

Fig. 1. Human CYP2C9 expression in the chimeric mice. Relative expression levels of human CYP2C9 mRNA (A), the expression of human CYP2C9 protein (B), and DICOH (C) were determined as described under *Materials and Methods*. C, DICOH catalyzed by CYP2C9 measured at 30 μ M diclofenac. Each column represents the mean of duplicate determinations except in uPA^{+/+}/SCID mice and uPA^{-/-}/SCID mice. The columns of uPA^{+/+}/SCID mice and uPA^{-/-}/SCID mice represent the mean \pm S.D. ($n = 3$). The sample numbers are described in Table 2. H, HLM; A, donor A; ND, not detected.

(5–20 μ g) were separated on 7.5% polyacrylamide gel and transferred electrophoretically to a polyvinylidene difluoride membrane. Recombinant human P450s were also applied as the standards. Biotinylated anti-rabbit or mouse IgG and a VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA) were used for diaminobenzidine staining. It was confirmed that the human P450 antibodies in this experimental condition did not cross-react with the autologous murine P450 proteins.

Enzyme Assays. The typical incubation mixtures (total volume, 0.20 ml) consisted of microsomes in 100 mM potassium phosphate buffer (pH 7.4) containing an NADPH-generating system (0.5 mM NADP⁺, 5 mM glucose 6-phosphate, 5 mM MgCl₂, and 1 U/ml glucose-6-phosphate dehydrogenase) and a substrate. Coumarin 7-hydroxylase activity (COH) was measured as described previously (Yamazaki et al., 1999b). Briefly, the concentrations of microsomes and coumarin were 0.1 mg/ml and 1 μ M, respectively. The reaction mixture was incubated for 3 min at 37°C. The product formation was determined using high-performance liquid chromatography (HPLC) with a C₁₈ 5- μ m analytical column (4.6 \times 150 mm). Paclitaxel 6 α -hydroxylase activity (PTXOH) was determined by the method of Willey et al. (1993), with slight modifications. The concentrations of microsomes and paclitaxel were 0.2 mg/ml and 20 μ M, respectively. The reaction mixture was incubated for 10 min at 37°C. The mobile phase was acetonitrile/10 mM ammonium acetate = 40:60 (v/v). The product formation was determined using HPLC with a C₁₈ 5- μ m analytical column (4.6 \times 150 mm). The eluent was monitored at 227 nm with a noise-base clean Uni-3 (Union, Gunma, Japan). Diclofenac 4'-hydroxylase activity (DICOH) was determined by the method of Kobayashi et al. (2000), with slight modifications. The concentrations of microsomes and diclofenac were 0.2 mg/ml and 30 μ M, respectively. The reaction mixture was incubated for 30 min at 37°C. The mobile phase was 22% acetonitrile in 50 mM phosphate buffer (pH 7.4). The product formation was determined using HPLC with a C₁₈ 5- μ m analytical column (4.6 \times 150 mm). *S*-Mephenytoin 4'-hydroxylase activity (MPOH) was measured as described previously (Chiba et al., 1993), with slight modifications. The concentrations of microsomes and

S-mephenytoin were 0.2 mg/ml and 200 μ M, respectively. The reaction mixture was incubated for 30 min at 37°C. The product formation was determined using HPLC with a C₁₈ 5- μ m analytical column (4.6 \times 150 mm). The eluent was monitored at 204 nm with a noise-base clean Uni-3. The mobile phase was 18% acetonitrile in 50 mM potassium dihydrogen phosphate. Debrisoquine 4'-hydroxylase activity (DBOH) was determined by the method of Nakajima et al. (2002b). The concentrations of microsomes and debrisoquine were 0.2 mg/ml and 5 μ M, respectively. The eluent was monitored fluorometrically (excitation, 219 nm; emission, 286 nm) with a noise-base clean Uni-3. Dexamethasone 6-hydroxylase activity (DEXOH) was performed according to the method of Tomlinson et al. (1997), with slight modifications. The concentrations of microsomes and dexamethasone were 0.2 mg/ml and 100 μ M, respectively. The reaction mixture was incubated for 30 min at 37°C. The product formation was determined using HPLC with a C₉ 5- μ m analytical column (4.6 \times 150 mm). The mobile phase was 22% acetonitrile/0.018% formic acid = 18:82 (v/v). The eluent was monitored at 243 nm with a Uni-3. DEXOH was quantified using a standard curve of dexamethasone because we could not obtain authentic 6-hydroxydexamethasone. The retention time of 6-hydroxydexamethasone was confirmed using the incubation product of recombinant CYP3A4 and dexamethasone. The final concentration of the solvent in the incubation mixture was <1%.

Results

Chimeric Mice Used in the Present Study. Five chimeric mice generated using hepatocytes from donor A and seven chimeric mice generated using those from donor B were used in the present study. The hAlb concentration and RI are shown in Table 2. We confirmed that the expression of all hepatic mRNA and all enzyme activities in the chimeric mice were not affected by the administration of nafamostat mesilate (data not shown).

Expression of Human CYP2C9 in Chimeric Mice. The expres-

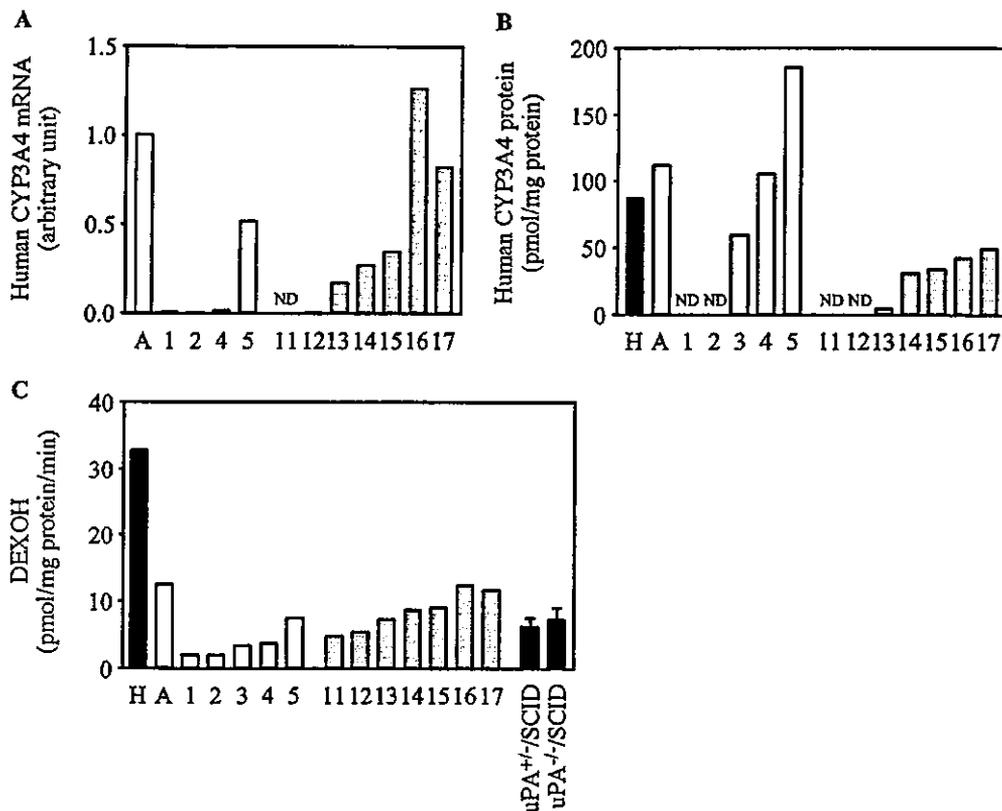


FIG. 2. Human CYP3A4 expression in the chimeric mice. Relative expression levels of human CYP3A4 mRNA (A), the expression of human CYP3A4 protein (B), and DEXOH (C) were determined as described under *Materials and Methods*. C, DEXOH catalyzed by CYP3A4 measured at 100 μ M dexamethasone. Each column represents the mean of duplicate determinations except in uPA^{+/}/SCID mice and uPA⁻/SCID mice. The columns of uPA^{+/}/SCID mice and uPA⁻/SCID mice represent the mean \pm S.D. ($n = 3$). The sample numbers are described in Table 2. H, HLM; A, donor A; ND, not detected.

sion of human CYP2C9 mRNA, the expression of human CYP2C9 protein, and DICOH in the chimeric mice were increased in a hAlb concentration-dependent manner (Fig. 1). The r values between mRNA, protein, or DICOH and a hAlb concentration in the donor A and donor B chimeric mice were 0.92 and 0.72, 0.97 and 0.95, and 0.98 and 0.93, respectively. In chimeric mouse 5 with hepatocytes from donor A, both the expression of human CYP2C9 mRNA and the expression of human CYP2C9 protein were almost the same as those in donor A. DICOH was mainly catalyzed by human CYP2C9 but not by murine Cyp2c. DICOH in the pooled HLM (1.80 nmol/mg of protein/min) was approximately 20-fold higher than that in uPA^{+/}/SCID mice (0.09 nmol/mg protein/min) and uPA⁻/SCID mice (0.09 nmol/mg protein/min). DICOH in microsomes from donor A (0.74 nmol/mg protein/min) was 41% compared with that in the pooled HLM.

Expression of Human CYP3A4 in Chimeric Mice. The expression of human CYP3A4 mRNA, the expression of human CYP3A4 protein, and DEXOH were increased in a hAlb concentration-dependent manner (Fig. 2). The r values between mRNA, protein, or DEXOH and a hAlb concentration in the donor A and B chimeric mice were 0.91 and 0.69, 0.96 and 0.96, and 0.97 and 0.87, respectively. DEXOH in the pooled HLM (32.6 pmol/mg protein/min) was approximately 5-fold higher than that in uPA^{+/}/SCID mice (6.2 pmol/mg of protein/min) and uPA⁻/SCID mice (7.2 pmol/mg protein/min). This activity in microsomes from donor A (12.5 pmol/mg protein/min) was 38% compared with that in the pooled HLM.

Expression of Human CYP2D6 in Chimeric Mice. The expression of human CYP2D6 mRNA, the expression of human CYP2D6 protein, and DBOH were increased in a hAlb concentration-dependent

manner (Fig. 3). DBOH in the pooled HLM (9.7 pmol/mg protein/min) was approximately 6.5-fold higher than that in uPA^{+/}/SCID mice (1.5 pmol/mg protein/min) and uPA⁻/SCID mice (1.5 pmol/mg protein/min). This activity in microsomes from donor A was lower compared with that in the pooled HLM.

Expression of Human CYP2C8 in Chimeric Mice. The expression of human CYP2C8 mRNA, the expression of human CYP2C8 protein, and PTXOH were increased in a hAlb concentration-dependent manner (Fig. 4). PTXOH in uPA^{+/}/SCID mice and uPA⁻/SCID mice could not be detected in this experimental condition. This activity in microsomes from donor A (100.6 pmol/mg protein/min) was 45% compared with that in the pooled HLM (224.4 pmol/mg protein/min).

Expressions of Human CYP1A2 and CYP3A5 in Chimeric Mice. The expressions of human CYP1A2 and CYP3A5 mRNA and the expressions of those proteins were increased in a hAlb concentration-dependent manner (Fig. 5). The content of human CYP1A2 and CYP3A5 protein in donor A was 1.1- and 0.2-fold higher than that in the pooled HLM, respectively. In donor A, the donor A chimeric mice, and the donor B chimeric mice, the content of human CYP3A5 protein was much lower than that in the pooled HLM.

Expression of Human CYP2C19 in Chimeric Mice. The expression of human CYP2C19 mRNA and MPOH in the chimeric mice are shown in Fig. 6. The expression of human CYP2C19 mRNA in the chimeric mice was increased in a hAlb concentration-dependent manner. MPOH in the pooled HLM (47.0 pmol/mg protein/min) was approximately 8.4- and 2.7-fold higher than that in uPA^{+/}/SCID mice (5.6 pmol/mg protein/min) and uPA⁻/SCID mice (17.3 pmol/mg protein/min), respectively. MPOH activity in donor A (10.9

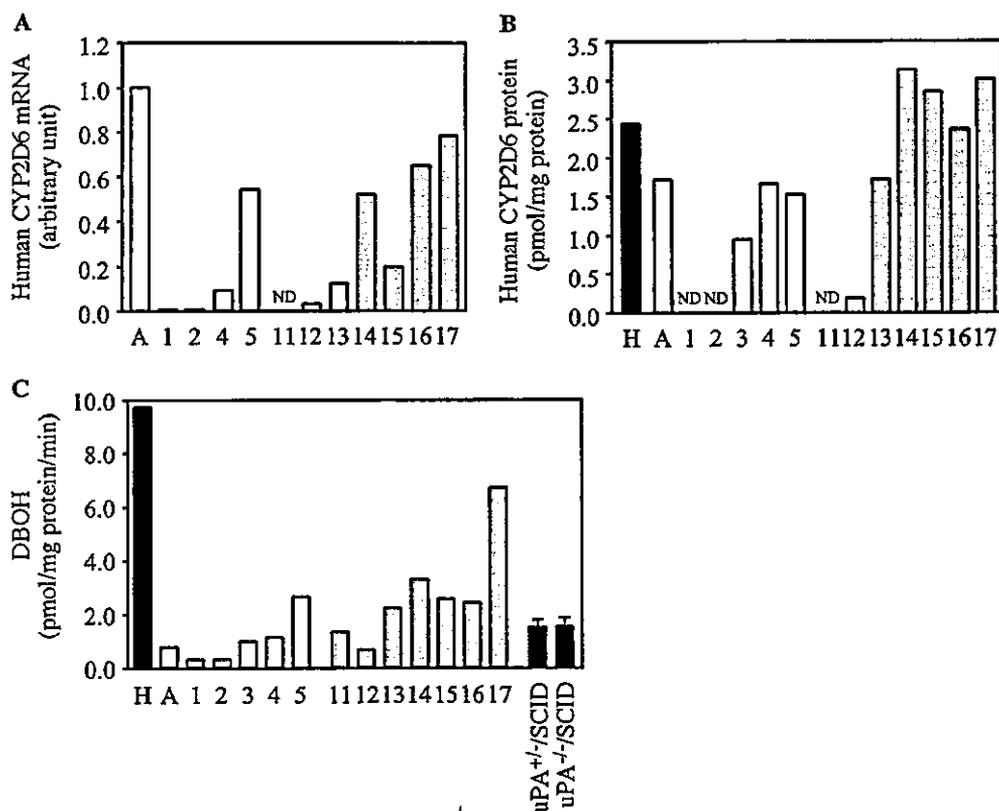


FIG. 3. Human CYP2D6 expression in the chimeric mice. Relative expression levels of human CYP2D6 mRNA (A), the expression of human CYP2D6 protein (B), and DBOH (C) were determined as described under *Materials and Methods*. C, DBOH catalyzed by CYP2D6 measured at 5 μ M debrisoquine. Each column represents the mean of duplicate determinations except in uPA^{+/-}/SCID mice and uPA^{-/-}/SCID mice. The columns of uPA^{+/-}/SCID mice and uPA^{-/-}/SCID mice represent the mean \pm S.D. ($n = 3$). The sample numbers are described in Table 2. H, HLM; A, donor A; ND, not detected.

pmol/mg protein/min) was 23% compared with that in the pooled HLM. Only chimeric mouse 5 exhibited MPOH among the chimeric mice with hepatocytes from donor A, although this activity could be detected in the chimeric mice with hepatocytes from donor B. The content of human CYP2C19 protein could not be quantified because there were no applicable primary antibodies commercially available.

Expression of Human CYP2A6 in Chimeric Mice. The expression of human CYP2A6 mRNA, the expression of human CYP2A6 protein, and COH in the chimeric mice are shown in Fig. 7. In the case of the donor A chimeric mice, neither the human CYP2A6 protein nor COH could be detected. However, in the donor B chimeric mice, the expression of human CYP2A6 protein and COH were increased in a hAlb concentration-dependent manner. COH in the pooled HLM (305.4 pmol/mg protein/min) was approximately 20.2- and 15.1-fold higher than in uPA^{+/-}/SCID mice (15.1 pmol/mg protein/min) and uPA^{-/-}/SCID mice (20.2 pmol/mg protein/min), respectively.

Genotyping of Human CYP2A6, Human CYP2C19, and Human CYP3A5 Alleles. By using genomic DNA extracted from liver, donor A and the chimeric mice with hepatocytes from the same donor were genotyped for *CYP2A6*4*, *CYP2C19*2*, and *CYP2C19*3*. Both donor A and the chimeric mice were genotyped as *CYP2A6*4/CYP2A6*4* and *CYP2C19*1/CYP2C19*2* (data not shown). Similarly, donor A, the donor A chimeric mice, and the donor B chimeric mice were genotyped for *CYP3A5*3*. All were genotyped as *CYP3A5*3/CYP3A5*3* (data not shown).

Discussion

P450 is a superfamily of the heme-containing protein that is involved in the metabolism of endogenous substrates and xenobiotics

including therapeutic drugs (Li et al., 1995). Some groups have successfully established transgenic mice with human P450s such as CYP2D6 and CYP3A4 (Corchero et al., 2001; Robertson et al., 2003; Zhang et al., 2003), which could be used to assess the toxicity of chemicals and to study the transcriptional regulation of the P450 genes. Since transgenic mouse models usually express only one human P450 with many murine P450s still retaining normal activities, their usefulness for predicting drug metabolism in humans in vivo is limited. On the other hand, human hepatocytes and hepatoma cell lines are frequently used as in vitro systems to investigate drug metabolism. Human hepatocytes retain near-normal hepatocellular morphology and the expression of the entire hepatic drug-metabolizing enzyme system in an integrated form. However, the drug-metabolizing capacity in human hepatocytes decreases during culture due to a decrease of the P450 gene transcription (Rodríguez-Antona et al., 2002). Hepatoma cell lines have unlimited life span but very low P450 expression (Guillouzo, 1998). Recently, chimeric mice whose livers could be replaced by more than 80% with human hepatocytes were established by Tateno et al. (2004). In the present study, we investigated the major human hepatic P450s in detail in these chimeric mice in terms of the mRNA, protein, and enzyme activity.

The expression of human CYP2C9 mRNA, the expression of CYP2C9 protein, and DICOH tended to correlate with the hAlb concentrations. The expression of human P450 in the chimeric mice could be monitored by the specific enzyme activity, catalyzed by human P450 but not by murine P450. DICOH was reported to be detectable in humans but not in mice (Mankowski et al., 2000). The V_{max} values of DICOH in humans were reported to be 10- or 20-fold higher than those in mice, although the K_m values were similar

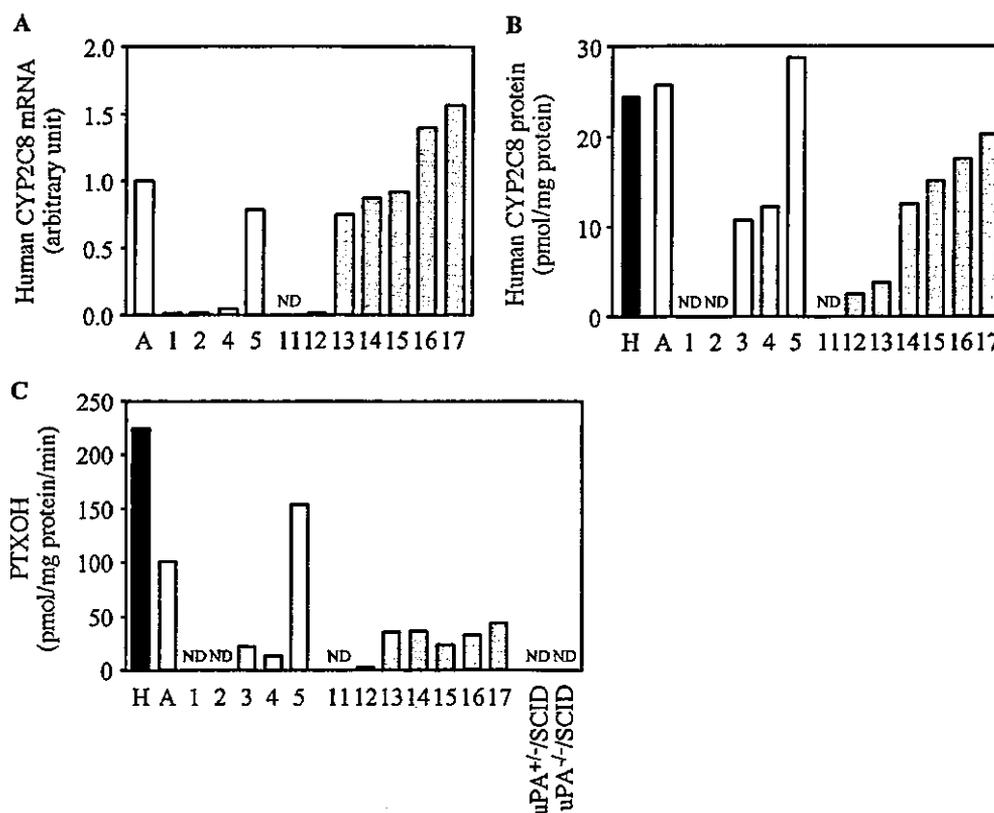


FIG. 4. Human CYP2C8 expression in the chimeric mice. Relative expression levels of human CYP2C8 mRNA (A), the expression of human CYP2C8 protein (B), and PTXOH (C) were determined as described under *Materials and Methods*. C, PTXOH catalyzed by CYP2C8 measured at 20 μ M paclitaxel. Each column represents the mean of duplicate determinations except in uPA^{+/-}/SCID mice and uPA^{-/-}/SCID mice. The sample numbers are described in Table 2. H, HLM; A, donor A; ND, not detected.

(Bogaards et al., 2000). In this study, DICOH in humans was approximately 20-fold higher than that in mice, consistent with previous reports. The investigation of DICOH in liver microsomes is suggested to be a more suitable way to distinguish human P450 from murine P450. DICOH in chimeric mouse 5 was approximately 2-fold higher than that in donor A, although the contents of CYP2C9 protein were similar. The enzyme activity in donor A microsomes might become lower during the preparation and/or storage, or human CYP2C9 protein might have been easy to express in chimeric mouse 5. The reason remains to be clarified.

The expression of human CYP3A4, human CYP2D6, and human CYP2C8 demonstrated a similar tendency to that of CYP2C9 in terms of the mRNA, protein, and DEXOH, DBOH, or PTXOH. Dexamethasone is primarily metabolized to 6-hydroxydexamethasone in humans but to 6-hydroxy-9 α -fluoro-androsta-14-diene-11 β -hydroxy-16 α -methyl-3,17-dione in mice (Tomlinson et al., 1997). DBOH is a typical reaction catalyzed by human CYP2D6, but lower DBOH was exhibited in the various strains of mice (Masubuchi et al., 1997). PTXOH has been reported the species difference in *in vitro* study using human, rat, minipig, and pig microsomes (Vaclavikova et al., 2004). DEXOH, DBOH, and PTXOH in the microsomes of uPA^{+/-}/SCID mice or uPA^{-/-}/SCID mice were lower compared with the pooled HLM. DEXOH in donor A was lower than that in the pooled HLM; thus, these activities in all of the donor A chimeric mice were low, thereby diminishing the differences between humans and mice. In CYP2D6 and CYP2C8, the enzyme activity in chimeric mouse 5 was higher than that in donor A, although the contents of protein were similar, as in CYP2C9.

From the results of CYP2C9, CYP3A4, CYP2D6, and CYP2C8, if

mRNA and protein were expressed, human P450 would exhibit the drug metabolism potency in the chimeric mouse liver. Although we could not determine the specific activities catalyzed by human CYP1A2 and CYP3A5, mRNA and the protein of human CYP1A2 and CYP3A5 were detectable in a hAlb concentration-dependent manner. Among all human P450s, the mean *r* values between mRNA, protein, or enzyme activity and a hAlb concentration were 0.82, 0.93, and 0.87, respectively. Therefore, these human P450s also retained the catalytic activity of drug metabolism. It is difficult to determine whether the ratio of each P450 content in the liver of a chimeric mouse would be the same as that of the donor. Further studies using various donors are needed to clarify this point. From our data, the amount of mRNA, the protein content, and the enzyme activity would be similar between the donor and chimeric mice with hepatocytes from the same donor with a higher concentration of hAlb.

The uPA^{+/-}/SCID mice showed hepatic failure due to proteolytic damage and could not remain alive long. In chimeric mice no. 1 and no. 12 (12–14 weeks old), some P450 activities were detected, which might be due to the partial repopulation of murine hepatocytes rather than human hepatocytes.

Another important aspect in drug metabolism for which the chimeric mice are useful is in reflecting the human polymorphic phenotypes and genotypes. Polymorphisms of P450s can cause adverse effects and interindividual variability in the metabolism of drugs in humans. It is important to confirm whether the chimeric mice have the same genotype as the donor. Until now, several CYP2A6 alleles have been reported (Nakajima et al., 2002a). One of the alleles, CYP2A6*4, deletes the whole CYP2A6 gene. Both donor A and the donor A chimeric mice were genotyped as CYP2A6*4/CYP2A6*4 using

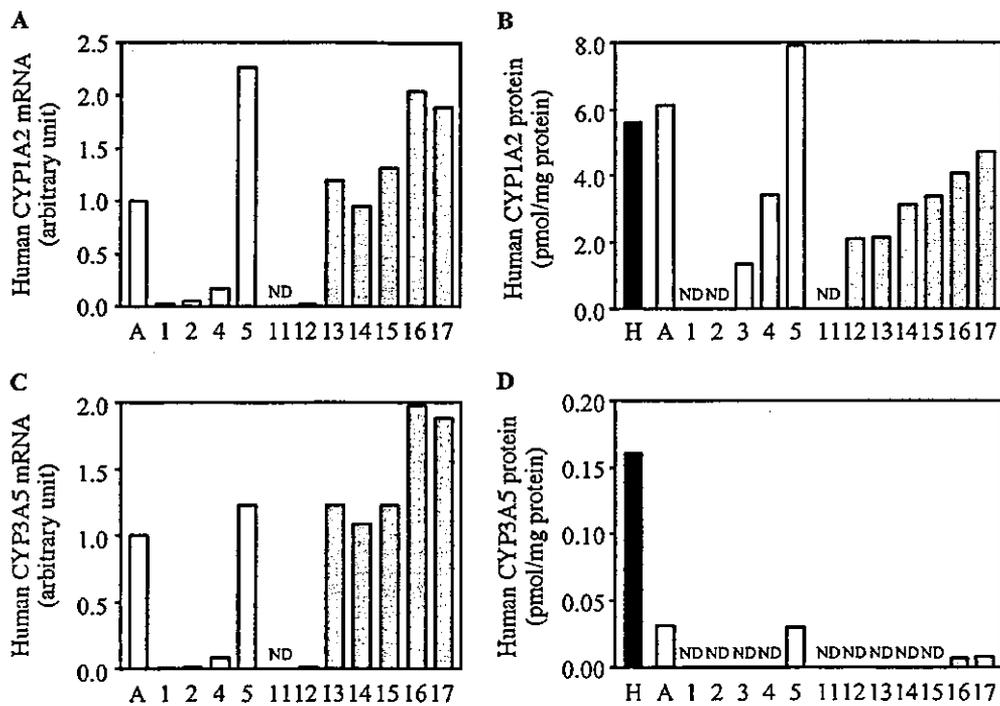


Fig. 5. Human CYP1A2 and CYP3A5 expressions in the chimeric mice. Relative expression levels of human CYP1A2 (A) and CYP3A5 (C) mRNA and human CYP1A2 (B) and CYP3A5 (D) protein were determined as described by Western blotting under *Materials and Methods*. The sample numbers are described in Table 2. H, HLM; A, donor A; ND, not detected.

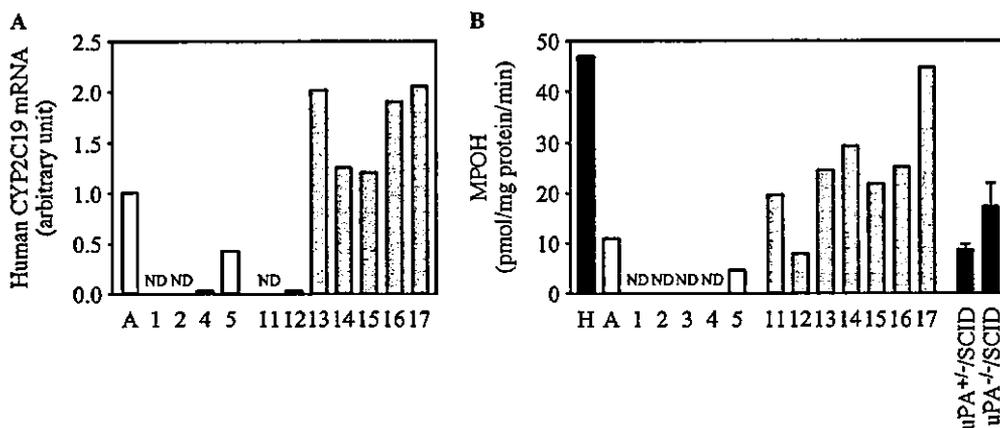


Fig. 6. Human CYP2C19 expression in the chimeric mice. Relative expression levels of human CYP2C19 mRNA (A) and MPOH (B) were determined as described under *Materials and Methods*. B, MPOH catalyzed by CYP2C19 measured at 200 μ M 5-mephenytoin. Each column represents the mean of duplicate determinations except in uPA^{+/-}/SCID mice and uPA^{-/-}/SCID mice. The columns of uPA^{+/-}/SCID mice and uPA^{-/-}/SCID mice represent the mean \pm S.D. ($n = 3$). The sample numbers are described in Table 2. H, HLM; A, donor A; ND, not detected.

genomic DNA extracted from liver. It is reasonable that the donor A chimeric mice could not detect CYP2A6 protein and COH, whereas the donor B chimeric mice exhibited CYP2A6 protein and COH. It has been reported that the V_{max} value of COH in humans was 25-fold higher than that in mice (Bogaards et al., 2000), which is consistent with the result of the present study (15–20-fold). COH is also suitable for estimating the humanization of the chimeric mice as DICOH.

The phenotype of CYP2C19 as a poor metabolizer correlates with the *CYP2C19* genotype when any combination of *CYP2C19* alleles *CYP2C19*2*, *CYP2C19*3*, *CYP2C19*4*, *CYP2C19*5*, *CYP2C19*6*, *CYP2C19*7*, and *CYP2C19*8* are present (Wedlund, 2000). Two major mutations, *CYP2C19*2* and *CYP2C19*3*, account for almost 100% of poor metabolizers in the Japanese population (de Morais et al., 1994). Both donor A and the donor A chimeric mice were

genotyped as *CYP2C19*1/CYP2C19*2* using the genomic DNA extracted from liver. Therefore, the lower MPOH activities in the donor A chimeric mice might be due to the genetic polymorphism.

In the chimeric mice with hepatocytes from donor A and donor B, the expression of human CYP3A5 protein was very low, suggesting that the expression of CYP3A5 protein would be lower in both donors. The genotype of donor A, the donor A chimeric mice, and the donor B chimeric mice were homozygous for the *CYP3A5*3* allele, which was clarified to decrease CYP3A5 protein compared with *CYP3A5*1* (Kuehl et al., 2001). The lower CYP3A5 protein levels were due to the genetic polymorphism. Taking these points into consideration, it is noteworthy to confirm that the chimeric mice retained the genotype and phenotype of the donor.

In conclusion, the livers of the chimeric mice used in the present

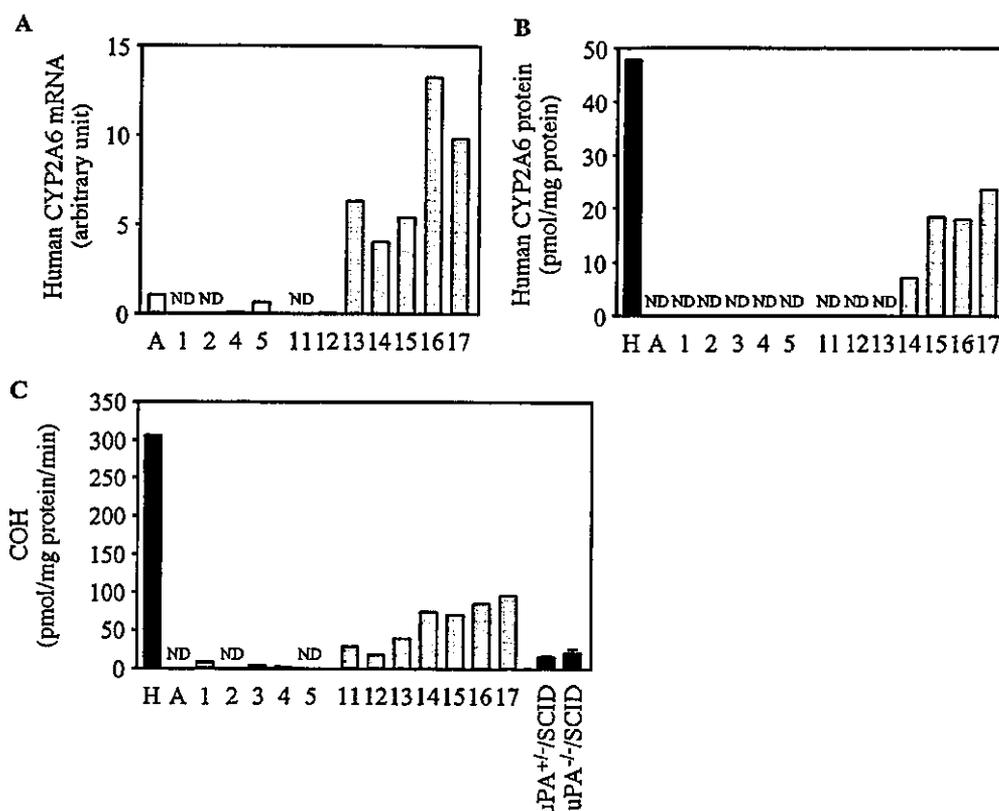


FIG. 7. Human CYP2A6 expression in the chimeric mice. The relative expression levels of human CYP2A6 mRNA (A), the expression of human CYP2A6 protein (B), and COH (C) were determined as described under *Materials and Methods*. C, COH catalyzed by CYP2A6 measured at 1 μ M coumarin. Each column represents the mean of duplicate determinations except in uPA^{+/-}/SCID mice and uPA^{-/-}/SCID mice. The columns of uPA^{+/-}/SCID mice and uPA^{-/-}/SCID mice represent the mean \pm S.D. ($n = 3$). The sample numbers are described in Table 2. H, HLM; A, donor A; ND, not detected.

study expressed human P450s and exhibited a similar capacity for drug metabolism in humans. Moreover, the liver in the chimeric mice exhibited the same genotype and phenotype as the donor, indicating that the interindividual variability due to the genetic polymorphism could be evaluated using the data from the chimeric mice. The metabolic activity in the chimeric mice with higher hAlb concentrations may be catalyzed by human P450s, and there is minimal influence by murine P450s. Further study is needed to clarify the induction and inhibition of human P450s and the expressions of phase II enzymes and transporters. In our laboratory, these studies are underway. The chimeric mice with humanized liver would become a useful model in studies of drug metabolism. We hope that the present study will contribute to the future study in drug development.

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Regular Article

Induction of Human CYP1A2 and CYP3A4 in Primary Culture of Hepatocytes from Chimeric Mice with Humanized Liver

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Summary: Chimeric mice with near-completely humanized liver were constructed by transplanting hepatocytes from a Japanese and Caucasian donor. In the present study, we investigated the induction of human CYP1A2 and CYP3A4 mRNA in a primary culture of the cryopreserved chimeric mouse hepatocytes. β -naphthoflavone (β -NF) and rifampicin (Rif) were used as typical cytochrome P450 (CYP) inducers for CYP1A2 and CYP3A4, respectively. Analysis was performed by the real-time reverse-transcription polymerase chain reaction method. CYP1A2 mRNA in the primary culture of chimeric mouse hepatocytes in mice No. 1, 2, and 3 was significantly increased 3.8-, 6.3-, and 3.3-fold by 5 μ M β -NF exposure, respectively, compared with the 0.1% DMSO treated control ($p < 0.01$). CYP3A4 mRNA in the primary culture of chimeric mouse hepatocytes in mice No. 1, 2, and 3 was significantly increased 8.4-fold ($p < 0.001$), 2.2-fold ($p < 0.01$), and 2.3-fold ($p < 0.05$) by 50 μ M Rif exposure, respectively, compared with the 0.1% DMSO treated control. The present study demonstrated that a primary culture of cryopreserved hepatocytes from chimeric mice with humanized liver could be used for evaluating the induction of drug metabolizing enzymes in human. This *in vitro* method may be a useful method for screening the induction potency of new drug candidates on drug metabolizing enzymes.

Key words: chimeric mouse liver; human hepatocytes; CYP1A2; CYP3A4; induction; real-time RT-PCR

Introduction

Drug-drug interactions are an important consideration in drug development. Human hepatocytes are used for the evaluation of induction potencies on drug-metabolizing enzymes after exposure to new drug candidates.¹⁻⁴ β -Naphthoflavone (β -NF) is a potent inducer of human CYP1A1 and CYP1A2 in primary cultures of human hepatocytes.¹¹ Rifampicin (Rif) is a potent inducer of CYP3A4 in primary cultures of human

hepatocytes.^{2,3} We previously reported a method for evaluating the mRNA induction of drug-metabolizing enzymes and transporters after exposure to probe drugs such as Rif in primary cultures of cryopreserved human hepatocytes.⁵ Garcia *et al.*⁶ and Roymans *et al.*⁷ also reported methods for evaluating the induction of CYPs after exposure of probe drugs such as β -NF and Rif in primary cultures of cryopreserved human hepatocytes.

The supply of cryopreserved human hepatocytes from donors for evaluating the induction of drug-metaboliz-

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ing enzymes is very limited. Chimera mice with near-completely humanized liver, showing a greater than 80% substitution rate from mouse liver to human liver, were constructed by the transplantation of human hepatocytes into urokinase-type plasminogen activator uPA^{-/-}/SCID mice.⁸⁾ The present study was undertaken to investigate the induction potency of human CYP1A2 and CYP3A4 mRNA in primary cultures of hepatocytes from chimeric mice with humanized liver.

Materials and Methods

Materials: β -NF and Rif was purchased from Wako Pure Chemical Industries (Osaka, Japan). Hepatocyte Culture Medium (CC-3198) was purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD, USA). Rneasy[®] Mini kit and QIAshredder[™] were purchased from QIAGEN (Hilden, Germany). Yeast tRNA was purchased from Life Technologies (Rockville, MD, USA). TaqMan One-Step RT-PCR Master Mix reagents kit, TaqMan GAPDH control reagents, TaqMan β -actin control reagents were purchased from Applied Biosystems (Foster City, CA, USA). All other chemicals and reagents used were of analytical grade.

Preparation of cryopreserved human hepatocytes using chimeric mice: The present study was approved by the Ethics Committees of Otsuka Pharmaceutical Factory, Inc. and the Hiroshima Prefectural Institute of Industrial Science and Technology Ethics Board. The human liver sample from donor A (Japanese, male, 12 years old) was obtained at autopsy after receiving written informed assent. The cryopreserved human hepatocytes from donor B (Lot number NLR, Caucasian, male, 13 years old) were purchased from *In Vitro* Technologies (Baltimore, MD, USA). The construction of the chimeric mice with near-completely humanized liver using human hepatocytes was performed according to the method of Tateno *et al.*⁹⁾ Liver cells were prepared from a chimeric mouse by the modified two-step collagenase perfusion method for rats.⁹⁾ Briefly, a 27G indwelling syringe was inserted into the portal vein and secured with a ligature. The posterior vena cava was cut, allowing outflow of solution. The liver was then perfused at 38°C with Ca²⁺- and Mg²⁺-Hank's Balanced Salt Solution (CMF-HBSS) containing 200 μ g/mL EGTA, 1 mg/mL glucose, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (Hepes), and 10 μ g/mL gentamycin for 3 min at 1.5 mL/min. The perfusion solution was then changed to CMF-HBSS containing 0.05% collagenase, 0.6 mg/mL CaCl₂, 10 mM Hepes, and 10 μ g/mL gentamycin, and perfused for 9 min at 1.5 mL/min. The liver was dissected and transferred to a dish. Liver cells were gently disaggregated in the dish with CMF-HBSS containing 10% albumin, 10 mM Hepes, and 10 μ g/mL gentamycin. The disaggregated cells were

Table 1. Characteristics of chimeric mice and hepatocyte preparations

Mouse No.	1	2	3
Donor	A	B	B
Replacement index (%)	about 80	>80	>80
Sex (mouse)	male	male	female
Viability (%)	60.1 \pm 3.8	70.1 \pm 1.1	57.8 \pm 3.6

A, Japanese, male, 12 years old; B, Caucasian, male 13 years old. Viability was determined by trypan blue dye exclusion. Viability data represent mean \pm SD of three independent experiments.

centrifuged 3 times at 50 \times g for 2 min. The pellet was suspended in medium consisting of Dulbecco's modified Eagle's medium (DMEM), 10% FBS, 20 mmol/L Hepes, 30 μ g/mL L-proline, 0.5 μ g/mL insulin, 10⁻⁷ mol/L dexamethasone, 44 mmol/L NaHCO₃, and antibiotics of 100 IU/mL penicillin G and 100 μ g/mL streptomycin. The hepatocytes from the chimeric mice were cryopreserved using a programmed deep freezer.

Monolayer culture of hepatocytes: Monolayer cultures of the cryopreserved hepatocytes were made according to the method of Nishimura *et al.*⁵⁾ Cell suspensions with viability rates of 57.8 to 70.1% as assessed by trypan blue dye exclusion were used for the experiments (Table 1).

Expose of inducers: After 48 h of inoculation, the hepatocytes were treated with inducers for 24 h. During inducer-treatment, the hepatocytes were cultured without hEGF, gentamicin, or amphotericin B under 5% CO₂ and 95% air at 37°C for exposure. β -NF (1 and 5 μ M) and Rif (10 and 50 μ M) were dissolved in DMSO (0.1%). Total RNA was extracted from the hepatocytes using the QIAshredder[™] and Rneasy[®] Mini kit.

Oligonucleotides: The pairs of forward and reverse primers and the TaqMan probes for human CYP1A2 and CYP3A4 used in the RT-PCR sequences have been reported previously.⁵⁾ The primers and TaqMan probes were synthesized by QIAGEN (Tokyo, Japan). The TaqMan probes contained 6-carboxyfluorescein (FAM) at the 5' end and 6-carboxytetramethylrhodamine (TAMRA) at the 3' end and were designed to hybridize to a sequence located between the PCR primers.

TaqMan RT-PCR conditions: Total RNA was diluted to about 4 μ g/mL with 50- μ g/mL yeast tRNA. The RT-PCR assay was performed in 50 μ L of TaqMan One-Step RT-PCR Master Mix reagents containing 300 nM forward primer, 900 nM reverse primer, 200 nM TaqMan probe, and about 20 ng of total RNA according to the method described previously.⁵⁾ Amplification and detection were performed using an ABI PRISM 7700 Sequence Detector system (Applied Biosystems) with the following profile: 1 cycle of 48°C for 30 min, 1 cycle of 95°C of 10 min, and 40 cycles each of 95°C

for 15 second and 60°C for 1 min. For human GAPDH, 200 nM forward primer, 200 nM reverse primer, and 100 nM TaqMan probe were used, and for human β -actin, 300 nM forward primer, 300 nM reverse primer, and 200 nM TaqMan probe were used.

Statistical analysis: Data analyses were performed with the ABI PRISM sequence detection software. The relative expression of each mRNA was calculated by the Δ Ct (the value obtained by subtracting the Ct value of the β -actin mRNA from the Ct value of the target mRNA), which was employed in our previous studies.¹⁰⁻¹² Namely, the amount of target relative to the β -actin mRNA was expressed as $2^{-\Delta\text{Ct}}$. Data are expressed as the ratio of the target mRNA to the β -actin mRNA. Further, the values indicate percentages relative to control. Experiments with the hepatocyte cultures were performed in duplicate and the means were calculated. Further, data represent the mean \pm SD of three independent experiments. Statistical analysis was performed using the Student's *t*-test with a significance level of $p < 0.05$.

Results and Discussion

Chimeric mice with near-completely humanized liver were constructed by transplanting hepatocytes from a Japanese (donor A) and Caucasian (donor B) (Table 1). The present study investigated the induction of human CYP1A2 and CYP3A4 mRNA in primary cultures of cryopreserved hepatocytes from chimeric mice with humanized liver. Analysis was performed by the real-time RT-PCR with TaqMan probe system.

The specificities of the RT-PCR of the primer sets and probes for the human β -actin, GAPDH, CYP1A2 or CYP3A4 mRNA to the human and mouse liver total RNA were determined. The data of the primer sets and probes using human mRNA are expressed as the ratio of target mRNA in the 4 $\mu\text{g}/\text{mL}$ uPA^{-/-}/SCID mouse liver total RNA to the target mRNA in the 4 $\mu\text{g}/\text{mL}$ Japanese (donor A) liver total RNA. The cross-reactivity of the primer sets and probes used to measure human β -actin, GAPDH, CYP1A2 or CYP3A4 mRNA was < 0.000002 (data not shown). The ability of this method to discriminate between human CYP1A1 gene expression and human CYP1A2 gene expression has already been reported.¹³ The ability of this method to discriminate between human CYP3A4 gene expression and human CYP3A5 gene expression has already been reported.¹⁴ Therefore, the specificity of the RT-PCR of the primer sets and probes for human mRNA was validated.

The ratio of GAPDH mRNA to β -actin mRNA in mouse No. 1, the endogenous control, at 0 h and 3 h of culture was 12.0 ± 0.50 and 2.46 ± 0.49 , respectively (Fig. 1). Although the GAPDH mRNA in mouse No. 1 decreased to $11.6 \pm 1.1\%$ of the initial expression level

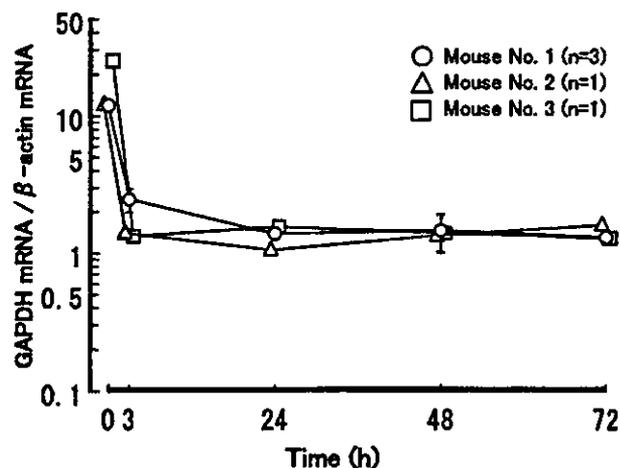


Fig. 1. Changes of human GAPDH mRNA expression in primary culture of hepatocytes from chimeric mice with humanized liver. Data are expressed as the ratio of human GAPDH mRNA to human β -actin mRNA. Data represent the mean \pm SD of three independent experiments in mouse No. 1 and individual values in mice No. 2 and 3.

at 24 h, the ratio remained constant from 24 to 72 h of culture. The profiles of the change of the ratio of GAPDH mRNA to β -actin mRNA in mice No. 2 and 3 were also similar to that in mouse No. 1, suggesting a similar profile in cryopreserved human hepatocytes as reported in our previous study.¹⁰ The ratio of GAPDH mRNA to β -actin mRNA in all chimeric mice was constant at all periods of culture for all the compounds used in the present study (data not shown). The experimental condition in this study was found to be suitable for the evaluation of the change of mRNA expression after the first 48 h of the culture.

The ratio of CYP1A2 mRNA to β -actin mRNA in mouse No. 1, 2 and 3 decreased to $0.474 \pm 0.041\%$, 0.362% and 0.910% of the initial level during the first 24 h, respectively, but the ratio remained constant from 24 to 72 h of culture (Fig. 2A). The CYP1A2 mRNA level (CYP1A2 mRNA/ β -actin mRNA ratio: 0.0736 ± 0.0082 , 0.0515 and 0.104) after 72 h culture in cryopreserved hepatocytes from chimeric mouse in mouse No. 1, 2 and 3 (Fig. 2A) was 2 to 5 times higher than that (0.0204 ± 0.0166) in cryopreserved human hepatocytes after 72 h culture.¹⁰ The ratio of CYP3A4 mRNA to β -actin mRNA in mouse No. 1, 2 and 3 decreased to $0.0346 \pm 0.0073\%$, 0.113% and 0.0402% of the initial level during the first 48 h, but the ratio remained constant from 48 to 72 h of culture (Fig. 2B). The expression of CYP3A4 mRNA was decreased to 0.13% of the initial level after 72 h culture in cryopreserved human hepatocytes as previously reported.¹⁰ Bowen *et al.*⁴ compared the initial expression level of CYP3A4 to that cultured 5 days with freshly prepared human hepatocytes and demonstrated that

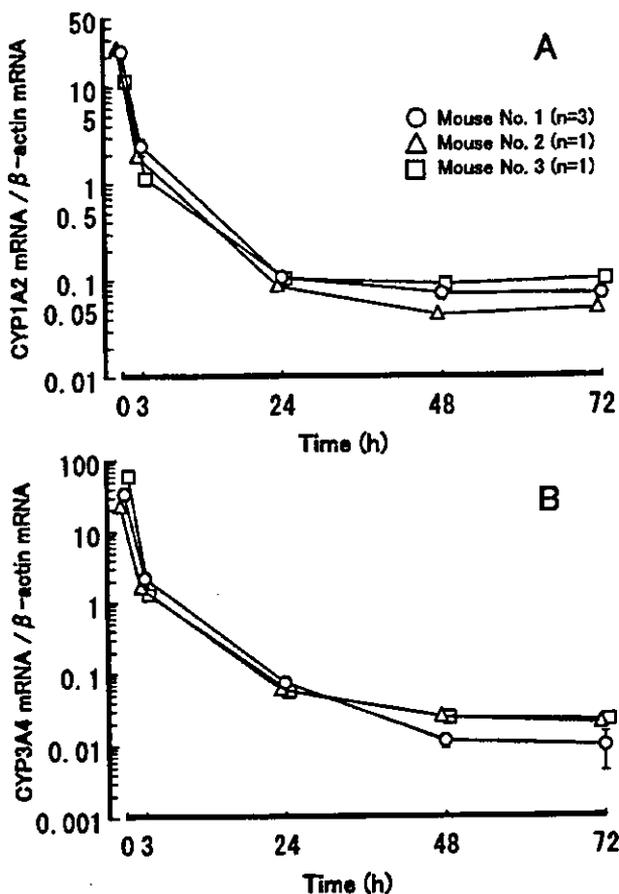


Fig. 2. Changes of human (A) CYP1A2 and (B) CYP3A4 mRNA expression in primary culture of hepatocytes from chimeric mice with humanized liver.

Data are expressed as the ratio of human CYP1A2 and CYP3A4 mRNA to human β -actin mRNA. Data represent the mean \pm SD of three independent experiments in mouse No. 1 and individual values in mice No. 2 and 3.

the CYP3A4 mRNA expression was decreased to 1% of the initial value. The CYP3A4 mRNA level (CYP3A4 mRNA/ β -actin mRNA ratio: 0.0101 \pm 0.0056, 0.0205 and 0.0226) after 72 h culture in cryopreserved hepatocytes from chimeric mice in mice No. 1, 2 and 3 (Fig. 2B) was 3 to 6 times higher than that (0.00373 \pm 0.0026) after 72 h culture in cryopreserved human hepatocytes.¹⁰ However, Bowen *et al.*⁴ and Roymans *et al.*⁷ also reported that the expression levels of CYP1A2 and CYP3A4 differed by several-fold or more among donors. Therefore, we assume that the cryopreserved hepatocytes from chimeric mice and the cryopreserved human hepatocytes demonstrate the same characteristics.

Previously, we examined the effect of DMSO on the induction of human CYP1A2 and CYP3A4 mRNAs in primary cultures of cryopreserved human hepatocytes.¹⁰ Because 0.1% DMSO appeared to show no effect on the CYP induction, the same concentration of

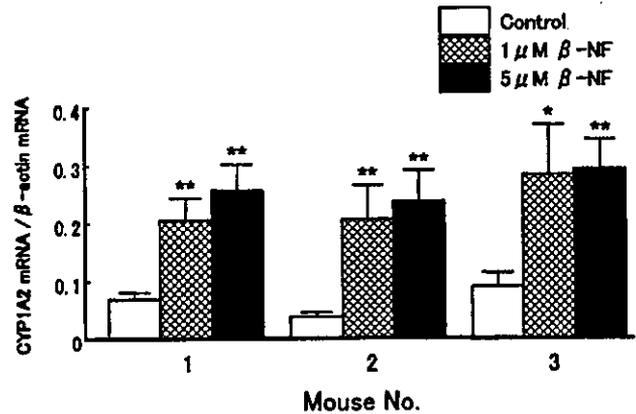


Fig. 3. Effect of β -NF exposure on the expression of human CYP1A2 mRNA in primary culture of hepatocytes from chimeric mice with humanized liver.

β -NF (1 and 5 μ M) were exposed for 24 hrs. Data are expressed as the ratio of human CYP1A2 mRNA to human β -actin mRNA. Data represent the mean \pm SD of three independent experiments. * p < 0.05 and ** p < 0.01 vs. the 0.1% DMSO control sample.

DMSO was adapted in the present study. CYP1A2 mRNA in the primary culture of chimeric mouse hepatocytes in mice No. 1, 2, and 3 was significantly increased 3.8-, 6.3-, and 3.3-fold by 5 μ M β -NF exposure, respectively, compared with the 0.1% DMSO treated control (p < 0.01) (Fig. 3), suggesting a similar increase of the CYP1A2 mRNA expression in cryopreserved human hepatocytes as reported by Garcia *et al.*⁶ CYP3A4 mRNA in mice No. 1, 2, and 3 was increased 1.9-fold, 2.0-fold (p < 0.05), and 2.8-fold (p < 0.05) with 10 μ M of Rif exposure, respectively, compared with the 0.1% DMSO control. Further, 50 μ M Rif exposure in mouse No. 1, 2, and 3 caused an 8.4-fold (p < 0.001), 2.2-fold (p < 0.01), and 2.3-fold (p < 0.05) increase in the expression of CYP3A4 mRNA, respectively, compared with the 0.1% DMSO control (Fig. 4), suggesting a similar increase of the expression of CYP3A4 mRNA in fresh human hepatocytes as reported by Bowen *et al.*⁴ and Sahi *et al.*,¹⁵ and in cryopreserved human hepatocytes as reported by Roymans *et al.*⁷ and Garcia *et al.*⁶ We also reported a similar increase in the expression of CYP3A4 mRNA in cryopreserved human hepatocytes.³

With fresh human hepatocytes, cells can only be used one time, making it difficult to compare data between studies. Cryopreserved primary human hepatocytes from a single donor could be used for multiple experiments at different times, or those from multiple donors could be used at the same time. However, the supply of cryopreserved human hepatocytes is limited. Both fresh and cryopreserved primary human hepatocytes showed high interindividual variability because of the influence of the many intrinsic and extrinsic factors. Chimeric mice with humanized liver can supply many

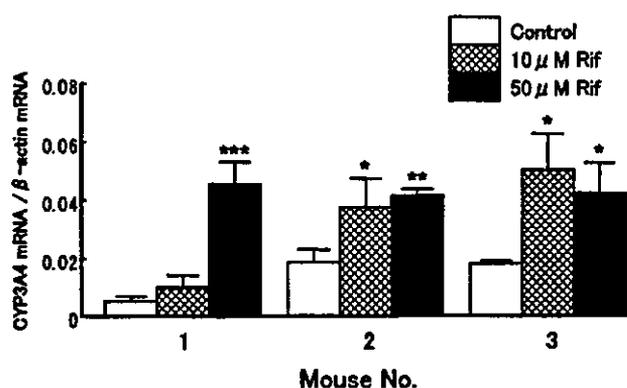


Fig. 4. Effect of Rif exposure on the expression of human CYP3A4 mRNA in primary culture of hepatocytes from chimeric mice with humanized liver.

Rif (10 and 50 μ M) were exposed for 24 hrs. Data are expressed as the ratio of human CYP3A4 mRNA to human β -actin mRNA. Data represent the mean \pm SD of three independent experiments. * p < 0.05, ** p < 0.01 and *** p < 0.001 vs. the 0.1% DMSO control sample.

cryopreserved primary hepatocytes from the same donor without interindividual variability. In comparison to the method of *in vivo* induction of drug metabolizing enzymes using chimeric mice, this *in vitro* method would be useful for screening the induction potency of new drug candidates on drug metabolizing enzymes, because the induction method using primary cultures of cryopreserved hepatocytes can provide large numbers of hepatocytes at low cost.

In conclusion, the results of the present study demonstrated that primary cultures of cryopreserved hepatocytes from chimeric mice with humanized liver would be useful in preclinical drug development to evaluate candidates for the induction of drug-metabolizing enzymes in human.

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IN VIVO INDUCTION OF HUMAN CYTOCHROME P450 ENZYMES EXPRESSED IN CHIMERIC MICE WITH HUMANIZED LIVER

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ABSTRACT:

AQ: A The induction and inhibition of human cytochrome P450 (P450) enzymes are clinically responsible for drug interactions. Although the induction of P450s is investigated using human hepatocytes in the drug development process, there are some disadvantages, such as the decline of the enzyme activity during culture. In the present study, we examined the *in vivo* induction potency in chimeric mice with humanized liver, which was recently established in Japan to clarify whether this chimeric mouse model would be more suitable for human induction studies. Rifampicin and 3-methylcholanthrene (3-MC) were used *in vivo* as typical P450 inducers in the chimeric mice. The expression levels of human CYP3A4 mRNA and CYP3A4 protein and dexamethasone 6-hydroxylase activity,

specific for human CYP3A4, were increased 8- to 22-, 3- to 10-, and 5- to 12-fold, respectively, by treatment with rifampicin. In addition, the expression levels of human CYP1A2 mRNA and CYP1A2 protein were also increased 2- to 9- and 5-fold, respectively, by treatment with 3-MC. Although other human P450s are expressed in the chimeric mice, there were few effects by the treatment of rifampicin and 3-MC on the mRNA, protein, and enzyme activity of those P450s. It was demonstrated that human P450s expressed in the chimeric mice with humanized liver were induced by rifampicin and 3-MC. This chimeric mouse model may be a useful animal model to estimate and predict the *in vivo* induction of P450s in humans.

Fabb Studies of drug metabolism are important for the determination of pharmacokinetic behavior and interindividual variability. Cytochrome P450 (P450) enzymes play a central role in the oxidative, peroxidative, and reductive metabolism of numerous endogenous compounds as well as drugs, environmental chemicals, and pollutants (Li et al., 1995). CYP3A4 is the predominant isoform in human liver and small intestine (Shimada et al., 1994; Ding and Kaminsky, 2003) and is responsible for the metabolism of many clinical drugs (Li et al., 1995). In clinical practice, serious drug interactions are frequently caused by the induction and inhibition of P450s (Dresser et al., 2000; Niemi et al., 2003). Induction is a long-term consequence of chemical exposure, whereas inhibition is an acute decrease of metabolism by another drug or a time-dependent decrease in the amount of an enzyme by several factors (Pelkonen et al., 1998). Therefore, the prediction of drug interactions involving P450s is essential during drug development.

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Recently, human hepatocytes, human liver microsomes, and recombinant human P450 microsomes have become available as enzyme sources for *in vitro* experimental systems. The major limitation of microsomes is that they cannot be used for induction studies, whereas the limitation of *in vivo* studies using experimental animals is the existence of species differences. Human hepatocytes in primary culture are considered to be the most suitable model for induction studies but also have some problems, such as the inability to proliferate, the quick degradation of P450 activities during culture, and the requirement for specific culturing or technical conditions (Li et al., 1997). On the other hand, a transgenic mouse model containing human P450 was generated (Corchero et al., 2001; Robertson et al., 2003; Zhang et al., 2003). These transgenic mice were mainly used for studying transcriptional regulation. Thus, the development of a better model of the human liver is needed.

Recently, the generation of chimeric mice with humanized liver by the transplantation of human hepatocytes has been attempted (Dandri et al., 2001; Mercer et al., 2001). Using the urokinase-type plasminogen activator (uPA)/SCID mice, Tateno et al. (2004) succeeded in establishing chimeric mice whose livers could be replaced by more than 80% with human hepatocytes. At present, there are no reports of chimeric mice with as high a percentage of human hepatocytes as that reported by Tateno et al. (2004).

ABBREVIATIONS: P450, cytochrome P450; uPA, urokinase-type plasminogen activator; SCID, severe combined immunodeficient; 3-MC, 3-methylcholanthrene; TESOH, testosterone 6 β -hydroxylase activity; hAlb, human albumin; RI, replacement index; PCR, polymerase chain reaction; HPLC, high-performance liquid chromatography; COH, coumarin 7-hydroxylase activity; DICOH, diclofenac 4'-hydroxylase activity; MPOH, S-mephenytoin 4'-hydroxylase activity; DEXOH, dexamethasone 6-hydroxylase activity; hGAPDH, human glyceraldehyde-3-phosphate dehydrogenase.

TABLE 1

Sequence of the primers used in the present study

Primer	Sequence
CYP1A1 S	5'-ATGACCAGAAGCTATGGGTC-3'
CYP1A1 AS	5'-GCACGCTGAATCCACCC-3'
CYP1A2 S	5'-GCTTCTACATCCCCAAGAAAT-3'
CYP1A2 AS	5'-TCCCACITGGCCAGGACT-3'
CYP2A6 S	5'-AGCAACAGGCCTTTCAGTT-3'
CYP2A6 AS	5'-CCAATGAAGAGGTTCAAC-3'
CYP2C8 S ^a	5'-AGATCAGAATTTTCTCACCC-3'
CYP2C8 AS ^a	5'-AACTTCGTGTAAGAGCAACA-3'
CYP2C9 S	5'-CAGATCTGCAATAATTTTCTC-3'
CYP2C9 AS	5'-CTTCAATAGTAAATTCAGATG-3'
CYP2C19 S ^a	5'-ATTGAATGAAAACATCAGGATTG-3'
CYP2C19 AS ^a	5'-GAGGGTGTGATGTCATC-3'
CYP2D6 S	5'-GGTGTGACCCATATGACATC-3'
CYP2D6 AS	5'-CTCCCGAGGCATGCACG-3'
CYP3A4 S	5'-CCAAGCTATGCTCTCACCG-3'
CYP3A4 AS	5'-TCAGGCTCCACTTACGGTGC-3'
hGAPDH S	5'-CCAGGGCTTTTAACTC-3'
hGAPDH AS	5'-GCTCCCCCTGCAAAATGA-3'

S, sense primer; AS, antisense primer.
^a From Klose et al. (1999).

TABLE 2

Chimeric mice used in the present study

Mouse No.	Donor	hAlb ^a	Approximate RI	Inducer
		mg/ml	%	
1	A	2.1	60	None
2	A	9.3	70	None
3	A	13.7	90	None
4	B	3.5	60	None
11	A	4.2	60	Rifampicin
12	A	5.7	70	Rifampicin
13	A	11.4	80	Rifampicin
14	B	2.3	50	Rifampicin
15	B	4.2	50	Rifampicin
16	B	5.2	50	Rifampicin
21	A	2.2	70	3-MC
22	A	2.8	60	3-MC
23	A	4.6	80	3-MC
24	A	13.0	80	3-MC

A, 9-month-old white male; B, 12-year-old Japanese male.
^a Human albumin concentration in chimeric mouse.

In the livers of these chimeric mice, we investigated the expression of human P450s (Kato et al., 2004). In the present study, we investigated the *in vivo* induction of human P450s by the treatment of these chimeric mice with some model P450 inducers [rifampicin and 3-methylcholanthrene (3-MC)]. When we discuss the induction of P450, it is controversial whether the mRNA, protein, or enzyme activity is optimal to evaluate the induction. Roymans et al. (2004) reported that the induction ratios of CYP3A4 mRNA and testosterone 6 β -hydroxylase activity (TESOH) by rifampicin in human hepatocytes QKR were greater than those in human hepatocytes 130, but the induction ratios of CYP3A4 protein were almost the same. On the other hand, Nallani et al. (2004) reported that there was a significant correlation between CYP3A4 protein and the testosterone 6 β -hydroxylase activity as well as between CYP3A4 protein and its mRNA after paclitaxel treatment using human hepatocytes. In induction studies using human hepatocytes or animals, some reports showed only the expression levels of mRNA, but others showed the protein or the enzyme activity. In the present study, we measured the changes of mRNA, protein, and enzyme activity.

Primarily, the changes of hepatic P450s needed to be evaluated because the induction of human P450s in the livers of the chimeric mice led to changes in the pharmacokinetics of the drugs. Therefore, we measured the mRNA, protein, and enzyme activity in the liver of the chimeric mice after rifampicin and 3-MC treatment.

Materials and Methods

Materials. All primers shown in Table 1 were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). Polyclonal rabbit anti-human CYP1A2 antibody, polyclonal rabbit anti-human CYP2A6 antibody, and polyclonal rabbit anti-human CYP2C8 antibody were purchased from Nossan (Yokohama, Japan). Polyclonal rabbit anti-human CYP1A1 was purchased from Chemicon International (Temecula, CA). Polyclonal rabbit anti-human CYP2C9 antibody, monoclonal anti-human CYP2D6 antibody, and polyclonal rabbit anti-human CYP3A4 antibody were from BD Gentest (Worburn, MA). Pooled human liver microsomes and recombinant human CYP1A1, CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2D6, and CYP3A4 expressed in baculovirus-infected insect cells were also from BD Gentest. Diclofenac and 3-MC were purchased from Sigma-Aldrich (St. Louis, MO). Coumarin, 7-hydroxycoumarin, dexamethasone, and rifampicin were purchased from Wako Pure Chemical Industries (Osaka, Japan). *S*-Mephenytoin and (\pm)-4'-hydroxymephenytoin were obtained from Toronto Research Chemicals (Toronto, Canada) and Sigma-Aldrich, respectively. 4'-Hydroxydiclofenac was purchased from BD Gentest. Nicotinamide adenine dinucleotide phosphate (oxidized form,

NADP⁺) and glucose-6-phosphate dehydrogenase were purchased from Oriental Yeast (Tokyo, Japan). Nafamostat mesilate was kindly provided by Torii Pharmaceutical (Tokyo, Japan). All other chemicals and solvents were of the highest grade commercially available.

Generation of the Chimeric Mice with Humanized Liver. The present study was approved by the Ethics Committees of Kanazawa University and the Hiroshima Prefectural Institute of Industrial Science and Technology Ethics Board. The cryopreserved human hepatocytes from donor A (9-month-old white male) were purchased from In Vitro Technologies (Catoonsville, MD). The human liver sample from donor B (12-year-old Japanese male) was obtained at autopsy after receiving written informed assent. The chimeric mice with humanized liver were generated by the method described previously (Tateno et al., 2004). Briefly, uPA^{+/+}/SCID mice at 20 to 30 days after birth were injected with human hepatocytes through a small left-flank incision into the inferior splenic pole. When necessary, the chimeric mice were treated intraperitoneally with nafamostat mesilate. The role of nafamostat mesilate was to prolong survival and promote steady gains in body weight within the 2 months after transplantation, because the chimeric mice die due to complement-induced disorders in organs other than the liver (Tateno et al., 2004). The concentration of human albumin (hAlb) in the blood of the chimeric mice and the replacement index (RI; the rate of the replacement from mice to humans) were measured using enzyme-linked immunosorbent assay and antihuman specific cytokeratin 8 and 18 antibody, respectively. There was a good correlation between the hAlb concentration and RI (Tateno et al., 2004). The male chimeric mice used in this study were 11 to 14 weeks old (Table 2). The uPA^{+/+}/SCID mice, uPA^{+/-}/SCID mice, and uPA^{-/-}/SCID mice were obtained as previously reported (Tateno et al., 2004).

Animal Treatments. The chimeric mice, uPA^{+/-}/SCID mice, and uPA^{-/-}/SCID mice were intraperitoneally treated daily for 4 days with rifampicin (50 mg/kg/day) or 3-MC (20 mg/kg/day). The mice used in the present study are listed in Tables 2 and 3.

Hepatic RNA Extraction and Real-Time Reverse Transcription-PCR. Human P450 mRNA was quantified by real-time reverse transcription-PCR. Total hepatic RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan), and cDNAs were synthesized as described previously (Iwanari et al., 2002). The sequences of primers that were specific to each human P450 are shown in Table 1. PCR was performed using the Smart Cycler (Cepheid, Sunnyvale, CA) with Smart Cycler version 1.2b software. The PCR conditions were as follows. After an initial denaturation at 95°C for 30 s, amplification was performed by denaturation at 94°C for 4 s, and annealing and extension were performed at 64°C for 20 s for 45 cycles. Amplified products were monitored directly by measuring the increase of the dye intensity of the SYBR Green I (Molecular Probes, Eugene, OR) that binds to double-strand DNA amplified by PCR. The copy number of mRNA in the cDNA samples was calculated using standard amplification curves. It was confirmed that the primer for human P450s used in this study did not cross-react with murine P450 mRNA.

INDUCTION OF HUMAN P450s IN CHIMERIC MICE IN VIVO

TABLE 3
Control mice used in the present study

Mouse No.	Strain	Inducer
M1	uPA ^{+/+} /SCID	None
M2	uPA ^{+/+} /SCID	Rifampicin
M3	uPA ^{+/+} /SCID	3-MC
M4	uPA ^{-/-} /SCID	None
M5	uPA ^{-/-} /SCID	Rifampicin
M6	uPA ^{-/-} /SCID	3-MC

Liver Microsomes. Liver microsomes from the chimeric or control mice were prepared as described previously (Yamazaki et al., 1999) and stored at -80°C until analysis. The protein concentration was determined using Bradford protein assay reagent (Bio-Rad, Hercules, CA) with bovine γ globulin as the standard.

Immunoblot Analysis of Human P450 Isoforms. SDS-polyacrylamide gel electrophoresis and immunoblot analysis of human CYP1A1, CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2D6, and CYP3A4 were performed according to Laemmli (1970) with slight modifications. The liver microsomes were separated on 7.5% polyacrylamide gel and transferred electrophoretically to a polyvinylidene difluoride membrane. Recombinant human P450s were also applied as the standards. Biotinylated anti-rabbit or mouse IgG and a VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA) were used for diaminobenzidine staining.

Enzyme Assays. The typical incubation mixtures (total volume, 0.20 ml) consisted of microsomes in 100 mM potassium phosphate buffer (pH 7.4) containing an NADPH-generating system (0.5 mM NADP⁺, 5 mM glucose 6-phosphate, 5 mM MgCl₂, and 1 U/ml glucose-6-phosphate dehydrogenase) and a substrate. Coumarin 7-hydroxylase activity (COH) catalyzed by CYP2A6 was measured as described previously (Katoh et al., 2004). In brief, the concentrations of microsomes and coumarin were 0.1 mg/ml and 1 μ M, respectively. The reaction mixture was incubated for 3 min at 37°C. The product formation was determined using high-performance liquid chromatography (HPLC) with a C₁₈ 5- μ m analytical column (4.6 \times 150 mm). Diclofenac 4'-hydroxylase activity (DICOH) catalyzed by CYP2C9 was determined as described previously (Katoh et al., 2004). The concentrations of microsomes and diclofenac were 0.2 mg/ml and 30 μ M, respectively. The reaction mixture was incubated for 30 min at 37°C. The mobile phase was 22% acetonitrile in 50 mM phosphate buffer (pH 7.4). The product formation was determined using HPLC with a C₁₈ 5- μ m analytical column (4.6 \times 150 mm). *S*-Mephenytoin 4'-hydroxylase activity (MPOH) catalyzed by CYP2C19 was measured as described previously (Katoh et al., 2004). The concentrations of microsomes and *S*-mephenytoin were 0.2 mg/ml and 200 μ M, respectively. The reaction mixture was incubated for 30 min at 37°C. The product formation was determined using HPLC with a C₁₈ 5- μ m analytical column (4.6 \times 150 mm). The eluent was monitored at 204 nm with a noise-base clean Uni-3 (Union, Gunma, Japan). The mobile phase was 18% acetonitrile in 50 mM potassium dihydrogen phosphate. Dexamethasone 6-hydroxylase activity (DEXOH) catalyzed by CYP3A4 was examined according to the method described previously (Katoh et al., 2004). The concentrations of microsomes and dexamethasone were 0.2 mg/ml and 100 μ M, respectively. The reaction mixture was incubated for 30 min at 37°C. The product formation was determined using HPLC with a C₈ 5- μ m analytical column (4.6 \times 150 mm). The mobile phase was 22% acetonitrile/0.018% formic acid = 18:82 (v/v). The eluent was monitored at 243 nm with a Uni-3. DEXOH was quantified using a standard curve of dexamethasone because we could not obtain authentic 6-hydroxy-dexamethasone. The retention time of 6-hydroxydexamethasone was confirmed using the incubation product of recombinant CYP3A4 and dexamethasone. The final concentration of the solvent in the incubation mixture was <1%. Data were analyzed using the mean of duplicate determinations.

Effect of Nafamostat Mesilate of Human P450 on Induction by Rifampicin or 3-MC in Chimeric Mice. To investigate the effect of nafamostat mesilate of human P450 on the induction by rifampicin or 3-MC, the induction of human P450 was compared between chimeric mice treated with and without nafamostat mesilate. The chimeric mice without nafamostat mesilate were not treated from 3 days before the start of the inducer treatment to the last day of this study.

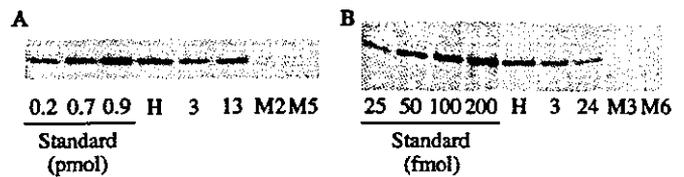


FIG. 1. Selectivity of human P450 antibodies to human P450 on immunoblot analysis. Immunoblot analyses of microsomes from pooled human liver, an induced chimeric mouse, and an induced uPA^{+/+}/SCID mouse were performed using human CYP3A4 antibodies (A) and human CYP1A2 antibodies (B). A, lanes of human liver microsomes (H) and chimeric mouse 3 were 10 μ g; lanes of chimeric mouse 13 and M2 and M5 were 2 μ g. The recombinant human CYP3A4 from BD Gentest (0.2, 0.7, and 0.9 pmol) was used as a standard. B, lanes of H and chimeric mouse 3 were 10 μ g; lanes of chimeric mouse 24 and M3 and M6 were 1 μ g. The recombinant human CYP1A2 from BD Gentest (25, 50, 100, and 200 fmol) was used as a standard. M2, rifampicin-treated uPA^{+/+}/SCID mouse; M3, 3-MC-treated uPA^{+/+}/SCID mouse; M4, rifampicin-treated uPA^{-/-}/SCID mouse; M5, 3-MC-treated uPA^{-/-}/SCID mouse.

Results

Chimeric Mice Used in the Present Study. Ten and four chimeric mice generated using hepatocytes from donor A and B were used in the present study (Table 2). The hAlb concentration, approximate RI, and inducer are shown in Table 2. The albumin concentration in the chimeric mice was measured 1 day before starting the inducer treatment. We confirmed that the expression of all hepatic mRNA and enzyme activities in uPA^{+/+}/SCID mice and uPA^{-/-}/SCID mice were not affected by the administration of nafamostat mesilate (data not shown). To investigate the induction of P450s, the expression levels of P450 mRNA and the protein content and enzyme activity in a chimeric mouse were compared to those in a chimeric mouse with a similar hAlb concentration or RI. The expression levels of human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) mRNA were increased in a hAlb concentration-dependent manner without the effect of inducers (data not shown).

Selectivity of Human P450 Antibodies to Human Microsomes in Immunoblot Analysis. The selectivities of human P450 antibodies, especially human CYP3A4 (Fig. 1A) and human CYP1A2 (Fig. 1B), were investigated. The amounts of microsomal protein used in those immunoblot analyses, which were determined not to cross-react with the autologous murine P450 proteins using induced murine liver microsomes, were as follows: CYP1A2, 10 μ g (no treatment) and 1 μ g (3-MC treatment); CYP2A6, 20 μ g; CYP2C9, 5 μ g; and CYP3A4, 10 μ g (no treatment) and 2 μ g (rifampicin treatment). Human CYP1A1 antibodies were raised against a C-terminal peptide of human CYP1A1 and did not cross-react with induced murine liver microsomes (data not shown).

Induction of Human CYP3A4 in Rifampicin-Treated Chimeric Mice. The effects of rifampicin treatment on CYP3A4 expression are shown in Fig. 2. The CYP3A4/hGAPDH mRNA ratios in rifampicin-treated chimeric mice with hepatocytes from donor A and B were 8.2- and 22.1-fold higher, respectively, than those in nontreated chimeric mice with similar concentrations of hAlb (Fig. 2A). The copy number of CYP3A4 mRNA was correlated with the hAlb concentration (Fig. 2B; none, $r = 0.77$; rifampicin, $r = 0.97$), and the slope of the fitted curve was increased in rifampicin-treated chimeric mice compared with nontreated chimeric mice. Treatment with rifampicin increased the levels of CYP3A4 protein by 9.8-fold in donor A chimeric mice and 3.0-fold in donor B chimeric mice (Fig. 2C). Rifampicin caused a significant enhancement of DEXOH in both donor A chimeric mice (5.1- to 5.3-fold; mean, 5.2) and donor B chimeric mice (10.0- to 14.6-fold; mean, 12.0) but no enhancement in uPA^{+/+}/SCID and uPA^{-/-}/SCID mice.

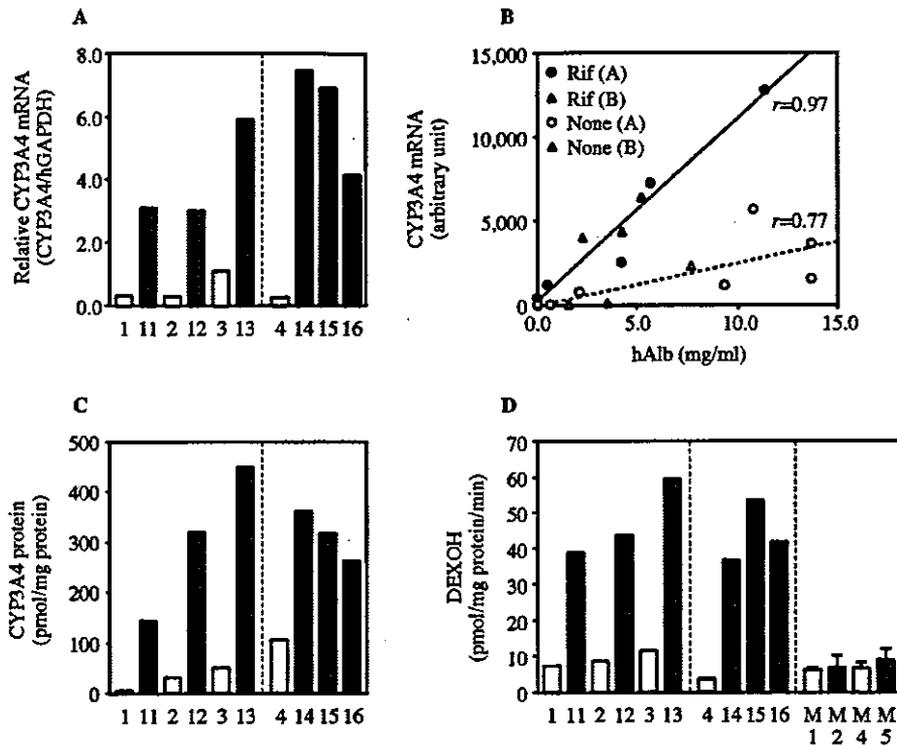


FIG. 2. Induction of human CYP3A4 expression in rifampicin-treated chimeric mice. Hepatic CYP3A4 mRNAs in the chimeric mice were measured by real-time PCR (A and B). The CYP3A4 mRNA was expressed as the relative expression to hGAPDH (A, CYP3A4/hGAPDH) and copy number (B, arbitrary unit). The CYP3A4 protein content (C) and DEXOH catalyzed by CYP3A4 (D) were determined by Western blot analysis and HPLC, respectively. A, C, and D, open and closed columns are expressed values of the non- and rifampicin-treated chimeric mice, respectively. B, circles and triangles represent the chimeric mice with hepatocytes from donor A and B, respectively. The open and closed symbols represent non- and rifampicin-treated chimeric mice, respectively. D, columns of M1, M2, M4, and M5 represent the mean \pm S.D. ($n = 3$). None, nontreated; Rif, rifampicin-treated; M1, nontreated uPA^{+/+}/SCID mouse; M2, rifampicin-treated uPA^{+/+}/SCID mouse; M4, nontreated uPA^{-/-}/SCID mouse; M5, rifampicin-treated uPA^{-/-}/SCID mouse.

Changes of Human CYP2A6 Expression in Rifampicin-Treated Chimeric Mice. The effects of rifampicin treatment on the CYP2A6 expression are shown in Fig. 3. The CYP2A6 mRNA, protein, and COH in the chimeric mice from donor B hepatocytes were not detected since these chimeric mice were genotyped as homozygous for the human CYP2A6*4 allele (Kato et al., 2004), which deletes the whole human CYP2A6 gene. The CYP2A6/hGAPDH mRNA ratios in rifampicin-treated chimeric mice with hepatocytes from donor A were 1.7-fold higher than those in nontreated chimeric mice with similar concentrations of hAlb (Fig. 3A). Rifampicin treatment increased the CYP2A6 protein and COH by 3.5- and 2.4-fold, respectively (Fig. 2, B and C). There were no changes of COH caused by rifampicin treatment in either uPA^{+/+}/SCID or uPA^{-/-}/SCID mice.

Changes of Human CYP2C9 Expression in Rifampicin-Treated Chimeric Mice. The effects of rifampicin treatment on CYP2C9 expression are shown in Fig. 4. The CYP2C9/hGAPDH mRNA ratio, protein content, and DICOH in rifampicin-treated chimeric mice with hepatocytes from donor B were 22.1-, 1.3-, and 1.8-fold higher, respectively, than those in nontreated chimeric mice with similar concentrations of hAlb (Fig. 4). On the other hand, the chimeric mice with hepatocytes from donor A did not exhibit evidence of the induction. No changes of DICOH were caused by rifampicin treatment in either uPA^{+/+}/SCID or uPA^{-/-}/SCID mice.

Changes of Human CYP2C19 Expression in Rifampicin-Treated Chimeric Mice. The effects of rifampicin treatment on CYP2C19 expression are shown in Fig. 5. The CYP2C19/hGAPDH mRNA ratios in rifampicin-treated chimeric mice with hepatocytes from donor A and B were higher than those in nontreated chimeric mice (Fig. 5A). The content of human CYP2C19 protein could not be quantified

because no applicable primary antibodies are commercially available. There was not enough MPOH to calculate the induction ratio in the chimeric mice with donor B hepatocytes, but there was a 2.4-fold increase by rifampicin treatment in the chimeric mice with donor A hepatocytes (Fig. 5B). No changes in MPOH were caused by rifampicin treatment in either uPA^{+/+}/SCID mice or uPA^{-/-}/SCID mice.

Changes of Other Human P450s in Rifampicin-Treated Chimeric Mice. The mRNA and protein expressions of human CYP1A2 and CYP2D6 were not affected by treatment with rifampicin. The expression levels of human CYP2C8 mRNA in rifampicin-treated chimeric mice were 1.4- to 3.7-fold higher than those in nontreated chimeric mice. Similarly, the expression of human CYP2C8 protein was increased 1.6- to 2.5-fold by rifampicin (data not shown).

Induction of Human CYP1A2 Expression in 3-MC-Treated Chimeric Mice. The effects of 3-MC treatment on human CYP1A2 (hCYP1A2) expression are shown in Fig. 6. The hCYP1A2/hGAPDH mRNA ratios in 3-MC-treated chimeric mice were 2.1- to 8.6-fold higher than those in nontreated chimeric mice (Fig. 6A). The slope of the fitted curve of human CYP1A2 mRNA was increased in 3-MC-treated chimeric mice compared with nontreated chimeric mice (Fig. 6B; none, $r = 0.86$; 3-MC, $r = 0.96$). The 3-MC treatment increased the content of human CYP1A2 protein by 4.9-fold (3.6-6.4) in donor A chimeric mice (Fig. 6C). Because of the lack of a substrate with specificity to human CYP1A2 that does not cross-react with murine Cyp1a, the induction of human CYP1A2 activity in the chimeric mice could not be evaluated.

Changes of Human CYP1A1 Expression in 3-MC-Treated Chimeric Mice. The effects of 3-MC on human CYP1A1 (hCYP1A1) expression are shown in Fig. 7. The hCYP1A1/hGAPDH mRNA

INDUCTION OF HUMAN P450s IN CHIMERIC MICE IN VIVO

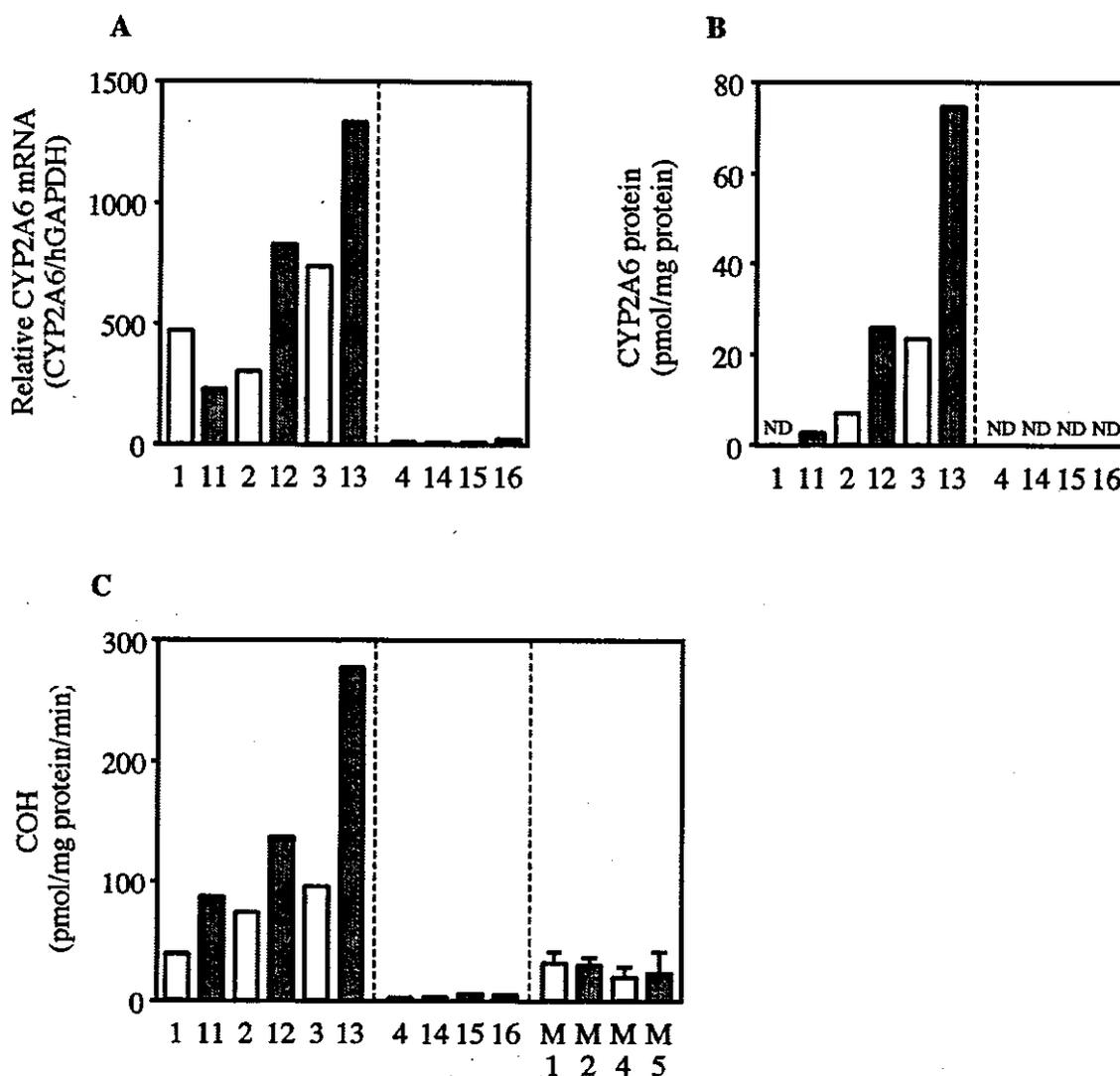


FIG. 3. Changes of human CYP2A6 expression in rifampicin-treated chimeric mice. Hepatic CYP2A6 mRNAs in the chimeric mice measured by real-time PCR were expressed as the relative expression to hGAPDH (A, CYP2A6/hGAPDH). The CYP2A6 protein content (B) and COH catalyzed by CYP2A6 (C) were determined by Western blot analysis and HPLC, respectively. Open and closed columns are the values of the non- and rifampicin-treated chimeric mice, respectively. C, columns of M1, M2, M4, and M5 represent the mean \pm S.D. ($n = 3$). ND, not detected; M1, nontreated uPA^{+/+}/SCID mouse; M2, rifampicin-treated uPA^{+/+}/SCID mouse; M4, nontreated uPA^{-/-}/SCID mouse; M5, rifampicin-treated uPA^{-/-}/SCID mouse.

ratios were increased 18.7-fold (10.0–37.7) by 3-MC treatment (Fig. 7A). The slope of the fitted curve of human CYP1A1 mRNA was increased in 3-MC-treated chimeric mice compared with nontreated chimeric mice (Fig. 7B; none, $r = 0.83$; 3-MC, $r = 0.82$). Human CYP1A1 proteins in both non- and 3-MC-treated chimeric mice were below 0.6 fmol/mg protein.

Changes of Other Human P450s in Chimeric Mice. The changes of CYP3A4 protein, CYP2A6 protein, CYP2C9 protein, DEXOH, COH, DICOH, and MPOH by 3-MC treatment are shown in Fig. 8. The treatment with 3-MC did not affect those enzyme activities. Similarly, in both uPA^{+/+}/SCID and uPA^{-/-}/SCID mice, there were no changes in DEXOH, COH, DICOH, and MPOH by 3-MC treatment. The expression levels of mRNA and protein in CYP2C8 and CYP2D6 exhibited no changes by 3-MC treatment (data not shown).

Effect of Nafamostat Mesilate of Human P450 on Induction by Rifampicin or 3-MC in Chimeric Mice. In the induction study of rifampicin, the induction of DEXOH was 38.7, 43.7, and 59.2 pmol/mg of protein per minute in chimeric mice 11, 12, and 13, respectively, which were treated with both rifampicin and nafamostat

mesilate (Fig. 3D). In the chimeric mice treated with rifampicin but not with nafamostat mesilate, DEXOH was 34.1 (hAlb concentration = 5.0 mg/ml), 43.3 (hAlb concentration = 7.6 mg/ml), and 45.8 (hAlb concentration = 9.6 mg/ml) pmol/mg of protein per minute. On the other hand, in the induction study of 3-MC, the expression levels of human CYP1A2 protein were 13.7, 17.9, and 17.8 pmol/mg of protein in chimeric mice 22, 23, and 24, respectively (Fig. 6C). In the chimeric mice treated with 3-MC but not with nafamostat mesilate, the expression levels of human CYP1A2 protein were 10.6 (hAlb concentration = 5.1 mg/ml), 17.0 (hAlb concentration = 7.6 mg/ml), and 20.0 pmol/mg of protein (hAlb concentration = 9.9 mg/ml). Nafamostat mesilate treatment caused no significant differences in the induction of DEXOH by rifampicin and CYP1A2 protein by 3-MC.

Discussion

The induction and inhibition of P450 enzymes are considered to cause many drug interactions. In the drug development process, it is extremely important to investigate whether a drug candidate will be an inducer or an inhibitor of P450s to predict potential drug interactions

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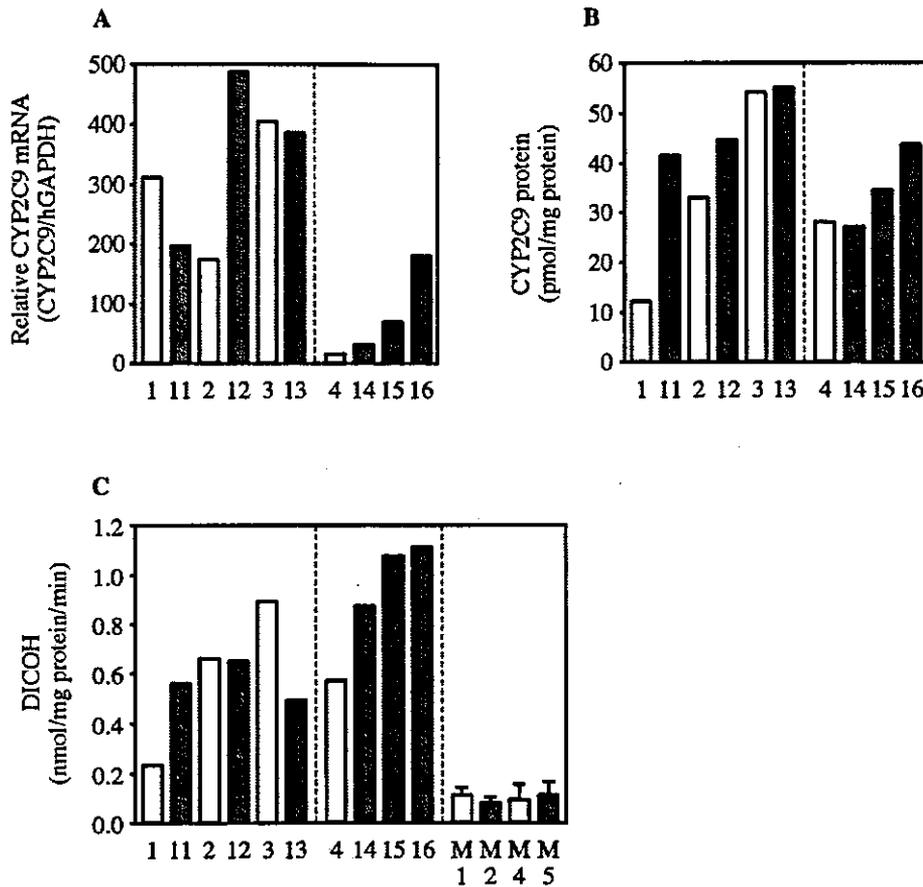


FIG. 4. Changes of human CYP2C9 expression in rifampicin-treated chimeric mice. Hepatic CYP2C9 mRNAs in the chimeric mice measured by real-time PCR were expressed as the relative expression to hGAPDH (A, CYP2C9/hGAPDH). The CYP2C9 protein content (B) and DICOH catalyzed by CYP2C9 (C) were determined by Western blot analysis and HPLC, respectively. Open and closed columns are the values of the non- and rifampicin-treated chimeric mice, respectively. C, columns of M1, M2, M4, and M5 represent the mean \pm S.D. ($n = 3$). M1, nontreated uPA^{+/+}/SCID mouse; M2, rifampicin-treated uPA^{+/+}/SCID mouse; M4, nontreated uPA^{-/-}/SCID mouse; M5, rifampicin-treated uPA^{-/-}/SCID mouse.

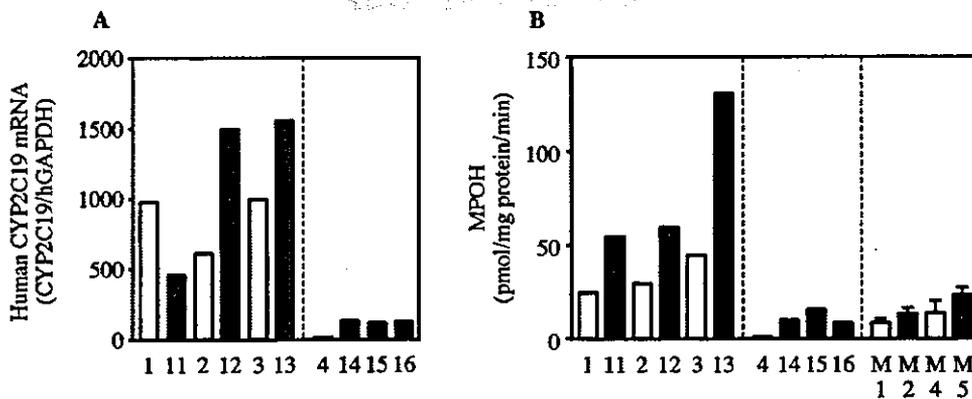


FIG. 5. Changes of human CYP2C19 expression in rifampicin-treated chimeric mice. Hepatic CYP2C19 mRNAs in the chimeric mice measured by real-time PCR were expressed as the relative expression to hGAPDH (A, CYP2C19/hGAPDH). The MPOH catalyzed by CYP2C19 (B) was determined by HPLC. Open and closed columns are the values of the non- and rifampicin-treated chimeric mice, respectively. B, columns of M1, M2, M4, and M5 represent the mean \pm S.D. ($n = 3$). ND, not detected; M1, nontreated uPA^{+/+}/SCID mouse; M2, rifampicin-treated uPA^{+/+}/SCID mouse; M4, nontreated uPA^{-/-}/SCID mouse; M5, rifampicin-treated uPA^{-/-}/SCID mouse.

in humans (Lin and Lu, 2001). The induction of P450 is defined as an increase in P450 activity associated with an increase in the intracellular P450 concentration (Ronis and Ingelman-Sundberg, 1999). At present, the inhibition of P450s in humans can be predicted relatively easily from in vitro approaches using recombinant human P450s and/or human liver microsomes; however, the results of P450 induction studies obtained from experimental animals are difficult to ex-

trapolate to humans due to species differences. Primary-cultured human hepatocytes are considered to be a more appropriate tool for the evaluation of the induction in humans; however, it has been reported that P450 mRNA in human hepatocytes declined rapidly after the isolation from liver (Gómez-Lechón et al., 2003). Moreover, the supply of human hepatocytes is sometimes limited.

Recently, chimeric mice with humanized liver were established by

INDUCTION OF HUMAN P450s IN CHIMERIC MICE IN VIVO

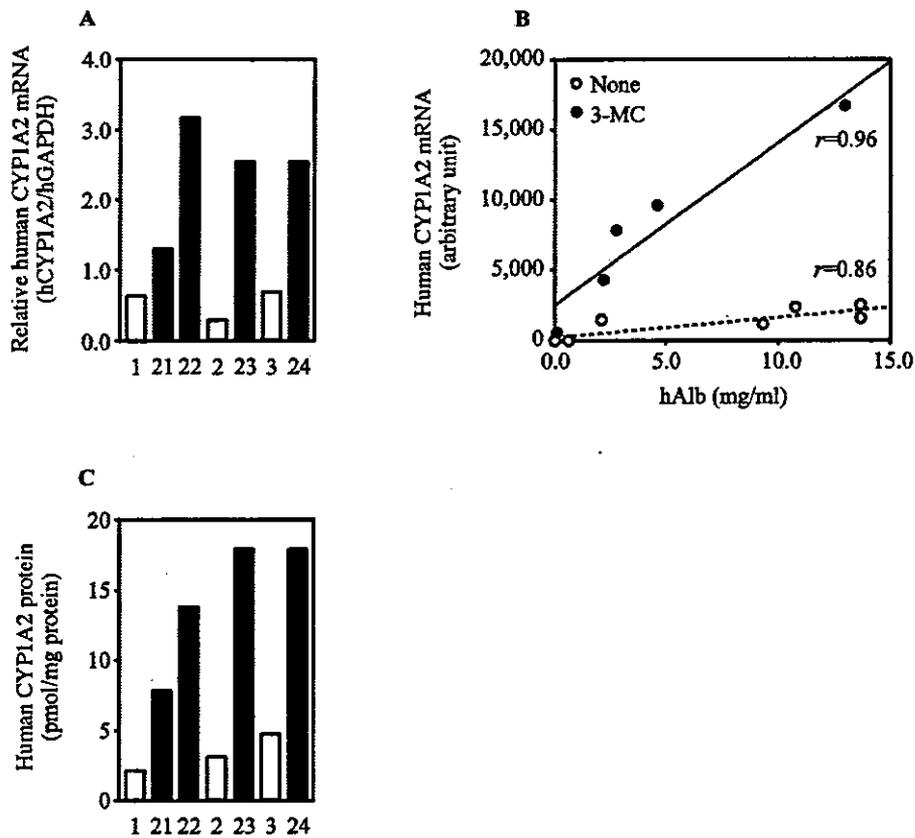


FIG. 6. Changes of human CYP1A2 expression in 3-MC-treated chimeric mice. Hepatic human CYP1A2 mRNAs in the chimeric mice were measured by real-time PCR (A and B). The human CYP1A2 mRNA was expressed as the relative expression to human GAPDH (A, hCYP1A2/hGAPDH) and the copy number (B, arbitrary unit). The human CYP1A2 protein content (C) was determined by Western blot analysis. A and C, open and closed columns are values of the non- and 3-MC-treated chimeric mice, respectively. None, nontreated; Rif, rifampicin-treated; ND, not detected.

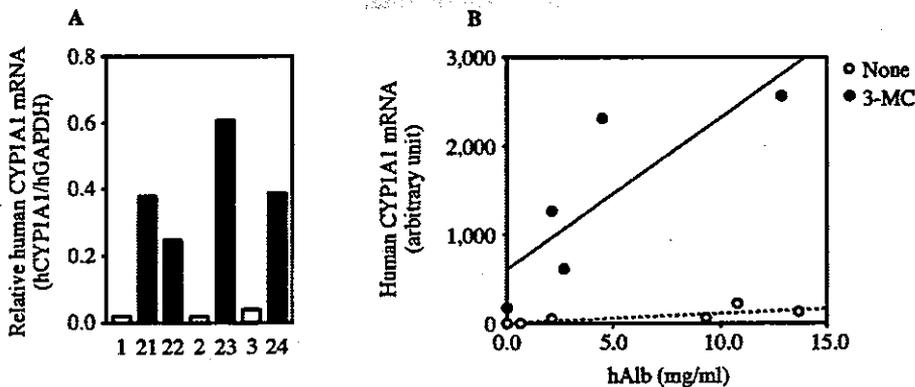


FIG. 7. Changes of human CYP1A1 expression in 3-MC-treated chimeric mice. Hepatic human CYP1A1 mRNAs in the chimeric mice were measured by real-time PCR (A and B). The human CYP1A1 mRNA was expressed as the relative expression to human GAPDH (A, hCYP1A1/hGAPDH) and the copy number (B, arbitrary unit). A, open and closed columns are the expressed values of the non- and 3-MC-treated chimeric mice, respectively. None, nontreated; Rif, rifampicin-treated.

Tateno et al. (2004). The livers of the mice could be replaced by more than 80% with human hepatocytes. We clarified that major human P450s were expressed in such livers and that the enzyme activities were almost the same as those of the donor (Katoh et al., 2004). In this study, we investigated the induction potency of human P450s in chimeric mice with humanized liver to determine whether the chimeric mice can be a useful tool in studies of human P450 induction.

There are many drug interactions with rifampicin caused by the induction of drug-metabolizing enzymes such as CYP3A4 (Niemi et al., 2003). It has been reported that the exposure of human hepatocytes

to rifampicin caused an approximately 10- and 5-fold increase in the expression levels of CYP3A4 mRNA and the protein content, respectively (Drocourt et al., 2001; Desai et al., 2002; Raucy et al., 2002). The induction effects of rifampicin on TESO_H catalyzed by CYP3A4 in human hepatocytes averaged 10-fold compared with the control, but large interindividual differences were reported (Desai et al., 2002; Madan et al., 2003). In the present study, rifampicin significantly induced CYP3A4 mRNA (8.2-fold in donor A chimeric mice and 22.1-fold in donor B chimeric mice) and increased the protein content (9.8- and 3.0-fold) and DEXOH (5.2- and 12.0-fold) in a manner