels and Activities of a Human Sample, HL-C6, with Those of Other Human Samples

The above results suggested that human sample HL-C6 has characteristics different from other human samples in the oxidation of CYP2C9 substrates in liver microsomes. To show that these defective catalytic activities of liver microsomes are not due to the storage of this liver sample before preparing the microsomal preparations, we determined levels of CYP2C9, CYP2C19, and CYP3A4 and activities of *S*-flurbiprofen 4′-hydroxylation, *S*-mephenytoin 4′-hydroxylation, and testosterone 6β-hydroxylation in different human samples (Table 1). HL-C6 had significant amounts of total P450, CYP2C9, CYP2C19, and CP3A4, and

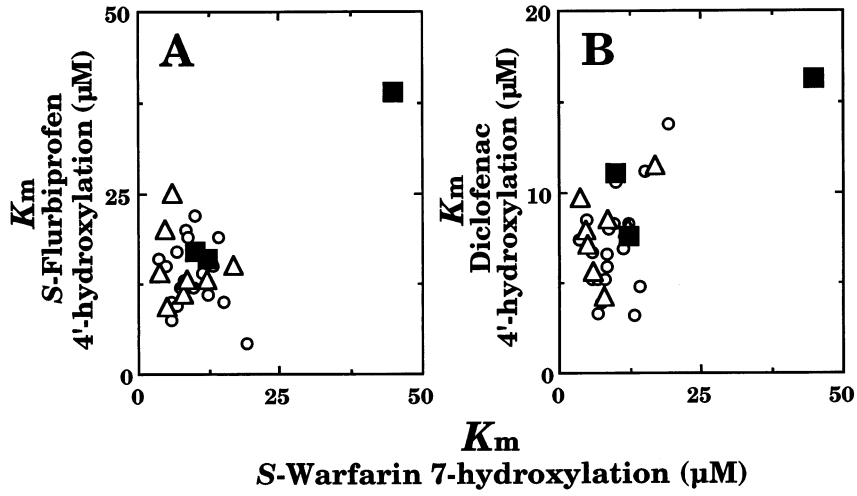

FIG. 3. Relationships of Km values between *S*-warfarin 7-hydroxylation and *S*-flurbiprofen 4′-hydroxylation (A) and diclofenac 4′-hydroxylation (B) activities in liver microsomes of 30 different humans. Genotypes for the *CYP2C9* gene are wild-type (○), heterozygous Cys allele (△), or heterozygous Leu allele (■). Other details are as in the legend to Fig. 2.

TABLE 1. Levels of total P450, CYP2C9, CYP2C19, and CYP3A4 and activities of *S*-flurbiprofen 4′-hydroxylation, *S*-mephenytoin 4′-hydroxylation, and testosterone 6β-hydroxylation in liver microsomes of humans genotyped for the *CYP2C9* gene

No. of human sample	*CYP2C9* genotype	Total P450*	Individual P450 forms†			*S*-Flurbiprofen 4′-hydroxlation	*S*-Mephenytoin 4′-hydroxylation	Testosterone 6β-hydroxylation
			CYP2C9	CYP2C19	CYP3A4			
		(pmol/mg protein)				(nmol products/min/nmol/P450		
C15	Wild	415	89	14.1	58	1.47	0.22	1.39
C19	Wild	690	110	9.2	315	0.57	0.09	4.56
C1	Cys	313	48	2.1	131	1.10	0.04	4.19
C31	Cys	488	55	6.9	182	0.81	0.10	3.72
C6	Leu	657	80	4.1	475	0.01	0.09	7.22
C29	Leu	204	25	3.6	73	0.16	0.11	3.58
C38	Leu	167	35	6.8	51	0.50	0.26	3.06

Substrate concentrations used for *S*-flurbiprofen 4′-hydroxylation, *S*-mephenytoin 4′-hydroxylation, and testosterone 6β-hydroxylation were 10, 400, and 200 μM, respectively. Data are means of duplicated determinations.

*Spectrally determined P450.

†Immunochemically determined P450.

activities of *S*-mephenytoin 4′-hydroxylation and testosterone 6β-hydroxylation in liver microsomes, although the activity for oxidation of CYP2C9 substrates such as *S*-flurbiprofen was very low.

Anti-CYP2C9 antibodies completely inhibited *S*-warfarin 7-hydroxylation, *S*-flurbiprofen 4′-hydroxylation, and diclofenac 4′-hydroxylation catalyzed by liver microsomes prepared from human samples HL-C15 (wild-type of CYP2C9), HL-C31 (Cys allele), and HL-C6 (Leu allele) (Table 2). Sulfaphenazole, a known selective inhibitor of CYP2C9, also suppressed the activities of oxidation of these substrates catalyzed by liver microsomes of humans irrespective of which of these three genotypes of *CYP2C9* were used (Table 2).

Characterization of Genotypes for CYP2C9 Gene in liver DNA and the Gene Products in Liver RNA and Microsomes in Different Individuals

To demonstrate the possible roles of CYP2C9 and its Cys- and Leu-variants in the oxidation of warfarin, flurbiprofen, and diclofenac by human liver microsomes, further analysis was carried out with selected human samples in which two samples (HL-C15 and -C19) were identified to be wild-type (Fig. 4, A and B; after digestion with *Ava*II and *Kpn*I, respectively), two (HL-C1 and -C31) were heterozygous for the Cys allele (Fig. 4A; with *Ava*II), and three (HL-C6, -C29, and -C38) were heterozygous for the Leu allele (Fig. 4B; with *Kpn*I). Heterozygous Ile359/Leu359 in samples HL-C6, C29, and C38 was also determined after digestion with an *Nsi*I enzyme (results not shown). Immunoblotting analysis with anti-CYP2C9 antibodies suggested that all of the seven human samples contained significant levels of CYP2C9 protein in liver microsomes (Fig. 5). It is also noteworthy that all three cDNA-expressed CYP2C9 allelic variants in yeast microsomes cross-reacted with antibodies raised against purified human CYP2C9 with almost equal intensities.

Total RNA was isolated from livers of these human samples and used to determine which Ile359 and Leu359 variants of CYP2C9 are more expressed in livers of humans genotyped for heterozygous Leu359 allele (Fig. 6). Two mismatched PCR primers, *g* and *i*, and the reverse primer

TABLE 2. Effects of anti-CYP2C9 and sulfaphenazole on *S*-warfarin 7-hydroxylation, *S*-flurbiprofen 4′-hydroxylation, and diclofenac 4′-hydroxylation catalyzed by liver microsomes of different humans genotyped for *CYP2C9* alleles

Anti-P450 IgG (mg/nmol P450) or sulfaphenazole (μM)	*S*-Warfarin 7-hydroxylation (pmol/min/nmol P450)			*S*-Flurbiprofen 4′-hydroxylation (nmol/min/nmol P450)			Diclofenac 4′-hydroxylation (nmol/min/nmol P450)		
	C15	C31	C6	C15	C31	C6	C15	C31	C6
None	5.0	4.3	0.21	1.74	1.16	0.067	1.60	1.19	0.17
Preimmune									
10 mg IgG	5.4	4.6	0.22	1.66	1.21	0.074	1.51	1.09	0.18
Anti-CYP2C9									
5 mg IgG	0.3	0.2	0.01	0.19	0.02	0.002	0.06	0.06	0.01
10 mg IgG	0.1	0.1	0.01	0.03	0.01	0.002	0.03	0.04	0.01
Sulfaphenazole									
10 μM	0.6	0.4	0.03	0.12	0.04	0.006	0.17	0.08	0.03
50 μM	0.2	0.1	0.01	0.09	0.02	0.005	0.06	0.03	0.01

S-Warfarin 7-hydroxylation, *S*-flurbiprofen, 4′-hydroxylation, and diclofenac 4′-hydroxylation activities were determined at concentrations of 10 μM with 0.04 μM of human liver microsomal P450. Data are means of duplicate determinations.

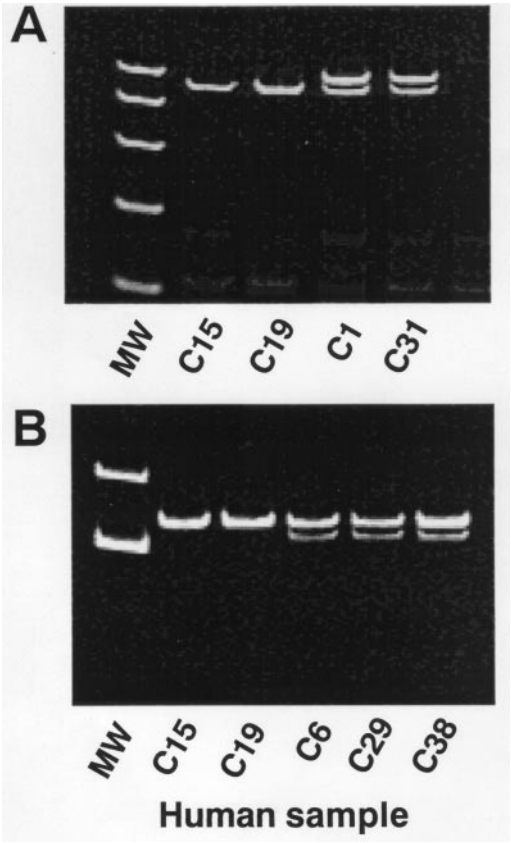

FIG. 4. Polyacrylamide gel electrophoresis of PCR products of the *CYP2C9* gene in human samples. The DNA fragments were digested with *Ava*II (in panel A for the detection of Arg144/Cys144) and *Kpn*I (in panel B for the detection of Ile359/Leu359). In panel A, the detection of the Arg/Cys · Ile *CYP2C9* gene was examined with liver samples C15, C19, C1, and C31, and in panel B, detection of the Arg · Ile/Leu *CYP2C9* gene was analyzed with human samples C15, C19, C6, C29, and C38. In both cases, C15 and C19 were the wild-type *CYP2C9* gene. Markers used were 50, 100, 200, 300, and 400 bp in panel A and 100 and 200 bp in panel B.

C6*R* were used for the detection of expression of Ile359 and Leu359 variants, respectively, as described in Materials and Methods. The number of cycles for the amplification was varied at 25, 30, and 40 (A, B, and C, respectively, in the figure). The DNA fragments thus produced were digested with *Nsi*I or *Kpn*I, and the expression of Ile359/Leu359 variants was determined after polyacrylamide gel electrophoresis (Fig. 6). It was determined that approximately 30 cycles were needed for sufficient amplification (Fig. 6, B and C). After 25 cycles for amplification (Fig. 6A), the DNA fragments from HL-C15 (wild-type) were completely digested with *Nsi*I, but not *Kpn*I, while the fragments from HL-C6, -C29, and -C38 (all were heterozygous Leu type) were digested with both enzymes. These findings were similar when the amplification was increased from 25 to 30 and 40 cycles (Fig. 6, B and C, respectively). Humans heterozygous for the Cys allele showed patterns similar to those with wild-type on RT–PCR analysis for the detection of expression of Ile/Leu359 variants (data not shown).

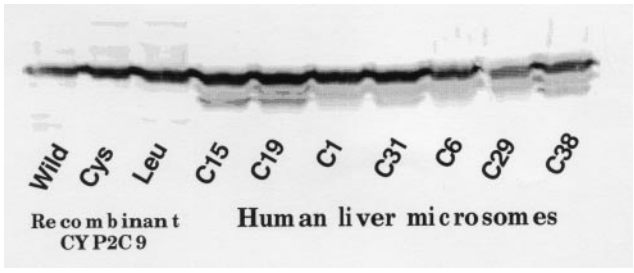

FIG. 5. Immunodetection of CYP2C proteins in yeast microsomes expressing wild (Arg · Ile)-type, Cys · Ile-type, and Arg · Leu-type of human CYP2C9 and in liver microsomes of human samples C15, C19, C1, C31, C6, C29, and C38. Immunodetection analysis was carried out with 1 pmol of yeast microsomal P450 and 5 pmol of human liver microsomal P450 using antibodies raised against purified human liver CYP2C9.

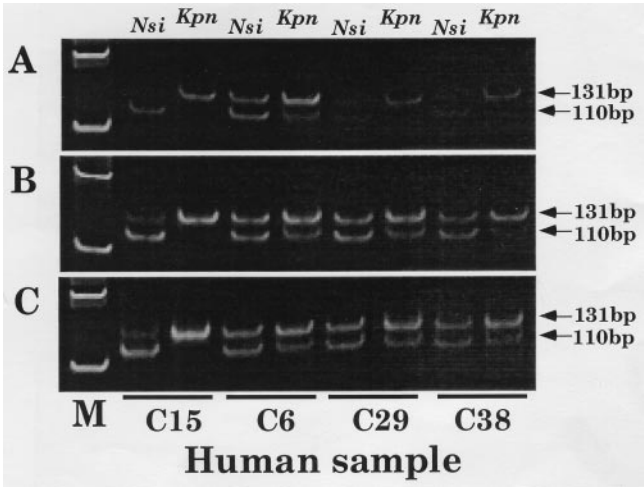

FIG. 6. Polyacrylamide gel electrophoresis of RT–PCR products from liver RNA of different human samples. RT–PCR amplification with liver RNA from human samples C15, C6, C29, and C38 was carried out as described in Materials and Methods, using 25 (A), 30 (B), and 40 (C) amplification cycles. The human sample C15 is a wild-type *CYP2C9* gene, while the other three samples are the heterozygous Leu variant of the *CYP2C9* gene. PCR products were digested with *Nsi*I or *Kpn*I (as indicated by *Nsi* or *Kpn* at the top) for the detection of Ile359 and Leu359 variants, respectively. M, molecular weight markers.

Catalytic Roles of Wild-Type, Cys Allele, and Leu Allele of CYP2C9 *Forms Expressed in Yeast*

Activities for *S*-warfarin 7-hydroxylation, *S*-flurbiprofen 4′-hydroxylation, and diclofenac 4′-hydroxylation were determined in yeast microsomes expressing wild-type CYP2C9 and the two allelic variants, CYP2C9(Cys) and CYP2C9(Leu) (Table 3). Kinetic analysis for oxidation of *S*-warfarin, *S*-flurbiprofen, and diclofenac by recombinant CYP2C9 and its allelic variants suggested that wild-type CYP2C9 had a lower K_m value and a higher V_{max} value than the Leu variant (Table 3). The CYP2C9 Cys variant had a lower V_{max} value than the wild-type CYP2C9 in the oxidations of *S*-warfarin and *S*-flurbiprofen. The Leu and Cys variants expressed in yeast were both less active than

TABLE 3. Kinetic analysis of *S*-warfarin 7-hydroxylation, *S*-flurbiprofen 4′-hydroxylation, and diclofenac 4′-hydroxylation by recombinant *CYP2C9* alleles expressed in yeast

	S-Warfarin 7-hydroxylation			*S*-Flurbiprofen 4′-hydroxylation			Diclofenac 4′-hydroxylation		
CYP2C9 variants	K_m (μM)	V_{max} (pmol/min/nmol P450)	V_{max}/K_m	K_m (μM)	V_{max} (nmol/min/nmol P450)	V_{max}/K_m	K_m (μM)	V_{max} (nmol/min/nmol P450)	V_{max}/K_m
Wild	18 ± 2	220 ± 7	12.2	5.3 ± 0.5	5.9 ± 0.2	1.1	1.8 ± 0.1	12.5 ± 0.2	6.9
Cys allele	22 ± 1	110 ± 3*	5.0	8.3 ± 1.3	3.1 ± 0.4†	0.4	2.5 ± 0.3	11.2 ± 0.4	4.5
Leu allele	53 ± 5*	67 ± 4*	1.3	31 ± 2.5*	2.6 ± 0.1*	0.1	11.1 ± 2.3*	8.1 ± 1.3	0.7

Oxidation of *S*-warfarin, *S*-flurbiprofen, and diclofenac were determined in substrate concentration ranges between 1 and 100 μM with 0.025 μM recombinant P450 in the incubation mixture. Data are means ± range of duplicate determinations.

*†Significantly different from wild type: *$P < 0.01$ and †$P < 0.05$.

wild-type CYP2C9, when the V_{max}/K_m ratio was calculated.

DISCUSSION

The results presented in this study support the previous view that *R*,*S*-, *R*-, and *S*-flurbiprofen 4′-hydroxylation, and diclofenac 4′-hydroxylation, as well as *S*-warfarin 7-hydroxylation, are catalyzed by CYP2C9 in human liver microsomes [17–19, 26–28]. There were good correlations between activities for the oxidation of these CYP2C9 substrates by liver microsomes of 30 human samples examined, and all of these microsomal activities of humans with wild-type and heterozygous Cys- and Leu-allele CYP2C9 were inhibited completely by anti-CYP2C9 antibodies and sulfaphenazole, a selective CYP2C9 inhibitor [29, 30]. Finally, yeast recombinant CYP2C9 catalyzed these oxidation reactions at rates higher than those measured by human liver microsomes.

Recently, CYP2C9 and its Cys and Leu variants have been expressed in several chimeric systems, and the recombinant proteins thus obtained were used to examine their abilities to oxidize prototypic substrates such as tolbutamide, phenytoin, and warfarin [6, 18, 27, 31–36]. It has generally been recognized that the Leu allele of CYP2C9 catalyzes the 6- and 7-hydroxylations of *S*-warfarin, the methyl hydroxylation of tolbutamide, and the *p*-hydroxylation of phenytoin at slower rates (with higher K_m values) than those of the wild type of CYP2C9 [6, 27, 36]. In contrast, the Cys allele of CYP2C9, another variant form, has been reported to have similar catalytic functions to wild-type CYP2C9 in the oxidation of tolbutamide, phenytoin, and warfarin [6, 36], although some studies suggested that this allelic variant has lower activities for *S*-warfarin 7-hydroxylation than those of wild-type CYP2C9 [31, 37].

In this study, the Leu variant of CYP2C9 is shown to have lower turnover numbers and higher K_m values for *S*-warfarin 7-hydroxylation, *S*-flurbiprofen 4′-hydroxylation, and diclofenac 4′-hydroxylation than in the case of those reactions catalyzed by wild-type and Cys alleles of CYP2C9 in yeast microsomes expressing CYP2C9 and its Cys- and Leu-variant forms (Table 3). Differences in the catalytic functions of wild-type and Cys variants were suggested to be minor for the oxidation of *S*-warfarin, *S*-flurbiprofen, and diclofenac in both human liver microsomes and recombinant CYP2C9 enzymes. We only used yeast microsomes expressing CYP2C9 and its variants, and further work will be required using other cDNA-expressed proteins, in order to better understand the basis of catalytic functions of Cys- and Leu-variants of CYP2C9.

Recently variant CYP2C9 forms with the homozygous Leu/Leu allele and heterozygous Arg/Cys and Ile/Leu alleles have been determined to associate with the poor metabolizer phenotypes *in vivo* for the oxidations of tolbutamide and phenytoin in humans [6, 10, 38]. Our previous data using genomic DNA of livers from 39 Japanese and 45 Caucasians suggest that the heterozygous Cys allele mutation is detected only in Caucasians, with frequencies of about 22%, while the Leu allele is detected in both Japanese and Caucasian populations, with frequencies of about 8%. In our human liver samples with 39 Japanese and 45 Caucasians, no samples were found to be homozygous Cys144/Cys144 or Leu359/Leu359 variants or the heterozygous Arg144Cys · Ile359Leu variant of *CYP2C9* [9]. The results are consistent with predictions based on the Hardy–Weinberg law that there are very few cases of homozygous variant forms of *CYP2C9* in humans. Our previous and present immunochemical studies indicated that all of the human samples with wild-type and heterozygous Cys and Leu alleles contain immunoreactive CYP2C9 protein, in liver microsomes, suggesting that the catalytic functions of individuals who express heterozygous Cys and Leu variants should be determined. It is interesting in this connection to note the results of Hall *et al.* [39], who showed that there was a lack of correlation between immunochemically determined CYP2C9 levels and tolbutamide methyl hydroxylation activities in liver microsomes of 12 human samples.

Among three human liver microsomal samples examined with a heterozygous Leu allele, one sample (C6) had very low activities for the oxidations of the CYP2C9 substrates *S*-warfarin, *S*-flurbiprofen, and diclofenac, although these microsomes contained significant levels of total P450 and CYP2C9 protein and activities toward other drug oxidations such as *S*-mephenytoin 4′-hydroxylation (catalyzed by CYP2C19) and testosterone 6β-hydroxylation (cata-

lyzed by CYP3A4). The results of K_m values of liver microsomes of a sample (HL-C6) for the oxidation of *S*-warfarin, *S*-flurbiprofen, and diclofenac were similar to those of recombinant CYP2C9(Leu).

The question arises if this human sample, HL-C6, expressed the Leu359 variant more abundantly than the Ile359 form in liver microsomes. Using RT–PCR analysis with specific oligonucleotide primers, we found that this human sample expressed both Ile359 and Leu359 variants at levels similar to those in the other two human samples expressing heterozygous Leu allele *CYP2C9*, although it is not known at present whether or not the protein is expressed. These results suggest that there were no significant differences in expressions of the Ile359 and Leu359 variants in livers of these humans, although only the HL-C6 individual had defective catalytic functions for CYP2C9 activities. It is not known why this individual (HL-C6) has low activity for CYP2C9 substrates. Conceivably, differing amounts of the Ile359 and Leu359 proteins might have been present in the liver at the time of sampling despite the presence of both mRNAs, or another genetic polymorphism could exist in the *CYP2C9* gene in humans. Although cDNA cloning studies suggest that two other genetic polymorphisms, namely Tyr358Cys and Gly417Asp alleles of *CYP2C9*, exist in humans, and whether or not another genetic polymorphism(s) exists in the *CYP2C9* gene in humans [5, 10], we and others have found no cases of such alleles in either the Japanese or the Caucasian samples examined [8–10].

We recently reported that warfarin 7-hydroxylation activities of human liver microsomes were lower when racemic warfarin, rather than *S*-warfarin, was used as a substrate and that *R*-warfarin (which is oxidized mainly by CYP1A2) noncompetitively inhibits *S*-warfarin 7-hydroxylation activities catalyzed by CYP2C9 in human livers [40]. In contrast, both *R*- and *S*-flurbiprofen were found in this study to be oxidized by the same enzyme (CYP2C9) in human liver microsomes. The findings that turnover numbers for the oxidation of flurbiprofen by liver microsomes of different human samples were very similar when *R*,*S*-, *R*-, and *S*-enantiomers were used as substrates indicate that *R*- and *S*-flurbiprofen do not affect the oxidation of the other isomers when racemic flurbiprofen is used as a substrate.

In conclusion, the present results show that *S*-flurbiprofen 4′-hydroxylation and diclofenac 4′-hydroxylation as well as *S*-warfarin 7-hydroxylation are catalyzed by CYP2C9 in human liver microsomes and that these activities may be affected by genotypes of *CYP2C9* in individual humans. The Cys144 variant had significant activities for these oxidation reactions in recombinant CYP2C9 and in liver microsomes of humans heterozygous for the Cys144 allele. The Leu variant of CYP2C9 appears to have decreased activities towards oxidations of these CYP2C9 substrates, with higher K_m values found in experiments with recombinant CYP2C9 enzymes in yeast microsomes. However, upon analyzing three human samples with heterozygous Leu allele *CYP2C9*, only one sample (HL-C6) was found to have very low activities for the oxidation of *S*-warfarin, *R*- and *S*-flurbiprofen, and diclofenac, even though this sample contained significant levels of CYP2C9 protein in liver microsomes and expressed both Ile359 and Leu359 variants in liver RNA. Further work using more human samples will be required in order to better understand the basis for *CYP2C9* genetic polymorphism in the oxidation of clinically used drugs in humans.

This work was supported, in part, by grants from the Ministry of Education, Science, and Culture of Japan, the Ministry of Health and Welfare of Japan, and the Developmental and Creative Studies from Osaka Prefectural Government and by United States Public Health Service Grants R35 CA44353 and P30 ES00267.

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