CYP2C9-Catalyzed Metabolism of S-Warfarin to 7-Hydroxywarfarin in Vivo and in Vitro in Chimeric Mice with Humanized Liver

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Received June 10, 2008; accepted September 8, 2008

ABSTRACT:
Chimeric mice having humanized livers were constructed by transplantation of human hepatocytes. In this study, we investigated whether these mice have a capacity for drug metabolism similar to that of humans by examining hydroxylation of S-warfarin, which is predominantly metabolized to S-7-hydroxywarfarin, catalyzed by CYP2C9, in humans but not mice. The 7-hydroxylating activity of chimeric mouse liver microsomes toward S-warfarin was approximately 10-fold higher than that of control (urokinase-type plasminogen activator-transgenic severe combined immunodeficient) mice. The 7-hydroxylase activity of chimeric mouse liver microsomes was markedly inhibited by sulfaphenazole, as was that of human liver microsomes, whereas the activity of control mice was unaffected. The CYP2C isoform in chimeric mouse liver was also confirmed to be the human isoform, CYP2C9, by immunoblot analysis. In the present in vivo study, the level of S-7-hydroxywarfarin in plasma of chimeric mice was approximately 7-fold higher than that in control mice, in agreement with the in vitro data. Thus, the CYP2C isoform in chimeric mice functions in vivo and in vitro as a human isoform, CYP2C9. These results suggest that chimeric mice with humanized liver could be useful for predicting drug metabolism in humans, at least regarding CYP2C9-dependent metabolism.

Chimeric mice have been constructed by transplantation of human hepatocytes into urokinase-type plasminogen activator-transgenic severe combined immunodeficient mice (Dandri et al., 2001; Mercer et al., 2001). It was suggested that these chimeric mice may be useful as an in vivo model for studies on human liver diseases and hepatotropic viruses. Prolonged infections with human hepatitis B or C virus can be maintained in these mice (Dandri et al., 2001; Mercer et al., 2001). However, the chimeric mice used in those experiments did not have a high level of replacement with human hepatocytes. In a recent study, Tateno et al. (2004) prepared chimeric mice in which the liver was almost completely repopulated with human hepatocytes. Furthermore, they reported that cytochrome P450 subtypes in liver microsomes of chimeric mice in which more than 80% of hepatocytes had been replaced with human hepatocytes were similar to those of the donor human liver (Tateno et al., 2004). Therefore, the chimeric mice constructed by Tateno et al. (2004) appear to be an excellent in vivo model for prediction of drug metabolism and drug-drug interactions due to drug induction and inhibition of drug metabolizing enzymes in humans. Katoh et al. (2004, 2005a) reported that cytochrome P450 (P450) isoforms and phase II enzymes in chimeric mice in which the human hepatocyte replacement rate was nearly 90% were almost the same as those in human liver, based on quantitation of enzyme proteins. They also showed that the chimeric mice are a useful animal model to estimate the inductive effect on P450 in humans (Katoh et al., 2005b). We have shown that aldehyde oxidase, a cytosolic drug-metabolizing enzyme, in chimeric mice has functional characteristics almost identical to those of human aldehyde oxidase (Kitamura et al., 2008). Furthermore, Nishimura et al. (2005) reported that hepatocytes from chimeric mice with nearly completely humanized liver are a useful tool for screening the potency of new drugs to induce drug-metabolizing enzymes in human. Here, we investigated whether these mice have a capacity for CYP2C9-dependent metabolism of S-warfarin similar to that of humans.

Warfarin is widely used as an anticoagulant drug in humans, but it has a narrow therapeutic index and requires tight control of the dosage regimen. Racemic warfarin has been used clinically as an oral drug and as an environmental rodenticide, but the two isomers differ in their pharmacodynamics. S-Warfarin is pharmacologically active, whereas the R-enantiomer is essentially inactive in humans (O’Reilly, 1974). R- and S-warfarin are metabolized via CYP1A1, 2B1, 2C6, 2C11, and 3A2 in rats (Kaminsky et al., 1983). In humans, S-warfarin is metabolized mainly to inactive 7-hydroxywarfarin and also to 6-hydroxywarfarin as a minor metabolite by CYP2C9 (Rettie et al., 1992; Kaminsky et al., 1993). CYP2C8, 2C18, and 2C19 expressed in yeast generated the 4’-hydroxyl metabolite from S-warfarin (Kaminsky et al., 1993). R-Warfarin is metabolized to 6-, 8- and 10-hydroxywarfarin via CYP1A2, 2C19, and 3A4 in humans (Rettie et al., 1989; Wienkers et al., 1996; Kaminsky and Zhang, 1997; Yamazaki and Shimada, 1997). S-Warfarin is metabolized more rapidly than the
R-enantiomer, and, in particular, 7-hydroxylation was 8-fold higher for S-warfarin than for R-warfarin (Park, 1988; Yamazaki and Shimagata, 1997). The interaction of warfarin with various drugs, natural substances, and foods via CYP2C9 is clinically significant (Toon et al., 1986; Rettie et al., 1992; Greenblatt and von Molké, 2005).

In this study, we estimated the activity of CYP2C isozyme in the livers of control [urokinase-type plasminogen activator-transgenic severe combined immunodeficient: (uPA+/−/SCID)] mice and chimeric mice with humanized liver by using S-warfarin as a substrate. The amounts of warfarin and its 7-hydroxyl derivative in the blood of chimeric mice dosed with warfarin were also measured by using high-performance liquid chromatography (HPLC). We demonstrated that oxidation of S-warfarin to S-7-hydroxylwarfarin was catalyzed by CYP2C9 in the chimeric mice, both in vivo and in vitro.

**Materials and Methods**

**Materials.** S-Warfarin was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). S-7-Hydroxywarfarin and S-6-hydroxywarfarin were purchased from Ultrafine Chemicals Ltd. (Manchester, UK). Pooled human liver microsomes and anti-CYP2C9 were purchased from BD Biosciences (San Jose, CA).

**Animals.** Chimeric mice with humanized liver were prepared according to the method of Tatenou et al. (2004). Human hepatocytes were transplanted into uPA+/−/SCID mice (20–30 days after birth) and progressively repopulated the mouse host liver. Hepatocytes from a male white (13 years old) were obtained from In Vitro Technologies (Catonsville, MD). For Western blot analysis, hepatocytes from a male white (4 years old) and a male white (6 years old) were used as donors. To estimate the humanization of the chimeric mice, the replacement index (RI) of the mice with the human hepatocytes was determined by measuring human albumin (hAlb) in blood collected periodically from the tail vein, using a Human Albumin ELISA Quantitation Kit (Bethyl Laboratories Inc., Montgomery, TX). The uPA+/−/SCID mice were generated by crossing uPA mice [B6SJ-Tg(AlbPlau)14Bri; The Jackson Laboratory, Bar Harbor, ME] with SCID mice (Fox Chase SCID C.B-17/Icr-scid Jcl; Clea Japan Inc., Tokyo, Japan). The mice were housed in caged on a 12-h light/dark cycle at 25°C, with free access to tap water and diets. Chimeric mice were given CRF1 containing vitamin C, sterilized by uPA+/−/SCID mice and chimeric mice with different levels of replacement with human liver microsomes toward 7-hydroxywarfarin were detected by means of HPLC, as described under Materials and Methods.

**Measurement of Warfarin and Its Metabolites in Plasma.** S-Warfarin and S-7-hydroxywarfarin in blood of mice were determined according to the method of Zhu and Shin (2005). Ten micrograms of 2-benzylphenol as an internal standard and 0.6 g of KCl were added to the plasma, and the mixture was extracted 3 times with 5 ml of diethyl ether. The amounts of S-warfarin and S-7-hydroxylwarfarin in the extract were determined by using HPLC as follows.

**HPLC.** HPLC was performed using a Hitachi L-6000 chromatograph (Hitachi Co. Ltd., Tokyo, Japan) fitted with an Inertsil ODS-3 column (150 cm × 4.6 mm; GL Science Inc., Tokyo, Japan). The mobile phase was acetonitrile-0.5% phosphoric acid (38:62 v/v). The chromatograph was operated at a flow rate of 1 ml/min, with a detection wavelength of 254 nm. The elution times of S-warfarin, S-7-hydroxywarfarin, S-6-hydroxywarfarin, and internal standard were 28.2, 13.3, 10.2, and 42.3 min, respectively. Standard curves of S-warfarin and S-7-hydroxywarfarin were linear in the ranges of 50 to 0.1 ng and 15 to 0.05 ng, respectively. For S-warfarin, y = 0.0911x with R² = 0.9800, whereas for S-7-hydroxywarfarin, y = 0.0417x with R² = 0.9919. For quantification of incubated samples, peak area ratios relative to that of the internal standard were used.

**Statistical Analysis.** Values are presented as means and S.D. Comparison of average area under the plasma concentration-time curve (AUC) values between chimeric mice and control mice were made using an unpaired Student’s t test with Excel software (Microsoft, Redmond, WA). p < 0.05 was considered statistically significant.

**Results**

**7-Hydroxylation Activity of Liver Microsomes from Control and Chimeric Mice.** 7-Hydroxylase activities of liver microsomes of control and chimeric mice toward S-warfarin were assayed. The calibration plot for S-7-hydroxywarfarin was linear in the range of 200 to 4 pmol/sample, and the detection limit was 0.1 pmol/min/mg protein. When S-warfarin was incubated with liver microsomes from control mice and from chimeric mice with a high level of hepatocyte replacement with human hepatocytes (>80%) in the presence of NADPH, marked differences in their activities were observed. The HPLC peak of S-7-hydroxywarfarin formed by liver microsomes of chimeric mice was much higher than that in the case of control mice, but no other peaks due to metabolites of S-warfarin were detected (data not shown). The 7-hydroxylating activity of the chimeric mouse liver microsomes toward S-warfarin was approximately 10-fold higher than for control mice. When the activities were compared among chimeric mice with different levels of replacement with human liver microsomes, the activity in livers of chimeric mice with various RI values for human hepatocytes is shown. Each column represents the mean of triplicate determinations of an individual animal. The oxidase activity was assayed by measuring S-7-hydroxywarfarin formed by means of HPLC, as described under Materials and Methods. HLM, human liver microsomes.

![FIG. 1. S-Warfarin oxidase activity of liver microsomes of chimeric mice. Oxidase activity in livers of chimeric mice with various RI values for human hepatocytes is shown. Each column represents the mean of triplicate determinations of an individual animal. The oxidase activity was assayed by measuring S-7-hydroxywarfarin formed by means of HPLC, as described under Materials and Methods. HLM, human liver microsomes.](https://www.aspetjournals.org)
hepatocytes (40–50, 60–70, and 80–90%), as judged from the hAlb concentration in the blood, clear differences were observed (Fig. 1). The 7-hydroxylating activity toward S-warfarin increased with an increasing replacement ratio with human hepatocytes. Furthermore, the 7-hydroxylase activity of liver microsomes of chimeric mice was markedly inhibited by sulfaphenazole, an inhibitor of CYP2C9 (Rettie et al., 1992; Yamazaki and Shimada, 1997), as is the case for human liver microsomes, but the activity of control mice was unaffected (Table 1). Further characterization of the CYP2C isoform in these liver microsomes was performed by Western blot analysis using CYP2C9 antibody. The CYP2C isoform in chimeric mouse liver was confirmed to be the human isoform, CYP2C9, by immunoblot analysis (Fig. 2). The density of the band in the case of liver microsomes of chimeric mice with a high level of displacement (>80%) with human hepatocytes was close to that of pooled human liver microsomes. These results suggest that P450 in livers of chimeric mice functions as a human-type P450, i.e., CYP2C9.

**In Vivo Metabolism of S-Warfarin to the 7-Hydroxyl Derivative in Chimeric Mice.** S-Warfarin and S-7-hydroxywarfarin in the plasma of control and chimeric mice after the administration of S-warfarin were assayed. The calibration plots for S-warfarin and S-7-hydroxywarfarin were linear in the ranges of 100 to 1 ng/ml plasma and 30 to 0.5 ng/ml plasma, respectively. The detection limits were 1 and 0.5 ng/ml plasma, respectively. When S-warfarin was administered to control and chimeric mice, peaks of warfarin and S-7-hydroxywarfarin were found in HPLC chromatograms of the extract of the plasma of these mice. No peak of S-6-hydroxywarfarin was seen at 10.2 min, the elution time of an authentic sample. The peak levels of S-warfarin in the plasma of control mice at various times after administration were approximately the same as those in chimeric mice. In contrast, the HPLC peaks of S-7-hydroxywarfarin in chimeric mice with high levels of human hepatocyte replacement were much higher than those in control mice (Fig. 3). The AUC of S-7-hydroxywarfarin was greater in chimeric mice than that in control mice. C_{max} in the plasma of control mice was also higher than that of chimeric mice (Fig. 4). Parameters of S-7-hydroxywarfarin in control (uPA^{−/−}/SCID and uPA^{wt/wt}/SCID) and chimeric mice were as follows: AUC 6.4, 4.4, and 36.4, C_{max} 1.0, 2.6, and 8.5, and T_{max} 1.0, 3.1, and 2.8.

### Table 1

<table>
<thead>
<tr>
<th>Liver Microsomes</th>
<th>7-Hydroxylase activity</th>
<th>% Control</th>
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<tr>
<td>uPA^{−/−}/SCID mouse liver microsomes</td>
<td>0.39</td>
<td>92</td>
</tr>
<tr>
<td>Chimeric mouse liver microsomes</td>
<td>5.46</td>
<td>23</td>
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<tr>
<td>Pooled human liver microsomes</td>
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**FIG. 2. Western blots probed with anti-rat CYP2C9 antibody for mouse liver microsomes.**

**FIG. 3.** HPLC chromatograms of the metabolites of S-warfarin in plasma of control (uPA^{−/−}/SCID) mice (A) and chimeric mice having a high level of replacement with human hepatocytes (B). I.S., internal standard.
HPLC as described under mg/kg to male control and chimeric mice. Blood (0.05 ml) was collected at intervals. Amounts of uPA and 243.2, respectively. Parameters of S-warfarin in control (uPA<sup>wt/wt</sup>/SCID and uPA<sup>wt/wt</sup>/SCID) and chimeric mice were as follows: AUC 170.6, 151.3, and 243.2, C<sub>max</sub> 56.5, 37.0, and 59.1, and T<sub>max</sub> 1.58, 1.58, and 1.58, respectively. The AUC values of S-warfarin showed no significant difference between chimeric mice and control mice, whereas those of S-7-hydroxywarfarin differed significantly by Student’s t test. This result confirms the fact that the in vivo metabolism of S-warfarin closely reflects the in vitro metabolism of S-warfarin in liver microsomes of chimeric mice.

These results suggest that the chimeric mice could be useful in predicting drug metabolism in humans, at least CYP2C9-dependent metabolism.

**Discussion**

In this study, we demonstrated that a microsomal drug-metabolizing enzyme in chimeric mice with a high level of hepatocyte replacement with human hepatocytes functions as human-type P450, CYP2C9, both in vivo and in vitro. Furthermore, the conversion of S-warfarin to the 7-hydroxyl metabolite in chimeric mice was similar to that in humans. The difference between the control mice and the chimeric mice reflects the differences in the CYP2C isoform between mice and humans. S-Warfarin is known to be oxidized mainly to the 7-hydroxyl metabolite by CYP2C9. However, in mice, S-warfarin is not oxidized as actively to 7-hydroxywarfarin as it is in humans. Therefore, S-warfarin is a good substrate to distinguish the P450 isoform in mice and humans. It seems to be possible to predict which new drugs would be metabolized by CYP2C9 by means of preclinical drug metabolic studies in these chimeric mice. We would not expect any marked difference between humanized chimeric mice and control mice regarding formation of S-4'-hydroxywarfarin and S-10-hydroxy warfarin by other P450s, because although the CYP2C subfamily shows different substrate specificities between humans and mice, other P450s do not.

We confirmed the usefulness of chimeric mice for studies of drug disposition. Until now, studies of drug metabolism in chimeric mice have been restricted to in vitro microsomal oxidase enzymes and phase II enzymes (Katoh et al., 2004, 2005b). In those reports, it was demonstrated that isoforms of P450 and phase II enzymes in chimeric mouse liver behaved similarly to those in human liver in in vitro experiments. In a recent study, we showed that aldehyde oxidase in chimeric mice functions as a human-type aldehyde oxidase in vivo and in vitro (Kitamura et al., 2008). In this study, we extended the studies in chimeric mice to in vivo and in vitro metabolism of S-warfarin by CYP2C9. This is the first report that a hepatic drug-metabolizing enzyme, P450, in chimeric mice acts as a human enzyme at the in vivo level. A good in vivo-in vitro correlation of S-warfarin oxidase activity due to P450 in chimeric mice was demonstrated in this study. The amounts of S-7-hydroxywarfarin found in plasma of chimeric mice were correlated with the in vitro warfarin oxidase activity of liver microsomes. Furthermore, the activity of chimeric mice varied in a hAlb concentration-dependent manner in both in vivo and in vitro studies. This fact suggests that the formation of the 7-hydroxyl derivative from S-warfarin in chimeric mice in vivo can be ascribed to human CYP2C9. This in vivo-in vitro correlation is important, because studies in chimeric mice would have various advantages for predicting drug interactions in humans. However, the role of enzyme activity in extrahepatic organs must also be considered. P450 is known to exist in organs other than liver. However, liver CYP2C9 is the major determinant of in vivo S-warfarin metabolism. In preclinical tests using chimeric mice, it would be important to confirm that the liver enzyme functions as the major metabolic en-

![Fig. 4. Plasma concentration-time profiles of S-warfarin (●) and the 7-hydroxylated metabolite (○) after oral administration of S-warfarin to chimeric mice (A) and control (uPA<sup>wt/wt</sup>/SCID and uPA<sup>wt/wt</sup>/SCID) mice (B and C). Each value represents the mean ± S.D. of four determinations. S-Warfarin was orally administered at a dose of 30 mg/kg to male control and chimeric mice. Blood (0.05 ml) was collected at intervals. Amounts of S-warfarin and S-7-hydroxywarfarin in the plasma were determined using HPLC as described under Materials and Methods.](https://example.com/)
zyme at the in vivo level, as seems to be the case for CYP2C9-catalyzed warfarin metabolism in these chimeric mice. In recent studies, transgenic mouse models containing individual human P450 isoforms, such as CYP2D6 and CYP3A4, have been generated (Corchero et al., 2001; Robertson et al., 2003; Zhang et al., 2003; Miksys et al., 2005). These mice could be useful for predicting the toxicity and pharmacological effects of various chemicals. In general, human hepatocytes are used in vitro for such studies, and cultured human hepatocytes express most of the hepatic drug-metabolizing enzymes found in vivo. However, the activities of these enzymes tend to decrease during culture because of a decrease in P450 concentration. This finding indicates that P450 changed from the murine form to the human form in parallel with the replacement ratio with hepatocytes. Katoh et al. (2004) also found that the expression of human CYP2C9 mRNA was correlated with the concentration of hAlb in chimeric mice. Diclofenac 4′-hydroxylation is catalyzed by CYP2C9 (Nakajima et al., 2002), and Tateno et al. (2004) reported that diclofenec 4′-hydroxylation activity in chimeric mice having a high replacement ratio with human hepatocytes was much higher than that of control untreated mice.

Functional induction in addition to increased expression of CYP2C9 mRNA and protein (not murine cyp2c) in chimeric mice has been reported (Katoh et al., 2005b). Many drug interactions with warfarin are caused by inhibition or induction of CYP2C9 (Rettie et al., 1992; Yamazaki and Shimada, 1997). Therefore, the present chimeric mouse model with humanized liver should be useful for estimation and analysis of in vivo drug-drug interactions involving warfarin and CYP2C9-metabolized drugs in humans.

References

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