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Roles of two allelic variants (Arg144Cys and Ile359Leu) of cytochrome P4502C9 in the oxidation of tolbutamide and warfarin by human liver microsomes

H. YAMAZAKI, K. INOUE and T. SHIMADA*

Osaka Prefectural Institute of Public Health, 3-69 Nakamichi 1-chome, Higashinari-ku, Osaka 537, Japan

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1. Tolbutamide methyl hydroxylation and racemic warfarin 7-hydroxylation activities were determined in liver microsomes of 39 Japanese and 45 Caucasians genotyped for the cytochrome P450 (P450 or CYP) 2C9 gene into three groups, namely the wild-type (Arg144·Ile359), and two heterozygous Cys allele (Cys144·Ile359) and Leu allele (Arg144·Leu359) variants.

2. Good correlations were found between tolbutamide methyl hydroxylation and racemic warfarin 7-hydroxylation activities in liver microsomes of Japanese and Caucasians. Humans with the Cys allele CYP2C9 variant, which was detected in 22% of Caucasians, were found to have similar catalytic rates to those of the wild-type in the oxidations of tolbutamide and racemic warfarin, whereas humans with the Leu allele, which was detected in 8% Japanese and 7% Caucasian samples, had lower catalytic rates than those of other two groups.

3. The rates of 6- and 7-hydroxylation of racemic warfarin were correlated well with those of *S*-warfarin, but not *R*-warfarin, in human liver microsomes.

4. Both human liver microsomes and recombinant CYP2C9 catalysed 7-hydroxylation of *S*-warfarin more extensively than those of *R*-warfarin. K_m's for the 7-hydroxylation of *S*-warfarin were not very different in liver microsomes of humans with these three genotypes. Anti-CYP2C9 antibodies and sulphaphenazole inhibited the 6- and 7-hydroxylation of *S*-warfarin, but not *R*-warfarin, by $> 90\%$ and the methyl hydroxylation of tolbutamide by about 50%.

5. These results suggest that humans with Leu allele of CYP2C9 have lower V_{max}'s for *S*-warfarin 7-hydroxylation and tolbutamide methyl hydroxylation than those with wild-type and Cys allele CYP2C9, although the K_m's are not very different in liver microsomes of these three groups of humans. *R*-warfarin hydroxylation may be catalysed by P450 enzymes other than CYP2C9 in man.

Introduction

Cytochromes P450 (P450 or CYP) in liver microsomes contribute significantly to the biotransformation of xenobiotic chemicals such as drugs, toxic chemicals, and carcinogens and some endobiotic chemicals including steroids, fatty acids, prostaglandins and vitamins (Gonzalez 1989, Guengerich 1991). P450 comprises a superfamily of these enzymes and individual forms of P450 have considerable, but overlapping, substrate specificities (Nelson *et al*. 1996). CYP2C subfamily members have been shown to constitute the major forms of P450 in microsomal fractions of human livers and to play an important role in the oxidation of clinically used drugs and other xenobiotic chemicals (Guengerich and Shimada 1991, Shimada *et al*. 1994).

In man, at least four CYP2C gene products (CYP2C8, CYP2C9, CYP2C18 and

* Author for correspondence.

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CYP2C19) have been shown to be expressed in the liver and, in some instances, in extrahepatic organs (Romkes *et al.* 1991, Goldstein and de Morais 1994). CYP2C9 and 2C19 proteins are the major CYP2C gene products that catalyze oxidation of the prototype substrates tolbutamide, warfarin and *S*-mephenytoin in human liver microsomes (Romkes *et al.* 1991, Goldstein and de Morais 1991). The molecular basis of *CYP2C19* genetic polymorphism has been studied extensively (de Morais *et al.* 1994, Goldstein and de Morais 1994).

Recent studies have shown that there are at least three genotypes of the *CYP2C9* gene, namely Arg144·Ile359 (wild-type), Cys144·Ile359 (Cys allele) and Arg-144·Leu359 (Leu allele) (Wang *et al.* 1995, Haining *et al.* 1996, Sullivan-Klose *et al.* 1996). By analysing genomic DNA isolated from human blood samples it has been found that these allelic variants may be expressed as the functional proteins in human liver microsomes. By analysing genomic DNA isolated from livers of Japanese and Caucasian populations, we have also found that the Cys allele *CYP2C9* is detected only in Caucasians with a frequency of ~22% (all heterozygotes), whereas the Leu allele was determined with frequencies of ~8% (all heterozygotes) in both races (Inoue *et al.* 1997). To determine if these allelic variants catalyse oxidation of several model substrates at similar rates to those of native P450 enzymes, many investigators have used recombinant P450 proteins expressed in heterologous systems into which cDNAs or the modified cDNA sequences of human P450 genes had been introduced (Gonzalez and Korzekwa 1995, Guengerich *et al.* 1996).

The recombinant *CYP2C9*-Leu^{359} allelic variant has been shown to have lower V_{max}'s and higher K_m's for the oxidation of tolbutamide and *S*-warfarin than those catalysed by recombinant CYP2C9 and CYP2C9-Cys^{144} (Veronese *et al.* 1993, Haining *et al.* 1996, Sullivan-Klose *et al.* 1996). Other studies have suggested that the recombinant CYP2C9-Cys^{144} variant catalyses the oxidation of *S*-warfarin 7-hydroxylation at lower rates with lower K_m's than those of wild-type CYP2C9, although such differences were not so significant when tolbutamide methyl hydroxylation is measured (Rettie *et al.* 1994). Our previous studies using liver microsomes of humans who were genotyped for *CYP2C9* have indicated that human samples with the heterozygous Leu allele have lower activities for tolbutamide methyl hydroxylation than those with the wild-type and heterozygous Cys allele of CYP2C9 in liver microsomes, although the K_m's were not very different in these three groups (Inoue *et al.* 1997).

To demonstrate that the allelic variants of CYP2C9 will affect the catalytic roles of CYP2C9 in liver microsomes, we further determined and compared the activities of oxidation of racemic and *R*- and *S*-warfarin as well as tolbutamide in human samples which were genotyped for the CYP2C9 gene into wild-type and two heterozygous Leu allele and Cys allele CYP2C9 variants (Inoue *et al.* 1997). Three Caucasians with the Leu allele and eight Caucasians with the Cys allele CYP2C9 were selected to examine in more detail by comparing the catalytic activities to those catalysed by humans with wild-type CYP2C9. Kinetic analysis was determined in liver microsomes of these human samples and by recombinant human P450 enzymes including CYP2C9 and CYP2C9(Cys). The effects of sulphaphenazole and anti-CYP2C9 antibodies on the oxidation of these model substrates are also reported.

Materials and methods

Chemicals

Tolbutamide and racemic warfarin were purchased from Sigma Chemical Co. (St Louis, MO, USA). *S*-warfarin and *R*-warfarin, and their 6- and 7-hydroxylated metabolites, and *S*-mephenytoin were obtained from Ultra Fine Chemicals Co. (Manchester, UK) and used without further purification. Other drug substrates, their metabolites and reagents used were obtained from sources as described previously or were of the highest quality commercially available (Shimada *et al*. 1989, 1994, Mimura *et al*. 1993, Yamazaki *et al*. 1994).

Enzyme preparation

Human liver samples were obtained from organ donors or patients undergoing liver resection as described previously (Mimura *et al*. 1993, Shimada *et al*. 1994). Liver microsomes were prepared as described and suspended in 10 mM Tris-Cl buffer (pH 7·4) containing 1·0 mM EDTA and 20% glycerol (v/v) (Guengerich 1994).

CYP2C9 was purified to electrophoretic homogeneity from human liver microsomes as described previously (Shimada *et al*. 1986). Microsomes from human lymphoblast cells expressing CYP2C9 and CYP2C9(Cys) were purchased from Gentest Co. (Woburn, MA, USA). Recombinant CYP2C8, 2C9, 2C18 and 2C19 expressed in yeast microsomes were obtained from Sumitomo Chemical Co. (Osaka, Japan). Rabbit anti-P450 antibodies raised against human CYP2C9 were prepared and the IgG fractions obtained as described (Kaminsky *et al*. 1981, Shimada *et al*. 1986). Anti-CYP2C9 antibodies have already been characterized and shown to inhibit CYP2C9-dependent tolbutamide methyl hydroxylation and CYP2C19-dependent *S*-mephenytoin 4′-hydroxylation activities in human liver microsomes (Shimada *et al*. 1986, Brian *et al*. 1989).

Enzyme assays

Liver microsomal incubations included microsomes (1·0 mg protein/ml) in 50 mM potassium phosphate buffer (pH 7·4) containing an NADPH-generating system consisting of 0·5 mM $NADP^+$, 5 mM glucose 6-phosphate and 0·5 unit of glucose 6-phosphate dehydrogenase/ml, and various concentrations of drug substrates (Shimada *et al*. 1989, 1994). Methyl hydroxylation of tolbutamide (substrate concentration of 2·5 mM), 4′-hydroxylation of *S*-mephenytoin (0·4 mM), 6β-hydroxylation of testosterone were determined using high-performance liquid chromatography as previously described (Shimada *et al*. 1986, 1994, Knodell *et al*. 1987, Yamazaki *et al*. 1996). 6- and 7-Hydroxylation of racemic and *R*- and *S*-warfarin were determined according to the original method with modification (Lang and Böcker 1995). The standard incubation mixture consisted of microsomal protein (0·5 mg protein/ml) with substrates (0·10 mM) in a final volume of 0·20 ml 50 mM potassium phosphate buffer (pH 7·4) containing the NADPH-generating system. Incubations were carried out at 37 °C for 30 min and terminated by adding 10 μl 60% $HClO_4$ (w/v). The separation of hydroxylated warfarin metabolites by HPLC was carried out with a C_{18} 5-μm analytical column (4·6 × 150 mm, Kanto Chemical, Tokyo, Japan) eluted with a mixture of 36% CH_3CN (v/v) containing 0·04% aqueous H_3PO_4 (Lang and Böcker 1995).

P450 contents were estimated spectrally by the methods of Omura and Sato (1964). The contents of human P450 proteins in liver microsomes were estimated by coupled sodium dodecyl sulphate-polyacrylamide gel electrophoresis/immunochemical development (Western-blotting) (Guengerich *et al*. 1982). The intensities of the immunoblots were measured with an Epson GT-8000 Scanner (Tokyo, Japan) equipped with NIH Image/Gel Analysis Program adapted for Macintosh computers. Protein concentrations were estimated by the method of Lowry *et al*. (1951).

Analysis of genetic polymorphisms in CYP2C9 *genes*

Genomic DNA was isolated from human livers as described previously (Inoue *et al*. 1997, Yamazaki *et al*. 1997). Briefly, about 2 g liver was suspended in 10 mM Tris-Cl buffer (pH 7·5) containing 10 mM EDTA and 100 mM NaCl, and homogenized with a Teflon-pestle homogenizer. The nuclear fractions, collected by centrifugation at 3500 *g* for 5 min at 4 °C, were washed twice with the latter buffer by centrifugation at 9000 *g* for 15 min. Genomic DNA was isolated from the nuclear fraction by a commercial Nuclei Acid Purification System (Perkin Elmer ABI 341, Norwalk, CT, USA).

The genotyping procedure for the detection of CYP2C9 genetic polymorphism was performed according to the methods as previously described (Wang *et al*. 1995, Inoue *et al*. 1997). Briefly, the incubation mixture (final volume 25 μl) for the PCR reaction consisted of 10x Ex Taq buffer, 4 dNTPs, primers, genomic DNA as a template (25 ng) and 0·5 U Ex Taq polymerase (Takara, Kyoto, Japan). The DNA fragments after PCR amplification were digested with appropriate restriction endonuclease enzymes, separated by electrophoresis on a 10% polyacrylamide gel (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and visualized after treatment with SYBRTM Green I nucleic acid gel stain (Wako, Osaka, Japan).

Statistical analysis

Kinetic parameters for the tolbutamide methyl hydroxylation and warfarin 7-hydroxylation by recombinant human P450 enzymes and by liver microsomes were estimated using a KaleidaGraph program from Synergy Software (Reading, PA, USA) designed for non-linear regression analysis. The correlations between activities of tolbutamide methyl hydroxylation and warfarin 7-hydroxylation and contents of individual forms of P450 in different human liver microsomal preparations were analysed using a linear regression analysis (InStat) program from GraphPad Software (San Diego, CA, USA). Statistical analysis was analysed by Student's *t*-test.

Results

Tolbutamide methyl hydroxylation and warfarin 7-hydroxylation activities by human liver microsomes

Genomic DNA was isolated from livers of 39 Japanese and 45 Caucasians and used to examine the genotypes of the *CYP2C9* gene using specific oligonucleotide primers as previously described (Inoue *et al*. 1997). Three genotypes were found in the *CYP2C9* gene, namely Arg144·Ile359 (wild-type), Arg144Cys·Ile359 (Cys allele) and Arg144·Ile359Leu (Leu allele). The Cys allele was detected only in Caucasians with a frequency of ~22%. The frequencies of the Leu allele of *CYP2C9* were determined to be ~8% in both Japanese and Caucasians. None of the human samples examined in this study had homozygous Cys144/Cys144 and Leu359/Leu359 variants nor the heterozygous Arg144Cys·Ile359Leu variant of CYP2C9 (Inoue *et al*. 1997).

Tolbutamide methyl hydroxylation and racemic warfarin 7-hydroxylation (in this case 20 μM warfarin concentration was used) activities were determined in these human liver microsomes in which three Japanese and three Caucasians were genotyped to be a heterozygous Leu allele, and ten Caucasians to be a heterozygous Cys allele of CYP2C9. In both races, humans with the Leu allele of CYP2C9 were in the lower range of turnover numbers for tolbutamide methyl hydroxylation and warfarin 7-hydroxylation activities than those with wild-type and Cys allele (figure 1). The Cys allele of humans detected only in Caucasians showed almost similar catalytic rates to those of wild-type of CYP2C9. In both races, there were good correlations between tolbutamide methyl hydroxylation and racemic warfarin 7-hydroxylation activities.

Activities of 7-hydroxylation of *S*-warfarin as well as racemic warfarin were determined in liver microsomes of 35 selected Caucasians in which three were genotyped to be the Leu allele, eight to be the Cys allele and 24 to be wild-type CYP2C9, and compared with the levels of total P450 and CYP2C9 and the activities of tolbutamide methyl hydroxylation (figure 2). *S*-mephanytoin 4′-hydroxylation and testosterone 6β-hydroxylation activities were also measured. There were no significant differences in the contents of total P450 and CYP2C9 in liver microsomes of humans with three CYP2C9 genotypes. In the Caucasian samples, humans with the Leu allele of CYP2C9 always exhibited very low tolbutamide methyl hydroxylation and racemic and *S*-warfarin 7-hydroxylations, whereas the Cys allele had almost the same rates as the wild-type of CYP2C9. *S*-mephenytoin 4′-hydroxylation and testosterone 6β-hydroxylation activities by human live microsomes were not very different in the three groups of CYP2C9 genotype.

Activities of *S*-warfarin 7-hydroxylation were correlated well with those of racemic warfarin 7-hydroxylation and *S*-warfarin 6-hydroxylation in liver microsomes of 35 Caucasian samples (figure 3). The turnover numbers of 7-hydroxylation

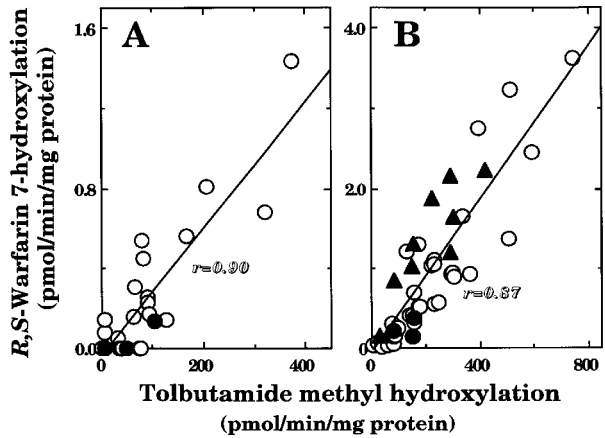

Figure 1. Correlation between tolbutamide methyl hydroxylation and racemic warfarin 7-hydroxylation activities in liver microsomes of Japanese (A) and Caucasian (B) populations. The genotypes of the CYP2C9 gene were shown to be wild-type (○), Cys allele (▲) and Leu allele (●).

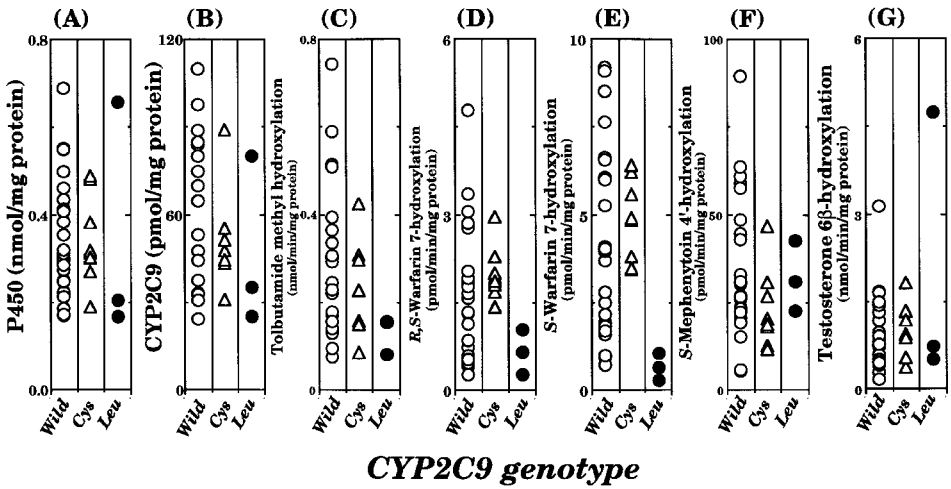

Figure 2. Contents of total P450 (A) and CYP2C9 (B) and activities of tolbutamide methyl hydroxylation (C), racemic warfarin 7-hydroxylation (D), *S*-warfarin 7-hydroxylation (E), *S*-mephenytoin 4′-hydroxylation (F), and testosterone 6*β*-hydroxylation (G) in liver microsomes of Caucasians who were genotyped for CYP2C9 gene into wild-type (○), Cys allele (△) and Leu allele (●).

of racemic warfarin was about one-third of those of *S*-warfarin in human liver microsomes and it was found that the ratio between activities of *S*-warfarin 7- and 6-hydroxylation was around 3–3·5 in these liver microsomes.

Kinetic analysis of warfarin 7-hydroxylation activities in 35 Caucasians

Kinetic analysis of 7-hydroxylation of racemic, and *R*- and *S*-warfarin was performed in liver microsomes of Caucasian samples genotyped for *CYP2C9* gene (table 1). Apparent K_m's for racemic warfarin 7-hydroxylation were found to be 27, 21 and 46 μM in liver microsomes of HL-C15 (wild-type), HL-C31 (Cys allele) and

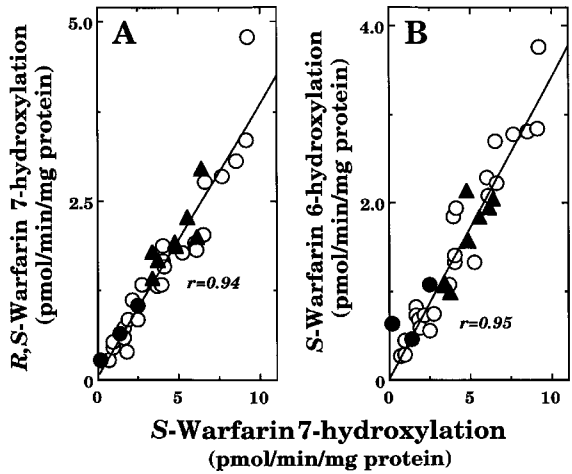

Figure 3. Correlation between activities of 7-hydroxylations of *S*-warfarin and racemic warfarin (A) and 6- and 7-hydroxylations of *S*-warfarin 7-hydroxylation (B) in liver microsomes of Caucasians who were genotyped for CYP2C9 gene to be wild-type (○), Cys allele (▲) and Leu allele (●).

Table 1. Kinetic analysis of warfarin 7-hydroxylation by liver microsomes of humans genotyped for *CYP2C9*.

Sample code no.	*CYP2C9* genotype	*R,S*-warfarin		*R*-warfarin		*S*-warfarin		*S/R* ratio of V_{max}
		K_m [a]	V_{max} [b]	K_m	V_{max}	K_m	V_{max}	
HL-C15	(wild)	27	1·4	107	1·0	32	5·8	5·8
HL-C19	(wild)			161	2·7	22	8·0	3·0
HL-C1	(Cys)			91	0·3	35	3·8	13
HL-C31	(Cys)	21	2·5	30	0·3	18	4·5	15
HL-C6	(Leu)	46	0·3	172	0·7	55	0·2	0·3
HL-C29	(Leu)			129	0·1	37	0·9	9·0
HL-C38	(Leu)			189	0·2	27	1·2	6·0

[a] K_m, μM.
[b] V_{max}, pmol/min/mg protein.

HL-C6 (Leu allele) respectively, with a lower V_{max} for the Leu allele of CYP2C9. K_m for the 7-hydroxylation of *R*-warfarin was lower in human samples of the Cys allele than those for the wild-type and Leu allele of CYP2C9. The K_m's were similar for three groups of the *CYP2C9* genotype.

The HPLC profile of *R*- and *S*-warfarin 6- and 7-hydroxylation showed that liver microsomes of human sample HL-C15 (wild-type of CYP2C9) catalysed the 7-hydroxylation of *S*-warfarin at much higher rates than those of *R*-warfarin 7-hydroxylation, whereas the rates of hydroxylation of *R*- and *S*-warfarin by liver microsomes of human sample HL-C6 (Leu allele) were found to be similar (figure 4).

Analysis for the *S*-warfarin 7-hydroxylation was also performed and compared in liver microsomes of HL-C19 (wild-type) and recombinant CYP2C9 (figure 5A), and of HL-C31 (Cys allele) and recombinant CYP2C9(Cys) (figure 5B). The K_m's for the 7-hydroxylation were very similar in liver microsomes of wild-type CYP2C9 (human sample of HL-C19) and recombinant CYP2C9(wild), whereas the V_{max} determined with recombinant CYP2C9 was much higher than those catalyzed by liver microsomes of a human sample of HL-C19. The K_m's and V_{max}'s for *S*-

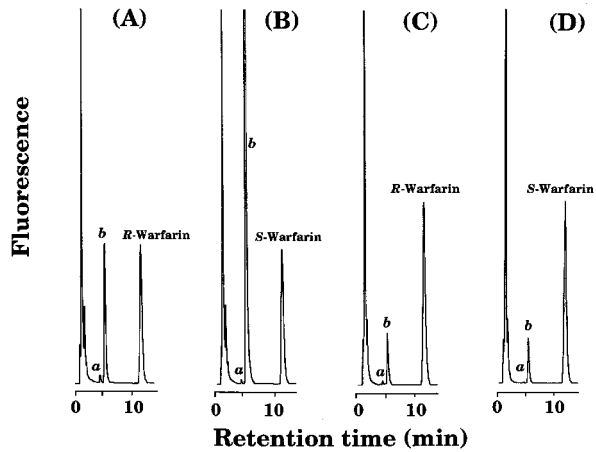

Figure 4. HPLC profile of hydroxylation of *R*-warfarin (A and C) and *S*-warfarin (B and D) by liver microsomes of HL-C15 (wild-type; A and B) and HL-C6 (Leu allele; C and D). 6-Hydroxylated (*a*) and 7-hydroxylated products (*b*) of *R*- and *S*-warfarin are also shown.

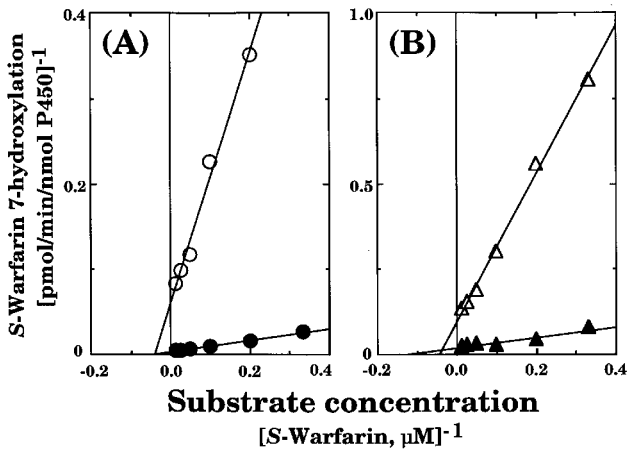

Figure 5. Kinetic analysis of *S*-warfarin 7-hydroxylation catalysed by liver microsomes of human samples HL-C19 (○) and HL-C31 (△), which were genotyped for the CYP2C9 gene to be a wild-type and Cys allele respectively, and by microsomes of human lymphoblastoid cells expressing CYP2C9 (●) and CYP2C9(Cys) (▲). Data are the mean of duplicate determinations.

warfarin 7-hydroxylation by recombinant CYP2C9(Cys) were lower and higher, respectively, than those catalysed by liver microsomes with HL-C31 (Cys allele). On analysis using Eadie–Hofstee plots, we found that a single kinetic parameter could be determined in the 7-hydroxylation of *S*-warfarin by this human sample (data not shown).

Tolbutamide methylhydroxylation and warfarin 6- and 7-hydroxylation by recombinant human CYP2C enzymes

Activities of methyl hydroxylation of tolbutamide and the 6- and 7- hydroxylation of *R*- and *S*-warfarin were determined in yeast microsomes expressing human CYP2C8, 2C9, 2C18 and 2C19, and microsomes of human lymphoblastoid

Table 2. Methyl hydroxylation of tolbutamide, and 6- and 7-hydroxylation of *R*- and *S*-warfarin by recombinant CYP2C proteins.

P450 enzyme	Tolbutamide methyl hydroxylation (nmol/min/nmol P450)	*R*-warfarin		*S*-warfarin	
		6-hydroxylation	7-hydroxylation	6-hydroxylation	7-hydroxylation
		(pmol/min/nmol P450)			
Yeast microsomes					
CYP2C8	12±2	< 10	2±1	< 10	1±1
CYP2C9	53±8	< 10	23±2	184±24	740±84
CYP2C18	14±2	< 10	5±1	< 10	49±3
CYP2C19	46±3	256±19	410±35	< 10	123±11
Human lymphoblast cells					
CYP2C9	26±3	< 10	13±2	43±3	244±15
CYP2C9(Cys)	17±2	< 10	4±1	< 10	43±3

Each value represents the mean and range of duplicate or triplicate determinations. Substrate concentrations used were 2·5 mM for tolbutamide and 100 μM for *R*- and *S*-warfarin.

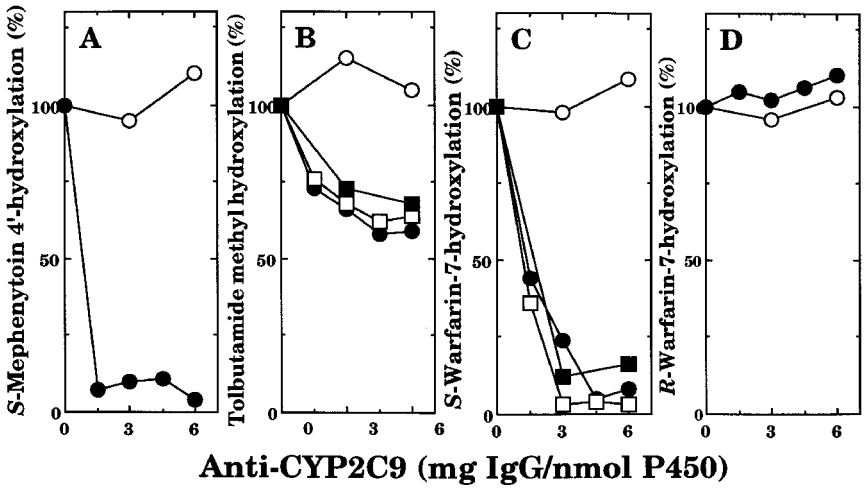

Figure 6. Effects of anti-CYP2C9 IgG on the activities of *S*-mephenytoin 4′-hydroxylation (A), tolbutamide methyl hydroxylation (B), *S*-warfarin 7-hydroxylation (C), and *R*-warfarin 7-hydroxylation (D) by liver microsomes of human samples HL-C15 (●), HL-C31 (□), and HL-C29 (■), which were genotyped for the CYP2C9 gene to be a wild-type, Cys allele and Leu allele respectively. The effects of preimmune IgG (○) were also determined. Control activities (pmol products/min/mg protein) in the absence of antibodies were 108 (*S*-mephenytoin 4′-hydroxylation by HL-C15), 711, 360 and 130 (tolbutamide methyl hydroxylation by HL-C15, HL-C31 and HL-C29), and 11, 8·7 and 1·1 (*S*-warfarin 7-hydroxylation by HL-C15, HL-C31 and HL-C29). *R*-warfarin 7-hydroxylation was determined only in liver microsomes of HL-C15; the turnover rate in the absence of antibodies was 0·8 pmol/min/mg protein. Data are the mean of duplicate determinations.

cells expressing CYP2C9 and CYP2C9(Cys) (table 2). Among the yeast microsomes expressing CYP2C enzymes examined, CYP2C9 and CYP2C19 were the most active in catalysing tolbutamide methyl hydroxylation. *S*-warfarin 6- and 7-hydroxylation were extensively catalysed by CYP2C9, whereas CYP2C19 was the most active for the hydroxylations of *R*-warfarin. In microsomes of human lymphoblastoid cells, CYP2C9(Cys) was found to be less active in catalysing *R*- and *S*-warfarin 7-hydroxylation as compared with those catalysed by CYP2C9(wild).

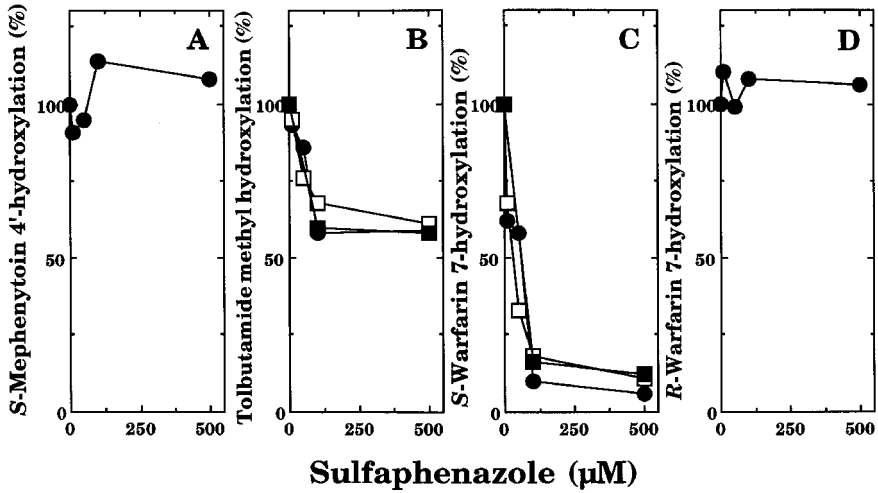

Figure 7. Effects of sulphaphenazole on the activities of *S*-mephenytoin 4′-hydroxylation (A), tolbutamide methyl hydroxylation (B), *S*-warfarin 7-hydroxylation (C), and *R*-warfarin 7-hydroxylation (D) by liver microsomes of human samples HL-C15 (●), HL-C31 (□) and HL-C29 (■), which were genotyped for the CYP2C9 gene to be a wild-type, Cys allele and Leu allele respectively. The effect of sulphaphenazole on the *R*-warfarin 7-hydroxylation was also determined only in liver microsomes of HL-C15. Data are the mean of duplicate determinations.

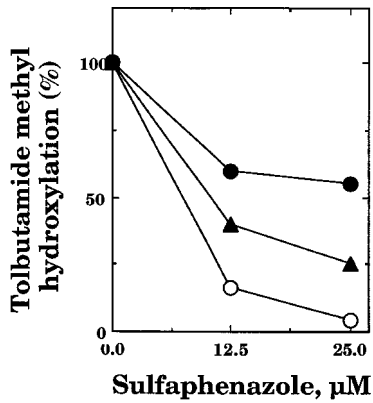

Figure 8. Effects of substrate concentration on the sulphaphenazole inhibition of tolbutamide methyl hydroxylation activities by liver microsomes of human samples HL-C15. Tolbutamide concentrations determined were 0·025 (○), 0·25 (▲) and 2·5 mM (●); control activities in the absence of sulphaphenazole were 25±5, 140±21 and 420±40 pmol/min/mg protein respectively. Data are the mean of duplicate determinations.

Effects of anti-CYP2C9 IgG and sulphaphenazole on the oxidation of S-*mephenytoin, tolbutamide and* S-*warfarin by human liver microsomes*

Effect of antibodies raised against purified human CYP2C9 on the tolbutamide methyl hydroxylation and *S*-warfarin 7-hydroxylation activities were determined in liver microsomes of human samples genotyped for CYP2C9 gene (figure 6). Effects of anti-CYP2C9 on the 4′-hydroxylation of *S*-mephenytoin and the 7-hydroxylation of *R*-warfarin were also measured in liver microsomes of a human sample HL-C15 (genotyped for CYP2C19wt/wt and wild-type of CYP2C9) and it was found that the antibodies inhibited activities very extensively (figure 6A). Anti-CYP2C9 IgG was also found to be very effective in inhibiting *S*-warfarin 7-hydroxylation catalysed by

liver microsomes of humans genotyped for the wild-type Cys- and Leu allele of CYP2C9 (figure 6C). However, the inhibition by anti-CYP2C9 antibodies of tolbutamide methyl hydroxylation by human liver microsomes were < 50% of the control activities (figure 6B). Anti-CYP2C9 was found to be non-inhibitory toward the *R*-warfarin 7-hydroxylation catalysed by human liver microsomes (figure 6D).

The effects of sulphaphenazole, a known inhibitor of CYP2C9 (Brian *et al.* 1989), were determined under the same experimental conditions described above (figure 7). Sulphaphenazole very significantly inhibited the 7-hydroxylation of *S*-warfarin and partially inhibited the methyl hydroxylation of tolbutamide, but not the 4′-hydroxylation of *S*-mephenytoin and 7-hydroxylation of *R*-warfarin, by human liver microsomes.

When the substrate (tolbutamide) concentrations were decreased from 2·5 to 0·025 mM, the effects of sulphaphenazole on the inhibition of tolbutamide methyl hydroxylation activities were much more extensive (figure 8).

Discussion

Recombinant protein engineering has been applied extensively to human P450 research in order to elucidate the mechanisms of expression and catalytic functions of individual P450 enzymes (Gonzalez 1989, Sandhu *et al.* 1993, Goldstein and de Morais 1994, Gonzalez and Korzekwa 1995, Guengerich *et al.* 1996). Many human P450 cDNAs and their modified DNA sequences have been introduced into heterologous organisms such as viruses, bacteria, yeasts, insects and mammalian cells and the expressed proteins have been used to examine the roles of these P450 enzymes in the oxidation of a number of xenobiotic and endobiotic chemicals and to study the genetic basis of P450 polymorphisms in man (Gonzalez *et al.* 1991, Guengerich *et al.* 1991, Goldstein and de Morais 1994, Gonzalez and Korzekwa 1995).

Recently, two allelic variant cDNAs of *CYP2C9*, Cys and Leu alleles, have been studied in several heterologous systems and the recombinant proteins thus obtained were used to examine their abilities to oxidize prototypic substrates such as tolbutamide, phenytoin and warfarin (Yasumori *et al.* 1991, Kaminsky *et al.* 1993, Lopez-Garcia *et al.* 1993, Sandhu *et al.* 1993, Veronese *et al.* 1993, Rettie *et al.* 1994, Tracy *et al.* 1995, Haining *et al.* 1996, Sullivan-Klose *et al.* 1996). Haining *et al.* (1996) reported that the Leu allele of CYP2C9 catalyses the 6- and 7-hydroxylation of *S*-warfarin at slower rates (with higher K_m's) than those of the CYP2C9 wild-type. Similar results have also been reported by others by measuring tolbutamide methyl hydroxylation, warfarin 7-hydroxylation and phenytoin p-hydroxylation (Veronese *et al.* 1993, Sullivan-Klose *et al.* 1996). In contrast, the Cys allele of CYP2C9, another variant form, has been reported to have similar catalytic functions to the wild-type of CYP2C9 for the oxidation of tolbutamide, phenytoin and warfarin (Veronese *et al.* 1993, Sullivan-Klose *et al.* 1996), although there is some controversy that this allelic variant has been shown to have lower activities for *S*-warfarin 7-hydroxylation than those of the CYP2C9 wild-type (Rettie *et al.* 1994).

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 for the oxidations of tolbutamide and phenytoin in man (Spielberg *et al.* 1996, Sullivan-Klose *et al.* 1996). The 6- and 7-hydroxylation activities of *S*-warfarin are lower in the CYP2C9 Leu allele protein than the wild

CYP2C9 protein, with the former protein having higher K_m's for these two reactions (Haining *et al*. 1996, Sullivan-Klose *et al*. 1996). Previously, we analysed genomic DNA of livers from 39 Japanese and 45 Caucasians to determine the genotypes for the CYP2C9 gene and found that the heterozygous Cys allele mutation is detected only in Caucasians, with frequencies of about 22%, whereas the Leu allele is detected in both Japanese and Caucasian populations, with frequencies of about 8% each. It should be mentioned, however, that none of the human samples examined had homozygous Cys144/Cys144 and Leu359/Leu359 variants nor heterogenous Arg144Cys·Ile359Leu variant of CYP2C9 (Inoue *et al*. 1997). In these studies we have shown that human samples with the heterozygous Leu allele have lower activities for tolbutamide methyl hydroxylation than those with the wild-type and heterozygous Cys allele of CYP2C9 in liver microsomes, although the K_m's were similar in these three groups (Inoue *et al*. 1997).

In this study we further examined the roles of variant forms of CYP2C9 in human liver microsomes towards the oxidation of tolbutamide, racemic, *R*-, and *S*-warfarin. The results presented showed that 6- and 7-hydroxylation of *S*-warfarin were catalysed principally by CYP2C9 in human liver microsomes with the latter reaction being catalysed better by human CYP2C9 proteins than the 6-hydroxylation reaction. The Leu allele of CYP2C9 was suggested to have lower turnover numbers for *S*-warfarin 7-hydroxylation than those catalysed by wild-type and Cys alleles of CYP2C9, although the K_m's in these groups of CYP2C9 genotypes were not very different. It was also determined that the apparent K_m's and V_{max}'s for *S*-warfarin 7-hydroxylation in man with the Cys- and wild-types were relatively similar. These results are in contrast with the previous findings that recombinant CYP2C9(Leu) and CYP2C9(Cys) have higher K_m's than those catalysed by recombinant CYP2C9 in the oxidation of *S*-warfarin (Veronese *et al*. 1993, Rettie *et al*. 1994, Haining *et al*. 1996).

Although there was a good correlation between the 7-hydroxylation of racemic warfarin and *S*-warfarin in liver microsomes from Caucasian samples, the turnover numbers of *S*-warfarin 7-hydroxylation were always higher than those of racemic warfarin 7-hydroxylation by these human liver microsomes. Since the CYP2C9 in human liver microsomes catalysed about 5–10-fold higher rates for 7-hydroxylation of *S*-warfarin than those of *R*-warfarin, it is possible that the *R*-enantiomer may be inhibitory for the hydroxylation of the *S*-enantiomer when the racemic warfarin was used as a substrate. Our results also suggested that *R*-warfarin was catalysed by other P450 enzymes rather than CYP2C9 in human liver microsomes since *R*-warfarin 7-hydroxylation activities were not inhibited by anti-CYP2C9 and sulphaphenazole. Of the recombinant human CYP2C enzymes examined, CYP2C19 was found to be the most active for the *R*-warfarin 7-hydroxylation. However, anti-CYP2C9 that inhibited *S*-mephenytoin 4′-hydroxylation very significantly did not affect the *R*-warfarin 7-hydroxylation by liver microsomes, suggesting that the reaction was catalysed principally by other P450 enzymes rather than the CYP2C enzymes (Rettie *et al*. 1992, Zhang *et al*. 1995).

In conclusion, the present results have shown that the Leu allele of CYP2C9 has lower V_{max}'s for *S*-warfarin 7-hydroxylation and tolbutamide methyl hydroxylation than those catalysed by wild-type and Cys allele, whereas the K_m's are similar in these three genotypes. CYP2C9 catalyses the 7-hydroxylation of *S*-warfarin better than that of *R*-warfarin, and *R*-warfarin hydroxylation may be catalysed by P450 enzymes other than CYP2C9 in man.

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