ている。

7. 今後の課題

　ヒト肝細胞キメラマウスは、カナダやドイツの研究者らが示したように、HBV と HCV を感染させることができ、HBV や HCV の感染のメカニズムの解明や、抗ウィルス剤の開発に利用可能と考えられる。私達が開発した高置換のキメラマウスにおいても、HBV や HCV の感染は可能であった。今後、これまで知られている抗ウィルス薬の効果について調べる予定である。

　これまでの私達の経験から、キメラマウスを作製できるドナー肝細胞は限られている。低年齢のドナーの方がマウス肝臓への生着や増殖が良いが、低年齢であってもマウスに生着しないドナー肝細胞も存在する。今後、マウスへのヒト肝細胞の生着および増殖のしやすさを決める因子を明らかにしたいと考えている。それが明らかになれば、さまざまな年齢の、また種々の病気を持つヒト肝細胞から構築されたマウス肝臓も作製できるのではないかと考えている。それが可能となれば、患者の肝細胞を持つキメラマウスを用いて、それぞれの患者にあった薬や医薬品や治療法の検討も行えるのではないかと期待している。

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表1 キメラマウス作製法

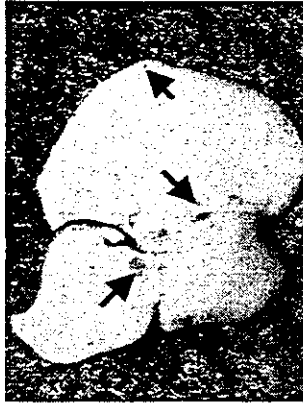| ホストマウス	ドナー肝細胞	移植時の マウス日齢	移植細胞数 (x10^{5}個)	置換率 (%,最高で)	用途	文献
uPA (+/-)	MT-lacZマウス	9-18	7.5-17	80	-	Rhimら (1994)[2]
FAH (-/-)	ROSA-26マウス	42-48	0.01	95	-	Overturf (1997)[3]
uPA (+/+)/nude (+/+)	ラット	10, 15	1-2	100	-	Rhimら (1995)[4]
uPA (+/-)/RAG2 (-/-)	ウッドチャック	10-18	5-10	95	WBV感染	Petersenら (1998)[5]
uPA (+/-)/RAG2 (-/-)	ヒト	13-21	5	15	HBV感染	Dandriら (2001)[6]
uPA (+/+)/SCID (+/+)	ヒト	5-14	10	50	HCV感染	Mercerら (2001)[7]
uPA (+/+)/SCID (+/+)	ヒト	20-30	5-10	96	薬物動態	Tatenoら (2004)[8]

uPA (+/-) /SCID, 6 wks

uPA (+/+) /SCID, 6 wks

図1．uPA/SCIDマウスの肝臓

ヘミマウス (uPA+/-/SCID) 肝臓は生後6週で導入遺伝子の欠失により生じたマウス肝細胞コロニーが多数観察される。一方、ホモマウス (uPA+/+/SCID) では、生後6週では小さなコロニーがわずかに観察されるのみである。矢印はマウス肝細胞コロニーを示す
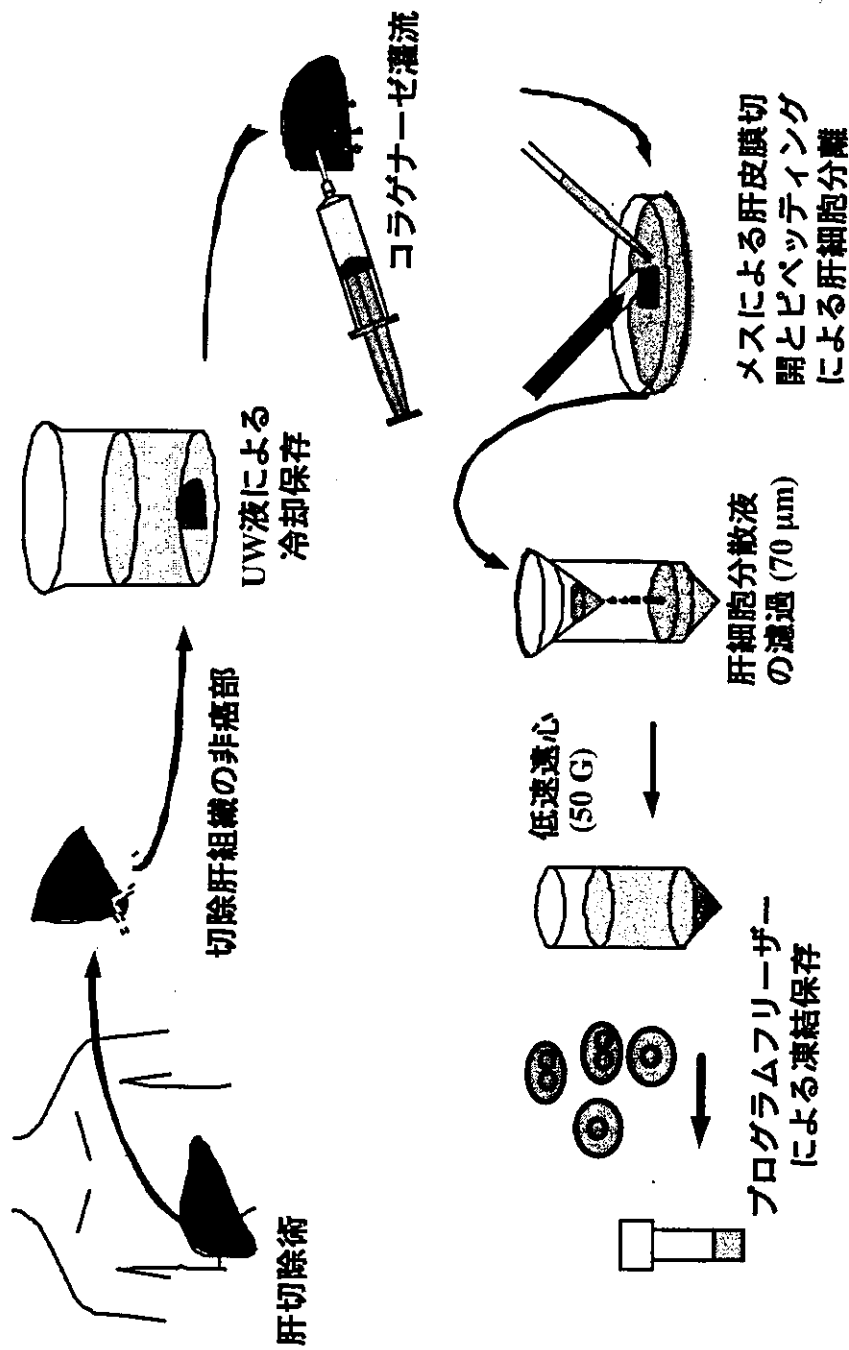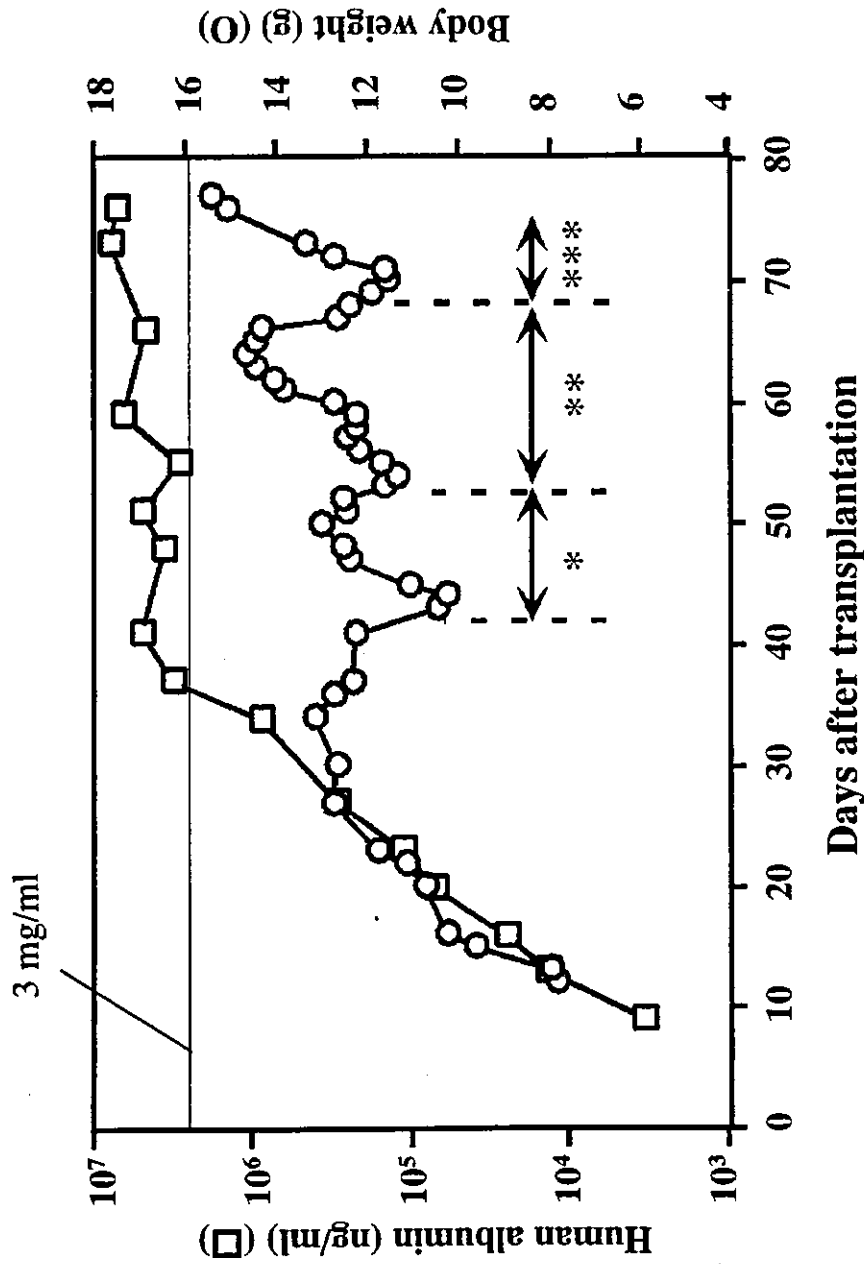
図 2. ヒト肝細胞分離の流れ

転移性肝癌などの切除肝から正常部位を切り出し、UW液で脱血する。EGTAを含む緩衝液に続いてコラゲナーゼを含む緩衝液で灌流し、柔らかくなったら細胞を分散させる。70 μmのフィルターで濾過後、低速遠心分離により沈殿を採取しプログラムフリーザーにより凍結し液体窒素下で保存する。

図3. 補体抑制剤投与におけるキメラマウス血中ヒトアルブミン濃度とマウス体重の変化

Futhan投与頻度：＊　1回／2日、＊＊1回／1日、＊＊＊2回／1日

生後3週のホモマウスに5 ×10⁵個のヒト肝細胞（12才、男児）を移植し、マウス血中ヒトアルブミン濃度と体重を経時的に測定した。その内の1例を示す。体重の減少が観察されたマウスにFuthanを腹腔内投与し、ヒトアルブミン濃度と体重の経過観察を行った。体重の減少が再び観察された場合、Futhan投与の頻度を増加した。このマウスの最終的はヒトアルブミン濃度は7.1 mg/mlで置換率は82%であった。

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移植後経過日数

縦軸: ヒトアルブミン濃度 (ng/ml)
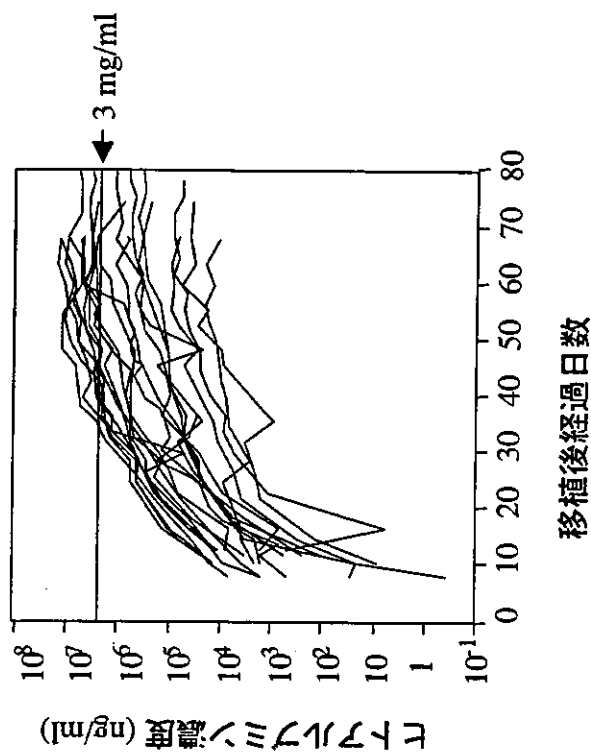
図4 キメラマウス血中のヒトアルブミン濃度の経時的変化

生後3週のホモマウス22匹に5-7.5 x10⁵個のヒト肝細胞（9ヶ月、男児）を移植し、マウス尾から2 μlの血液を採取し、マウス血中ヒトアルブミン濃度を経時的に測定した。ヒトアルブミン濃度が2 mg/mlを越えた11匹のマウスにはFuthanを腹腔内投与し、最終的に8匹のマウスにおいてヒトアルブミン濃度が3 mg/mlを越えた。
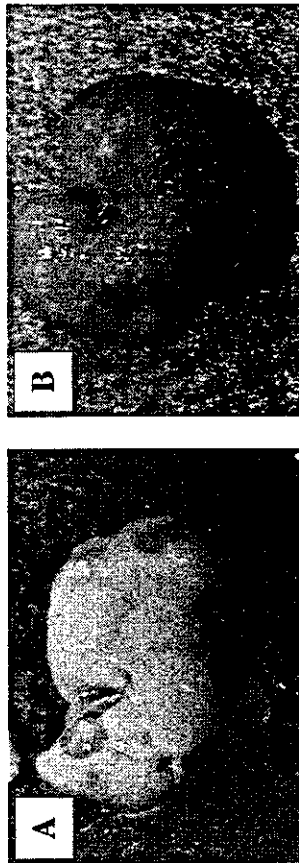
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図5　キメラマウス肝臓
生後3週のホモマウスに7.5-8 x10⁵個のヒト肝細胞（9ヶ月、男児）を移植し、移植後71日目(A)と86日目(B)に解剖した。ヒトアルブミン濃度が0 mg/mlのマウス(A)は置換率0%であった。ヒトアルブミン濃度が8.7 mg/mlのマウス(B)は置換率82%であった。
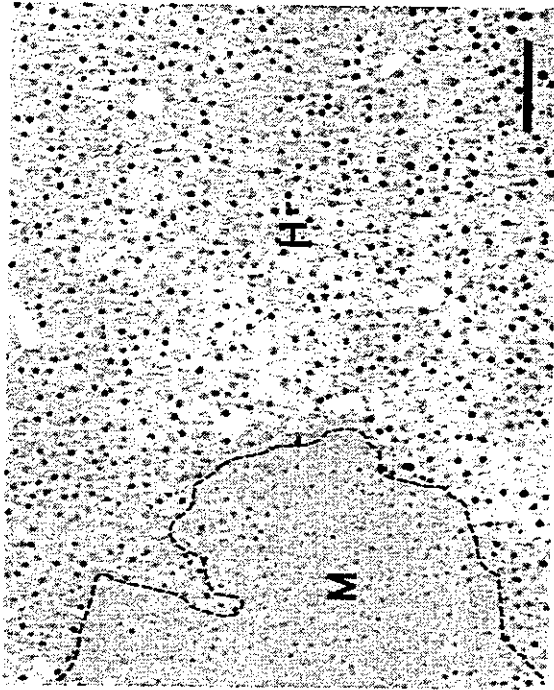
図6 ヒトcDNAを用いた*in situ* hybridizationによるキメラマウス肝臓における ヒト肝細胞の検出

生後3週のホモマウスに4×10⁵個のヒト肝細胞（12才、男児）を移植し、移植後71日目に解剖した。肝臓の凍結切片をヒトcDNAを用いて*in situ* hybridizationを行った。核が濃染している細胞領域（H）がヒト肝細胞に置換された部位で、核が薄い部分がマウス肝細胞領域（M）である。バーは100 μm。

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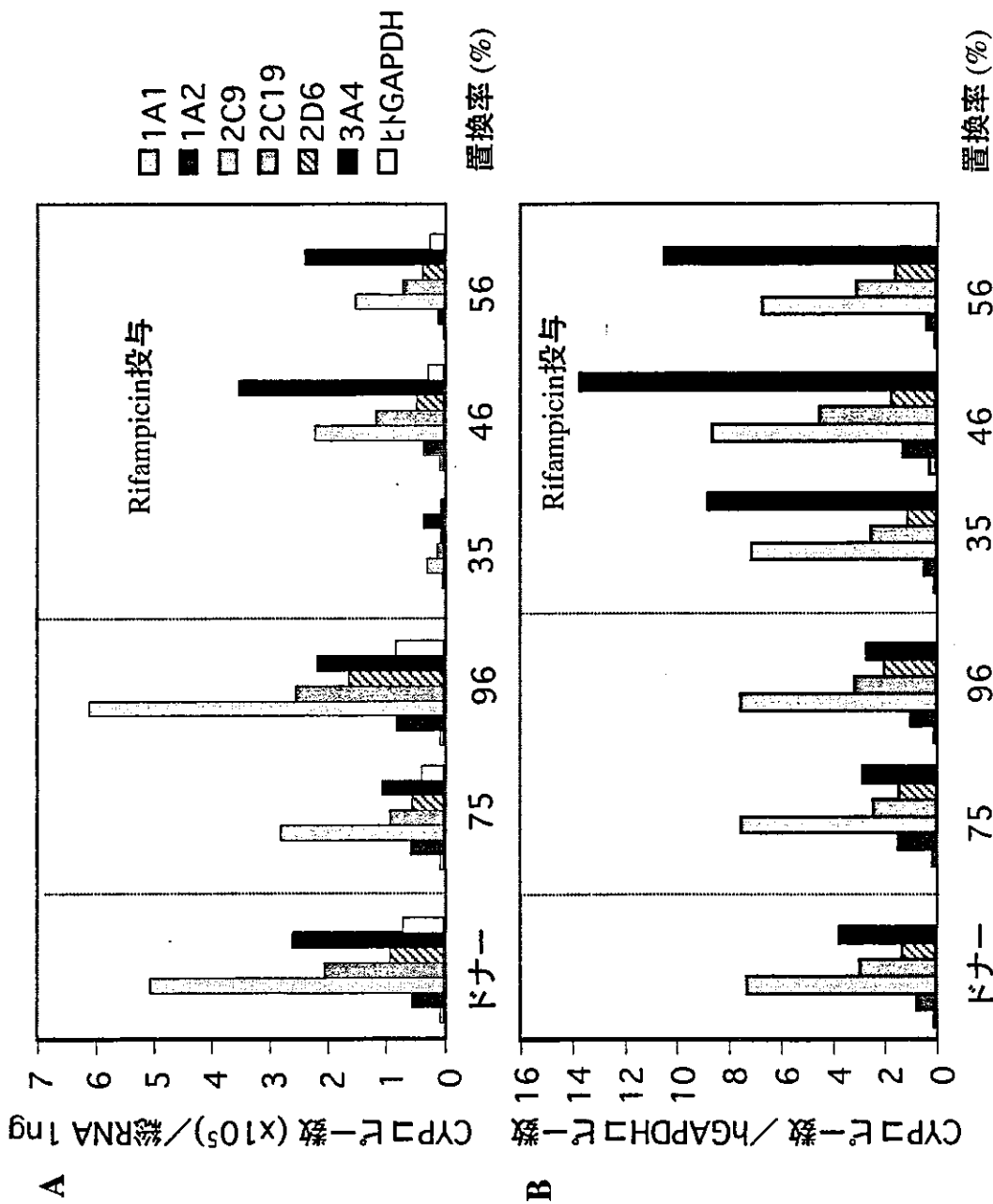
図7 キメラマウス肝臓におけるヒト特異的シトクロームP450mRNAの発現量

生後3週のホモマウスに4-7.2x10⁵個のヒト肝細胞（12才，男児）を移植し、移植後70-106日目に解剖した。(A) ドナーおよびキメラマウス肝臓から総RNAを抽出し、総RNA 1 ngにおけるヒトCYP分子種とヒトGAPDHの mRNAのコピー数をreal-time 定量性RT-PCRにより求めた。コピー数は置換率と相関があり、高置換率のキメラ マウスのコピー数はドナーのコピー数と近かった。(B) CYP分子種のmRNAのコピー数をヒトGAPDHのmRNA のコピー数で割り補正したところ、ドナーとキメラマウス肝臓で同じ発現パターンを示した。Rifampicinを投 与した (i.p., 50 mg/kg body weight/day, 4 days)キメラマウス肝臓ではCYP3A4のみ誘導された。

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EXPRESSION OF HUMAN CYTOCHROMES P450 IN CHIMERIC MICE WITH HUMANIZED LIVER

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ABSTRACT:

Recently, a chimeric mouse line in which the liver could be replaced by more than 80% with human hepatocytes was established in Japan. Because the chimeric mouse produces human albumin (hAlb), replacement by human hepatocytes could be estimated by the hAlb concentration in the blood of chimeric mice. In this study, we investigated human major cytochrome P450 (P450) in the livers of chimeric mice by mRNA, protein, and enzyme activity using real-time polymerase chain reaction, Western blot analysis, and high-performance liquid chromatography, respectively. Chimeric mice with humanized liver generated using hepatocytes from a Japanese and white donor were used. Human P450 mRNAs were expressed in the liver of chimeric mice, and major human P450 proteins such as CYP1A2, CYP2C9, and CYP3A4 were detected. The expression of P450 mRNA and protein was corre-

lated with the hAlb concentration in the blood. The enzyme activities such as diclofenac 4'-hydroxylase activity, dexamethasone 6-hydroxylase activity, and coumarin 7-hydroxylase activity, activities that are specific to human P450 but not to murine P450, were increased in a hAlb concentration-dependent manner. The chimeric mice with nearly 90% replacement by human hepatocytes demonstrated almost the same protein contents of human P450s and drug-metabolizing enzyme activity as those of the donor. It was confirmed that genomic DNA from the livers of the chimeric mice and that from the liver of the donor exhibited the same genotype. In conclusion, the chimeric mice exhibited a similarly efficient capacity of drug metabolism as humans, suggesting that they could be a useful animal model for drug development.

Recently, efforts have been made toward optimizing drug treatment by taking into account individual differences in drug metabolism, which are regarded as major determinants of the interindividual variability in pharmacokinetics, clinical efficacy, and toxicity of drugs (Lu, 1998). Cytochrome P450 (P450) plays a central role in drug metabolism in human liver. One of the isoforms, CYP3A4, is predominantly expressed in human liver and has been reported to be responsible for the metabolism of more than 50% of the clinical drugs (Pelkonen et al., 1998). In addition, several human P450s have been shown to be polymorphic due to single nucleotide polymorphisms, gene deletions, and gene duplications (Ingelman-Sundberg, 2002). These polymorphisms can also affect the pharmacokinetics, safety, and efficacy of the drugs. Detailed analyses of drug metabolism catalyzed by human P450s are very important in drug development.

Various approaches have been used to evaluate the metabolism of

drug candidates in humans at early preclinical stages. In vitro approaches using human liver microsomes (HLM), recombinant human P450 microsomes, human hepatocytes, and cDNA-expressing cells have been reported. These in vitro models have various advantages and limitations, as have been described previously (Gomez-Lechon et al., 2003; Rodrigues and Rushmore, 2003). Briefly, HLM are commercially available and can be stored for years with minimal or no loss of P450 enzyme activities, but it is impossible to use them for P450 induction studies. Although human hepatocytes retain all the drug-metabolizing enzymes, the enzyme activities are decreased during culture. These human-derived samples are limited in supply. In addition, although in vivo approaches using experimental animals are able to clarify the pharmacokinetics of drugs in the whole body, human and animal P450s are known to often be functionally distinct.

Several attempts have been made to develop bioartificial livers. Watanabe et al. (2003) established the immortalized human hepatic stellate scavenger cells that could be used as a bioartificial liver. Although this model was shown to express CYP3A4 and CYP2C9 proteins, the enzyme activities in these cells were not clarified. It was reported that the livers in urokinase-type plasminogen activator (uPA)/recombinant activation gene-2 mice could be repopulated with

TABLE 1

Sequence of the primers used in the present study

Primer	Sequence
CYP1A1 S	5'-ATGACCAGAAGCTATGGGTC-3'
CYP1A1 AS	5'-GCACGCTGAATTCCACCC-3'
CYP1A2 S	5'-GCTTCTACATCCCCAAGAAAT-3'
CYP1A2 AS	5'-TCCCACTTGGCCAGGACT-3'
CYP2A6 S	5'-AGCAACAGGCCTTTCAGTT-3'
CYP2A6 AS	5'-CCCAATGAAGAGGTTCAAC-3'
CYP2C8 S[a]	5'-AGATCAGAATTTTCTCACCC-3'
CYP2C8 AS[a]	5'-AACTTCGTGTAAGAGCAACA-3'
CYP2C9 S	5'-CAGATCTGCAATAATTTTTCTC-3'
CYP2C9 AS	5'-CTTTCAATAGTAAATTCAGATG-3'
CYP2C19 S[a]	5'-ATTGAATGAAAACATCAGGATTG-3'
CYP2C19 AS[a]	5'-GAGGGTTGTTGATGTCCATC-3'
CYP2D6 S	5'-GGTGTGACCCATATGACATC-3'
CYP2D6 AS	5'-CTCCCCGAGGCATGCACG-3'
CYP3A4 S	5'-CCAAGCTATGCTCTTCACCG-3'
CYP3A4 AS	5'-TCAGGCTCCACTTACGGTGC-3'
CYP3A5 S	5'-AGTGTTCTTTCCTTCACTTTA-3'
CYP3A5 AS	5'-TTCAACATCTTTCTTGCAAGT-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 conc.	RI
		mg/ml	*%*
1	A	0.0	0
2	A	1.6	31
3[a]	A	2.1	52
4	A	3.5	57
5	A	7.7	89
11	B	0.0	0
12	B	0.6	19
13	B	2.1	60
14	B	9.3	71
15	B	13.7	71
16	B	10.8	88
17	B	13.7	93

A, Japanese, male, 12 years; B, white, male, 9 months.
[a] Total hepatic RNA in no. 3 could not be obtained in this study.

approximately 15% normal human hepatocytes isolated from adult liver and that these mice could be inoculated with human hepatitis B virus (Dandri et al., 2001). Mercer et al. (2001) have reported the generation of chimeric uPA^{+/+}/SCID mice with human cells occupying a portion of the cross-sectional liver area, and these mice could be successfully infected with human hepatitis C virus. These studies were valuable for the development of new treatments against hepatitis virus and for the progress of regenerative medicine; however, the replacement of mouse liver with human hepatocytes was low and insufficient in these reports (Dandri et al., 2001; Mercer et al., 2001). Recently, chimeric mice in which the livers could be replaced by more than 80% with human hepatocytes were established by Tateno et al. (2004). We speculated that such mice could be used for predicting drug metabolism in humans, since the livers of the chimeric mice were almost completely replaced with human hepatocytes. In the present study, we investigated the expression of human P450s in the livers of such mice to determine whether they can be used as a new tool for studies in drug development.

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 Nosan (Yokohama, Japan). Polyclonal rabbit anti-human CYP2C9 antibody and human CYP2D6, CYP3A4, and CYP3A5 Western blotting kits were obtained from BD Gentest (Worburn, MA). Recombinant human CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2D6, and CYP3A4 expressed in baculovirus-infected insect cells were also obtained from BD Gentest. Recombinant human CYP3A5 was obtained from Invitrogen (Carlsbad, CA). Debrisoquine, diclofenac, and paclitaxel were purchased from Sigma-Aldrich (St. Louis, MO). Coumarin, 7-hydroxycoumarin, and dexamethasone were purchased from Wako Pure Chemical Industries (Osaka, Japan). S-Mephenytoin was obtained from Toronto Research Chemicals (Toronto, ON, Canada). (±)-4'-Hydroxydebrisoquine and (±)-4'-hydroxymephenytoin were purchased from Ultrafine Chemicals (Manchester, UK). Pooled HLM, 4'-hydroxydiclofenac, and 6α-hydroxypaclitaxel were 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 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 (white, male, 9 months old) were purchased from In Vitro Technologies (Baltimore, MD). 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 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 anti-human specific cytokeratin 8 and 18 antibody, respectively. There was a good correlation between the hAlb concentration and the 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).

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 software (version 1.2b). The PCR conditions were as follows. After an initial denaturation at 95°C for 30 sec, the amplification was performed by denaturation at 94°C for 4 s and annealing and extension 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. We could not obtain total RNA from chimeric mouse 3.

Genomic DNA Preparation from Livers. Genomic DNA from the liver of donor A and that of the chimeric mice were extracted with phenol/chloroform followed by ethanol precipitation (Brilliant et al., 1991). The genotyping of CYP2A6*4, CYP2C19*2, CYP2C19*3, and CYP3A5*3 was performed as described previously (de Morais et al., 1994; Fukuen et al., 2002; Nakajima et al., 2004).

Liver Microsomes. Liver microsomes were prepared as described previously (Yamazaki et al., 1999a) and stored at −80°C until analysis. The protein concentration was determined using Bradford protein assay reagent (Bio-Rad, Hercules, CA) with bovine gamma globulin as the standard. We could not obtain liver microsomes from donor B.

Immunoblot Analysis of Human P450 Isoforms. SDS-polyacrylamide gel electrophoresis and immunoblot analysis of human CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2D6, CYP3A4, and CYP3A5 were performed according to Laemmli (1970), with slight modifications. The liver microsomes
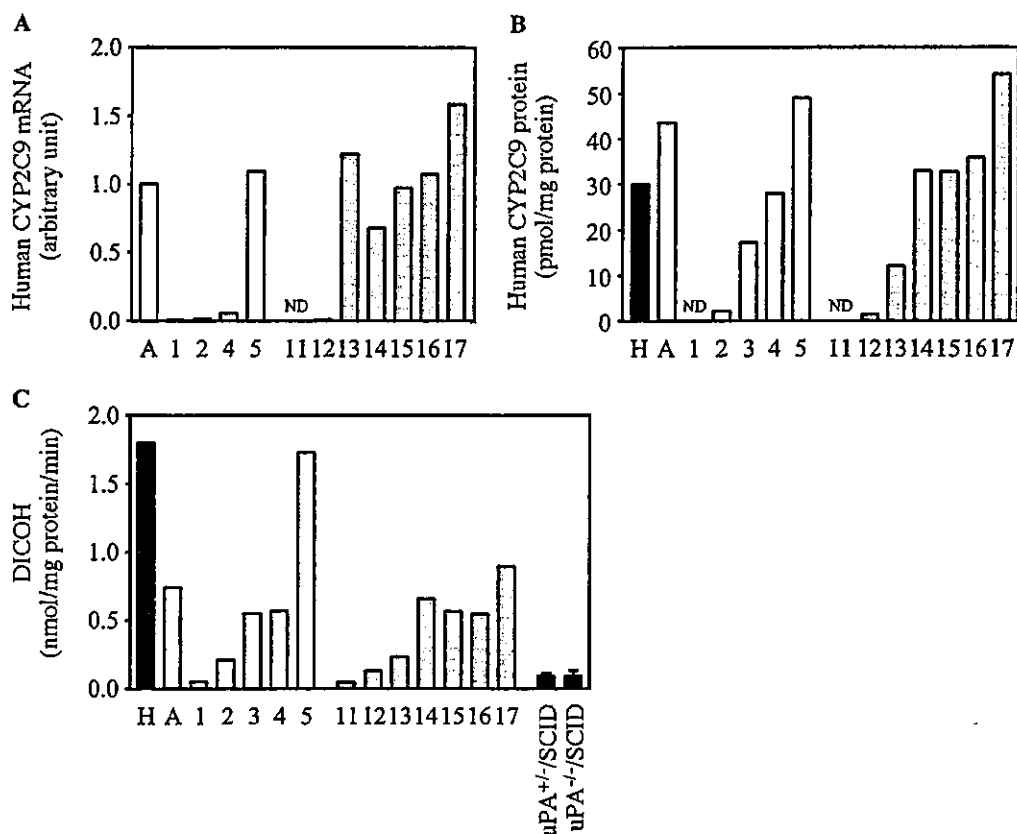
FIG. 1. Human CYP2C9 expression in the chimeric mice. Relative expression levels of human CYP2C9 mRNA (A), the expression of human CYP2C9 protein (B), and DICOH (C) were determined as described under *Materials and Methods*. C, DICOH catalyzed by CYP2C9 measured at 30 μM diclofenac. Each column represents 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 ± S.D. (*n* = 3). The sample numbers are described in Table 2. H, HLM; A, donor A; ND, not detected.

(5–20 μg) 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 MgCl$_2$, 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 μM, 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 C$_{18}$ 5-μm analytical column (4.6 × 150 mm). Paclitaxel 6α-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 μM, 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 C$_{18}$ 5-μm analytical column (4.6 × 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 μM, 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 C$_{18}$ 5-μm analytical column (4.6 × 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 μM, respectively. The reaction mixture was incubated for 30 min at 37°C. The product formation was determined using HPLC with a C$_{18}$ 5-μm analytical column (4.6 × 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 μM, 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 μM, respectively. The reaction mixture was incubated for 30 min at 37°C. The product formation was determined using HPLC with a C$_8$ 5-μm analytical column (4.6 × 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).

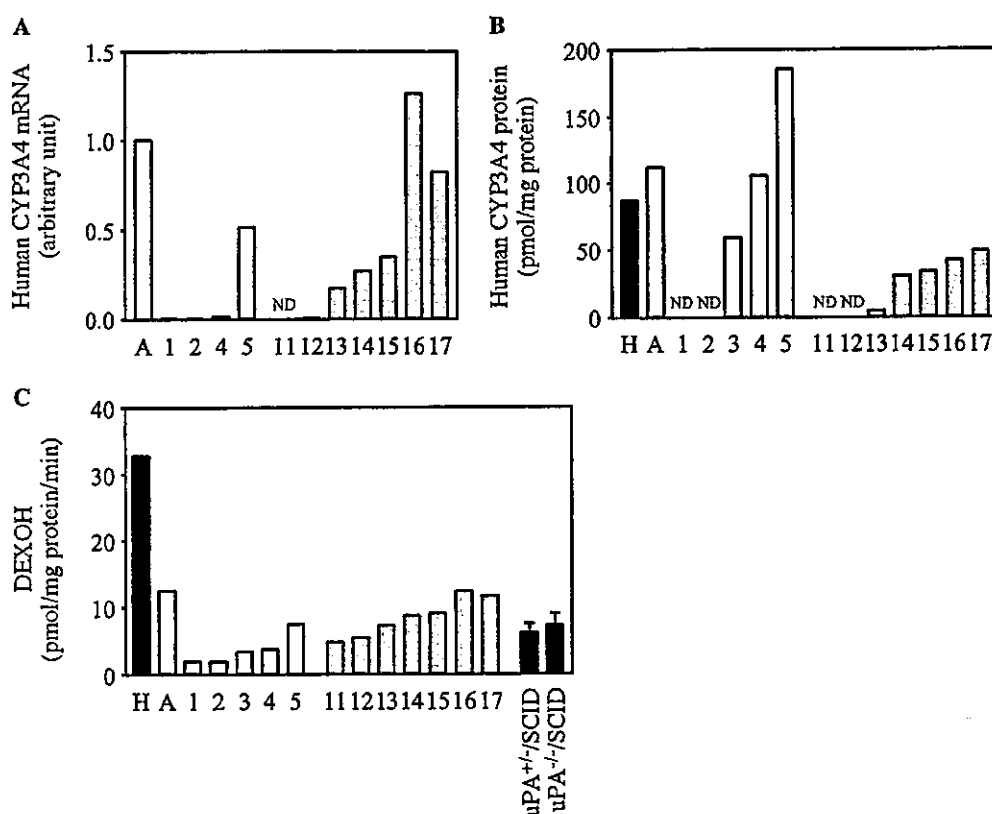**Expression of Human CYP2C9 in Chimeric Mice.** The expres-

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 μM 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 ± 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 CY1A2 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
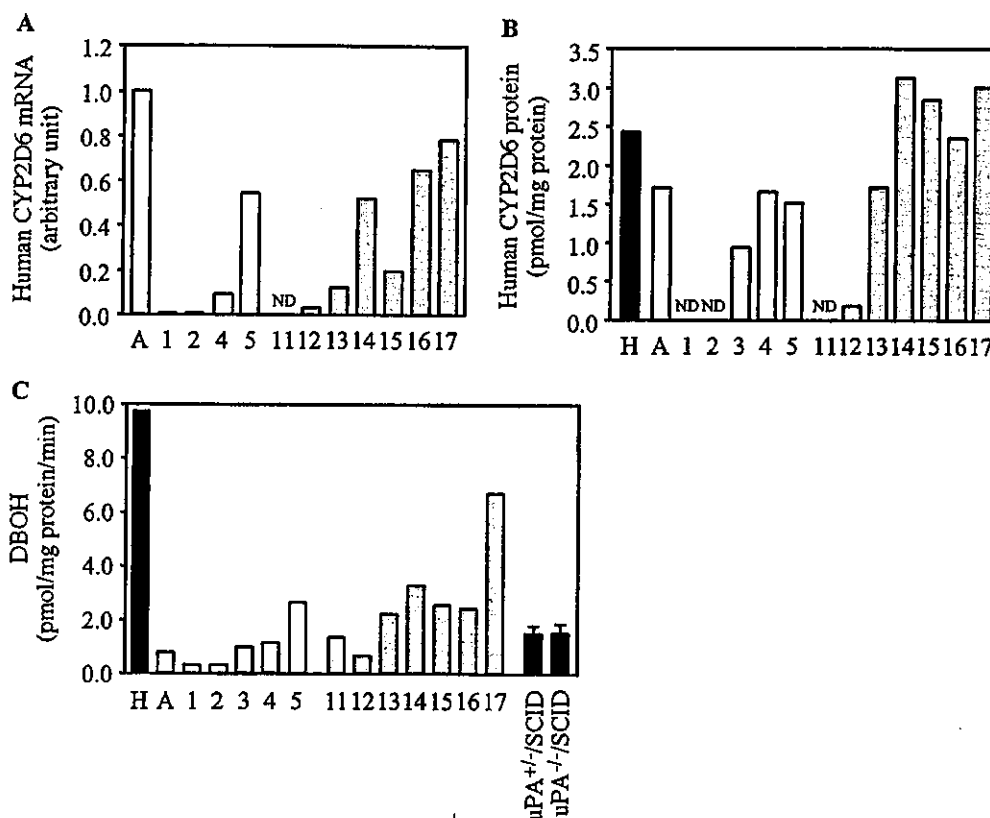
FIG. 3. Human CYP2D6 expression in the chimeric mice. Relative expression levels of human CYP2D6 mRNA (A), the expression of human CYP2D6 protein (B), and DBOH (C) were determined as described under *Materials and Methods*. C, DBOH catalyzed by CYP2D6 measured at 5 μM debrisoquine. Each column represents 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 ± 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 (Rodriguez-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
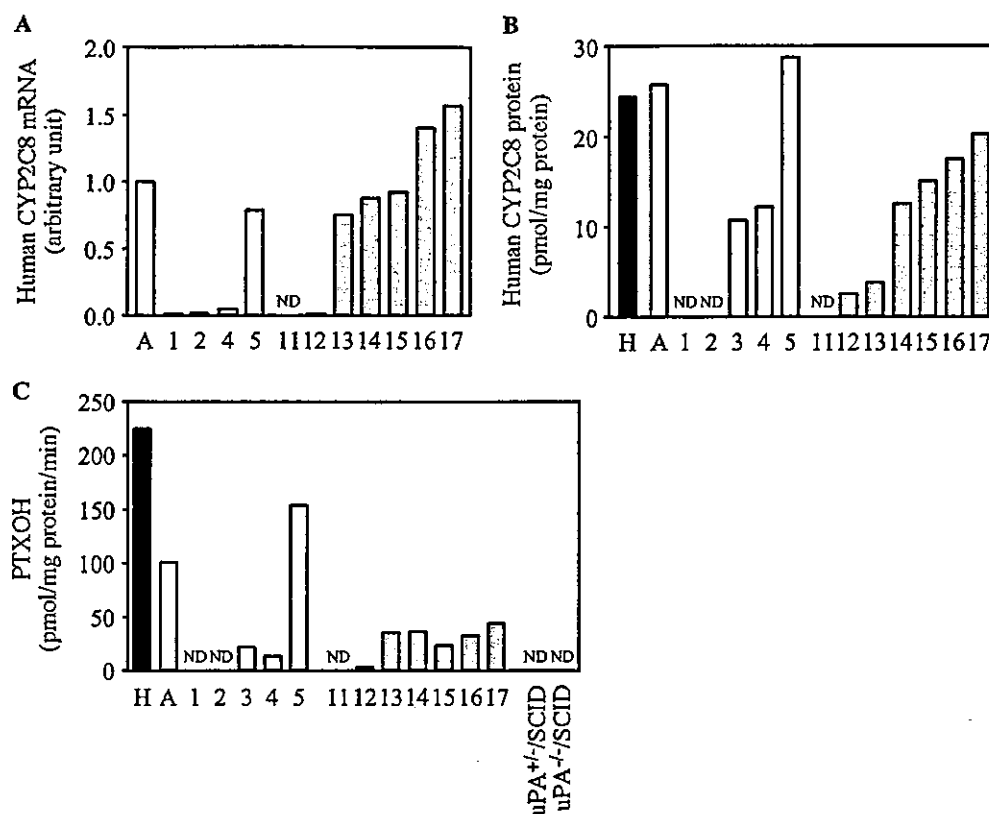
Fig. 4. Human CYP2C8 expression in the chimeric mice. Relative expression levels of human CYP2C8 mRNA (A), the expression of human CYP2C8 protein (B), and PTXOH (C) were determined as described under *Materials and Methods*. C, PTXOH catalyzed by CYP2C8 measured at 20 μM paclitaxel. Each column represents 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-9α-fluoro-androsta-14-diene-11β-hydroxy-16α-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
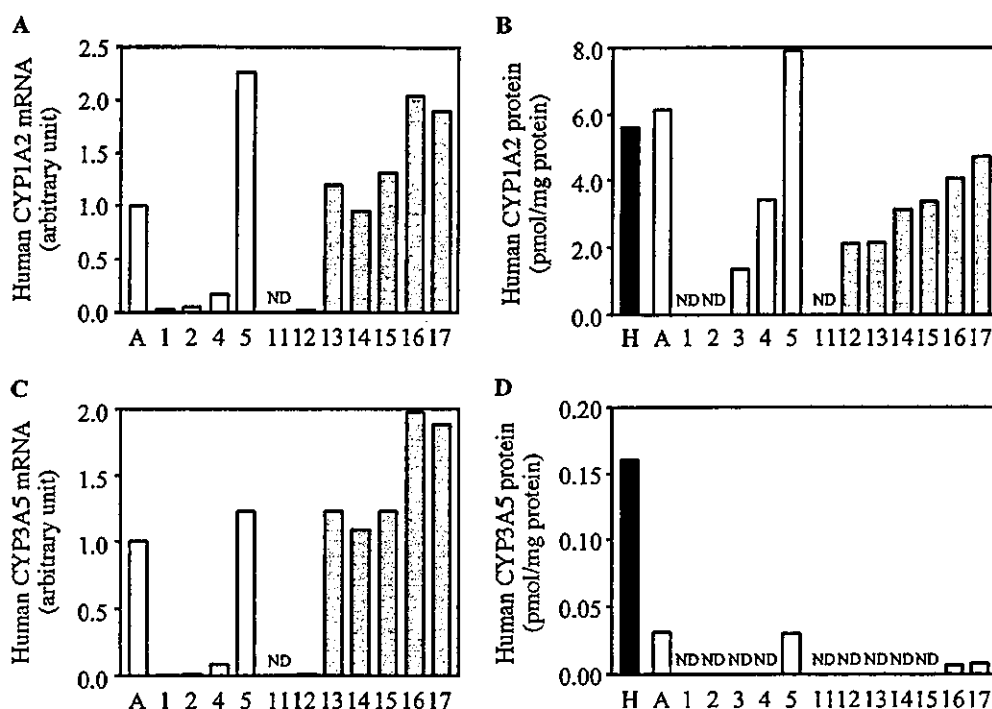
Fig. 5. Human CYP1A2 and CYP3A5 expressions in the chimeric mice. Relative expression levels of human CYP1A2 (A) and CYP3A5 (C) mRNA and human CYP1A2 (B) and CYP3A5 (D) protein were determined as described by Western blotting under *Materials and Methods*. The sample numbers are described in Table 2. H, HLM; A, donor A; ND, not detected.
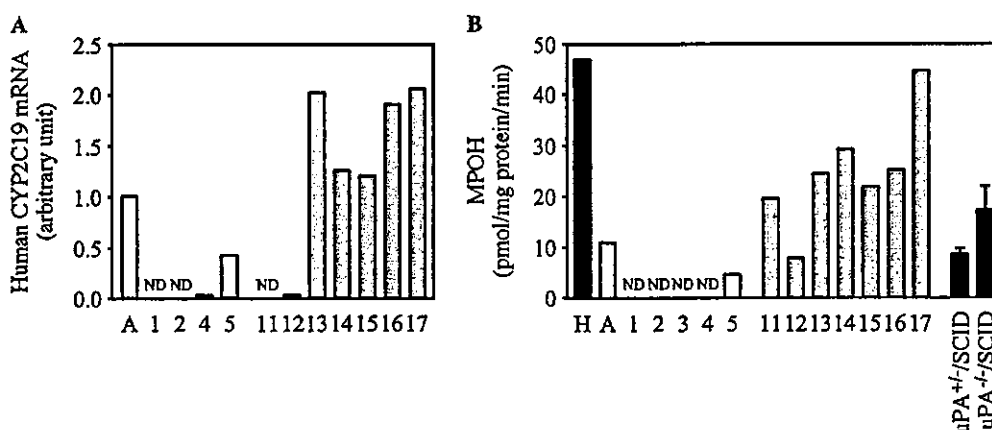

Fig. 6. Human CYP2C19 expression in the chimeric mice. Relative expression levels of human CYP2C19 mRNA (A) and MPOH (B) were determined as described under *Materials and Methods*. B, MPOH catalyzed by CYP2C19 measured at 200 μM S-mephenytoin. Each column represents the mean of duplicate determinations 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.

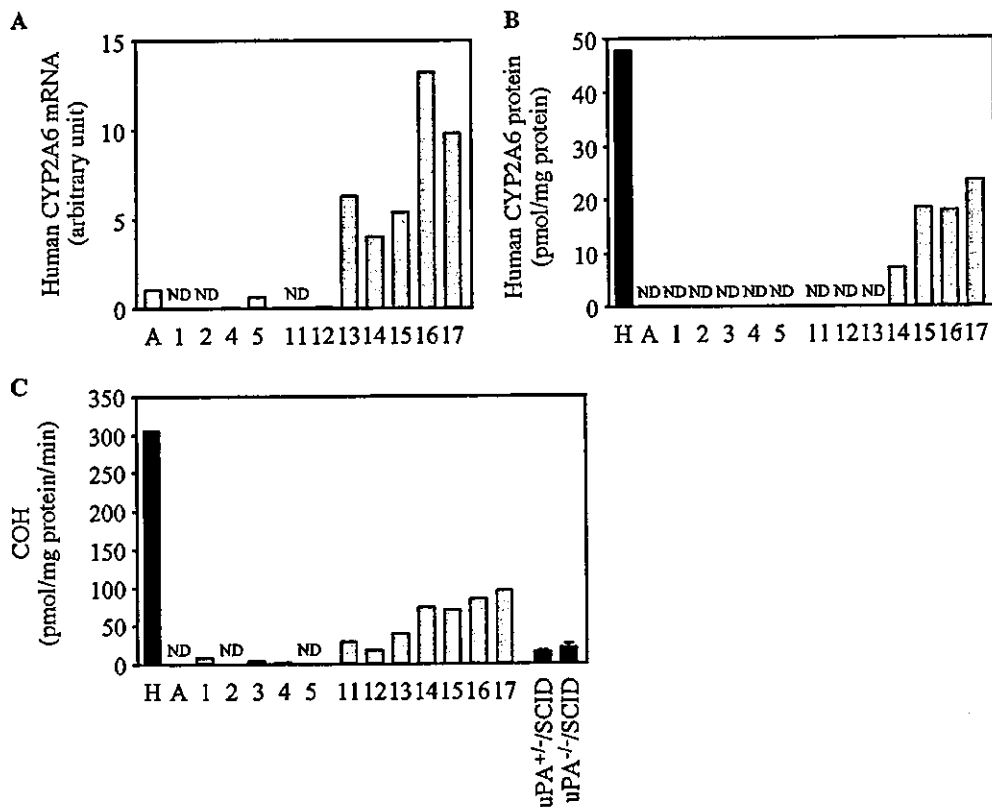In conclusion, the livers of the chimeric mice used in the present

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 μM 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 ± 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 transplantating 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 μM 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 μM 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.[1] 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-

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.*[8] 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 Ca^{2+}- and Mg^{2+}-Hank's Balanced Salt Solution (CMF-HBSS) containing 200 μg/mL EGTA, 1 mg/mL glucose, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (Hepes), and 10 μg/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 CaCl$_2$, 10 mM Hepes, and 10 μg/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 μg/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±3.8	70.1±1.1	57.8±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±SD of three independent experiments.

centrifuged 3 times at 50×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 μg/mL L-proline, 0.5 μg/mL insulin, 10^{-7} mol/L dexamethasone, 44 mmol/L NaHCO$_3$, and antibiotics of 100 IU/mL penicillin G and 100 μg/mL streptomycin. The hepatocytes from the chimeric mice were cryopreserved using a programed 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% CO$_2$ and 95% air at 37°C for exposure. β-NF (1 and 5 μM) and Rif (10 and 50 μM) 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 μg/mL with 50-μg/mL yeast tRNA. The RT-PCR assay was performed in 50 μL 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