for the activity of CYP2D6 to be almost abolished, at least in V79 cells. This combined effect may be a factor responsible for the poor metabolism of debrisoquine found in a subject carrying *CYP2D6*14* (Wang, 1992; Wang et al., 1999).

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Short communication

Frequent occurrence of *CYP2D6*10* duplication allele in a Japanese population

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Abstract

Allele-specific long-polymerase chain reaction (PCR), PCR-restriction fragment length polymorphism (RFLP) and haplotype analysis using *Xba*I and *Eco*RI were used to determine whether gene duplication of *CYP2D6*10* exists in a Japanese population of 162 healthy subjects. Based on the results of PCR and haplotype analysis, the frequencies of *CYP2D6*1X2*, *CYP2D6*2X2* and *CYP2D6*10X2* in the Japanese population were estimated to be 0.3, 0.3 and 0.6%, respectively. The results suggest that duplicated alleles of *CYP2D6*10* exist in the Japanese population and that it may be one of the factors affecting the capacity of Japanese to metabolize various CYP2D6 substrate drugs.

Keywords: *CYP2D6*10*; Duplication; Japanese; Ultrarapid metabolizer

1. Introduction

CYP2D6, an isoform of CYP catalyzing more than 50 clinically important drugs, is highly polymorphic. There are at least 41 different alleles of *CYP2D6*, which are associated with deficient, decreased, normal and increased enzyme activities. Moreover, the existence of duplication of the active *CYP2D6* gene has been reported [1]. Individuals carrying duplicated *CYP2D6* alleles require more-than-average doses of CYP2D6 substrate drugs because of their increased capacity of CYP2D6-mediated drug metabolism. Duplication involves mainly the active allelic variant *CYP2D6*2*, and individuals have been found to carry 2, 3 or 13 copies of *CYP2D6*2* [1–3].

On the other hand, the existence of a duplicated allele of *CYP2D6*10* has been reported in Hong Kong Chinese [4]. *CYP2D6*10* is a mutant allele of *CYP2D6* that produces an unstable enzyme, resulting in decreased capacity of CYP2D6 activity. Although this mutant allele has quite frequently been found in Chinese and Japanese populations [5–7], a duplicated allele of *CYP2D6*10* has not been detected in a Japanese population [6].

In this study, we analyzed 162 healthy Japanese subjects for the presence of duplicated *CYP2D6*10* alleles and found two subjects carrying duplicated alleles of *CYP2D6*10*.

Abbreviations: cDNA, complementary DNA; CYP, cytochrome P450; DIG, digoxigenin; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism

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Table 1
Detection of duplicated *CYP2D6* genes in a Japanese population

Subject no.	Long-PCR[a]	*Eco*RI haplotype[b] (kb)					*Xba*I haplotype[b] (kb)			PCR–RFLP[c] (bp)		*CYP2D6* genotype
		8.8	9.4	12.1	13.7	15.1	29	42	44	*Ban*II	*Hph*I	
I	+	+	+	+	−	+	+	+	−	231/33	362/71	**1X2/*2*
II	+	+	+	+	−	+	+	+	−	264	362/71	**1/*2X2*
III	+	+	+	+	−	+	+	+	−	264	262/100/71	**1/*10X2*
IV	+	+	+	+	+	+	−	+	+	264	262/100/71	**10/*10X2*

[a] PCR-based *CYP2D6* duplication assay with *cyp-32r* and *cyp207-f* primers [8].
[b] *Eco*RI or *Xba*I haplotype analysis with a DIG-labeled *CYP2D6* cDNA probe.
[c] PCR–RFLP assay with p2X2f/p2X2r, p2X2f/p92, and p11/p12 primers [9].

2. Methods

One hundred and sixty-two unrelated, healthy Japanese volunteers (95 males and 67 females), aged 19–61 years, were genotyped. The use of human genomic DNA in the present study was approved by the local Institution Review Board.

Long-PCR amplification was carried out using the combination of primers *cyp-32r* and *cyp-207f*, which yields a 3.2 kb product for only subjects carrying at least two copies of the *CYP2D6* gene [8]. Moreover, to distinguish different types of allele duplication, long-PCR and PCR–RFLP were performed using specific primers as described by Sachse et al. [9]. The resulting PCR products were separated and detected in ethidium bromide-containing 1 and 2% agarose gels.

For haplotype analysis, 5 μg of genomic DNA was digested with *Xba*I or *Eco*RI under conditions recommended by manufacturer. The samples were subjected to electrophoresis on 0.4% agarose gel at 1 V/cm for 65 h. Southern blotting was performed on PALL BIODYNE® (NIPPON Genetics Co. Ltd., Tokyo, Japan) and hybridized with a DIG-labeled *CYP2D6* cDNA probe.

3. Results

Long-PCR analysis using the pair of primers *cyp-32r* and *cyp-207f* showed the existence of the expected 3.2 kb fragment in 4 among of the 162 Japanese subjects, suggesting that these subjects are carriers of an extra *CYP2D6* gene. This was also suggested by the results of haplotype analysis using *Eco*RI showing the existence of a 12.1 kb a marker of an extra *CYP2D6* gene in these four subjects (Table 1). These four subjects had been genotyped as *CYP2D6*1/*2* (sample numbers, I and II), **1/*10* (sample number, III) and **10/*10* (sample number, IV), in two, one and one subject, respectively, in a previous study [7].

Haplotype analysis using *Xba*I showed the existence of two fragments, 29 and 42–44 kb fragments, in the subjects of I, II (**1/*2*) and III (**1/*10*). Since the subject of IV (**10/*10*) showed only a fragment of 42–44 kb (Table 1), this subject was judged to be a heterozygous carrier of duplicated **10*. Therefore, these four subjects were analyzed in order to distinguish the differences between **1*, **2* and **10* of allele duplication. Consequently, these four subjects were genotyped as *CYP2D6*1X2/*2* (I), **1/*2X2* (II), **1/*10X2* (III), and **10/*10X2* (IV) (Table 1). Based on our findings, we estimated that the frequencies (95% confidential interval) of *CYP2D6*1X2*, *CYP2D6*2X2*, and *CYP2D6*10X2* alleles in the Japanese population are 0.3 (0.1–1.7), 0.3 (0.1–1.7) and 0.6% (0.2–2.2), respectively.

4. Discussion

The frequency of duplicated *CYP2D6* alleles found in the present study is similar to that reported previously for Japanese subjects [6]. However, it has been reported no duplication of *CYP2D6*10* was found in Japanese subjects.

*CYP2D6*10* is the most frequently observed mutated allele of *CYP2D6* in Mongolian populations [5–7]: the reported frequencies of this mutation are more than 50.7% in Chinese [5] and 38.1–38.6% in

Japanese populations [6,7]. In addition, duplicated alleles of *CYP2D6*10* have been reported to be present in Hong Kong Chinese [4]. Therefore, it is not surprising that duplicated alleles of *CYP2D6*10* were seen in our Japanese subjects.

In conclusion, the present study showed the existence of duplicated alleles of *CYP2D6*10* in a Japanese population. Since, *CYP2D6*10* is the most frequently observed mutated allele of *CYP2D6* and is regarded as a factor affecting the capacity of Japanese to metabolize substrate drugs of CYP2D6, the presence of duplicated *CYP2D6*10* may also be an additional factor affecting CYP2D6 activity in the Japanese population.

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Biochemical Pharmacology

Substrate specificity for rat cytochrome P450 (CYP) isoforms: screening with cDNA-expressed systems of the rat

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Abstract

In this study, we performed a screening of the specificities of rat cytochrome P450 (CYP) isoforms for metabolic reactions known as the specific probes of human CYP isoforms, using 13 rat CYP isoforms expressed in baculovirus-infected insect cells or B-lymphoblastoid cells. Among the metabolic reactions studied, diclofenac 4-hydroxylation (DFH), dextromethorphan *O*-demethylation (DMOD) and midazolam 4-hydroxylation were specifically catalyzed by CYP2C6, CYP2D2 and CYP3A1/3A2, respectively. These results suggest that diclofenac 4-hydroxylation, dextromethorphan *O*-demethylation and midazolam 4-hydroxylation are useful as catalytic markers of CYP2C6, CYP2D2 and CYP3A1/3A2, respectively. On the other hand, phenacetin *O*-deethylation and 7-ethoxyresorufin *O*-deethylation were catalyzed both by CYP1A2 and by CYP2C6. Benzyloxyresorufin *O*-dealkylation and pentoxyresorufin *O*-dealkylation were also catalyzed by CYP1A2 in addition to CYP2B1. Bufuralol 1′-hydroxylation was extensively catalyzed by CYP2D2 but also by CYP2C6 and CYP2C11. *p*-Nitrophenol 2-hydroxylation and chlorzoxazone 6-hydroxylation were extensively catalyzed by CYP2E1 but also by CYP1A2 and CYP3A1. Therefore, it is necessary to conduct further study to clarify whether these activities in rat liver microsomes are useful as probes of rat CYP isoforms. In contrast, coumarin 7-hydroxylation and *S*- and *R*-mephenytoin 4′-hydroxylation did not show selectivity toward any isoforms of rat CYP studied. Therefore, activities of coumarin 7-hydroxylation and *S*- and *R*-mephenytoin 4′-hydroxylation are not able to be used as catalytic probes of CYP isoforms in rat liver microsomes. These results may provide useful information regarding catalytic probes of rat CYPs for studies using rat liver microsomal samples.

Keywords: CYP; Rat; Substrate specificity; cDNA-expression system; Probe; Drug metabolism

1. Introduction

CYP enzymes comprise a large family of hemoprotein [1], and enzymes from three families (CYP1, CYP2, and CYP3) are mainly involved in the biotransformation of xenobiotics in both humans and rats [2]. However, considerable differences exist in the expression and catalytic activities between human and rat CYP orthologues [2]. Although CYP enzymes make xenobiotics less toxic, the reactions frequently involve the formation of reactive intermediates or allow the leakage of free radicals capable of causing toxicity [3,4]. Therefore, differences in the levels of individual CYP isoforms and indeed the expression of distinct isoforms significantly contribute to interspecies differences in the toxicity of chemicals and the susceptibility to chemically induced cancer [2]. Nevertheless, drug safety assessment studies, including pharmacological and toxicological studies, have been performed using rodents although they are not necessarily excellent surrogate models for man. To assess the effects and toxicities of xenobiotics (i.e. developmental drugs and environmental chemicals), it is necessary to identify CYP isoforms responsible for the metabolism of drugs in each species.

Currently, many different strategies are employed for the unambiguous identification of CYP isoforms responsible for the biotransformation of therapeutic agents in humans.

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Abbreviations: CYP, cytochrome P450; POD, phenacetin *O*-deethylation; EROD, 7-ethoxyresorufin *O*-deethylation; BROD, 7-benzyloxyresorufin *O*-dealkylation; PROD, 7-pentoxyresorufin *O*-dealkylation; CRH, coumarin 7-hydroxylation; DFH, diclofenac 4-hydroxylation; SMH, S-mephenytoin 4′-hydroxylation; RMH, *R*-mephenytoin 4′-hydroxylation; DMOD, dextromethorphan *O*-demethylation; BLH, bufuralol 1′-hydroxylation; PNPH, *p*-nitrophenol 2-hydroxylation; CZH, chlorzoxazone 6-hydroxylation; MD1H, midazolam 1′-hydroxylation; MD4H, midazolam 4-hydroxylation.

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These include the use of CYP isoform-selective inhibitors, immunoinhibitory antibodies, studies with purified and heterogeneously expressed CYP protein, and correlation analyses of metabolic rates of drugs with immunoquantified CYP levels or metabolic rates of specific substrates for each isoform. The use of cDNA-expressed human CYPs has greatly facilitated the evaluation of the metabolic specificity of probe substrates and the identification of individual CYP isoforms involved in metabolism of drugs. On the other hand, criteria and approaches for unambiguous identification of individual CYP isoforms responsible for the metabolism of drugs in rats have not been established. This is because the specificities of metabolic reactions used as probes of rat CYP isoforms have not been thoroughly evaluated.

In the present study, therefore, the metabolism of model substrates for human CYP-isoforms was examined using 13 rat CYP isoforms expressed in baculovirus-infected insect cells or human B-lymphoblastoid cells.

2. Materials and methods

2.1. Chemicals

Bufuralol hydrochloride, 1′-hydroxybufuralol and 4-hydroxydiclofenac were purchased from Gentest. Midazolam, 1′- and 4-hydroxymidazolam were gifts from F. Hoffmann-La Roche. Zaltoprofen was a gift from Zeria Pharmaceutical. Dextrorphan, *R*- and *S*-mephenytoin, 4-hydroxymephenytoin, 6-hydroxychlorzoxazone, 6β-, 7α- and 16α-hydroxytestosterone were purchased from Ultrafine Chemicals. 7-Benzyloxyresorufin, chlorzoxazone, dextromethorphan hydrobromide, 7-ethoxyresorufin, 7-pentoxyresorufin and resorufin were purchased from Sigma. Cyclobarbital was purchased from Tokyo Kasei Kogyo. $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Oriental Yeast. *p*-Nitrophenol was purchased from Nacalai Tesque Inc. Acetaminophen, caffeine, coumarin, diclofenac, 7-hydroxycoumarin, 7-hydroxy-4-methylcoumarin, *p*-nitrocatechol, phenacetin, testosterone and HPLC-grade acetonitrile and methanol were purchased from Wako Pure Chemical Industries.

2.2. Microsomes

Microsomes prepared from baculovirus-infected insect cells expressing CYP1A2 (lot 1), CYP2A2 (lot 1), CYP2B1 (lot 2), CYP2C6 (lot 1), CYP2C11 (lot 1), CYP2C12 (lot 1), CYP2C13 (lot 1), CYP2D1 (lot 2), CYP2D2 (lot 1), CYP3A1 (lot 1) and CYP3A2 (lot 1) were obtained from Gentest. All recombinant CYPs were coexpressed with NADPH-CYP oxidoreductase. Recombinant CYP2A2, CYP2B1, CYP2C6, CYP2C11, CYP2C12, CYP2C13, CYP3A1 and CYP3A2 were coexpressed with cytochrome b_5. Microsomes prepared from human B-lymphoblastoid cells expressing CYP2A1 (lot 7) and CYP2B1 (lot 6) were obtained from Gentest. Recombinant CYP2A1 and CYP2E1 were coexpressed with NADPH-CYP oxidoreductase. Control microsomes were from insect cells infected with wild-type baculovirus and from human B-lymphoblastoid cells containing the expression vector without cDNA. The ratios of cytochrome *c* reductase activity (nmol/min/mg) to CYP content (pmol CYP/mg) in each recombinant CYP isoform used in this study were 6 for CYP1A2, 4 for CYP2A1, 42 for CYP2A2, 7 for CYP2B1, 10 for CYP2C6, 6 for CYP2C11, 27 for CYP2C12, 2 for CYP2C13, 13 for CYP2D1, 22 for CYP2D2, 10 for CYP2E1, 22 for CYP3A1 and 17 for CYP3A2, respectively.

2.3. Incubation conditions

Activities of phenacetin *O*-deethylation (POD), 7-ethoxyresorufin *O*-deethylation (EROD), coumarin 7-hydroxylation (CRH), 7-benzyloxyresorufin *O*-dealkylation (BROD), 7-pentoxyresorufin *O*-dealkylation (PROD), DFH, *R*- and *S*-mephenytoin 4′-hydroxylation (RMH and SMH), DMOD, bufuralol 1′-hydroxylation (BLH), *p*-nitrophenol 2-hydroxylation (PNPH), chlorzoxazone 6-hydroxylation (CZH), midazolam 1′- and 4-hydroxylation (MD1H and MD4H), and testosterone 6β-, 7α- and 16α-hydroxylation were determined. A typical incubation mixture (0.25 mL total volume) contained 0.1 mM EDTA, 100 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system (0.5 mM $NADP^+$, 2 mM glucose-6-phosphate, 1 IU/mL of glucose-6-phosphate dehydrogenase, and 4 mM $MgCl_2$), cDNA-expressed rat CYPs and a substrate. The reaction was initiated by the addition of the NADPH-generating system following a 1-min pre-incubation at 37°. All reactions were performed in the linear range with respect to CYP concentration and incubation time. After the reaction was stopped by the addition of 100 μL of ice-cold acetonitrile, an internal standard was added. The mixtures were centrifuged at 13,000 *g* for 10 min, and the supernatants (100 μL) were analyzed by HPLC as described below. The substrate concentration, incubation time, content of cDNA-expressed CYP and amount of internal standard used for each assay are listed in Table 1. 7-Ethoxyresorufin, 7-benzyloxyresorufin and 7-pentoxyresorufin were dissolved in dimethylsulfoxide, and this mixture was added to the incubation mixture at a final dimethylsulfoxide concentration of 1%. Testosterone was dissolved in methanol and added to the incubation mixture at a final methanol concentration of 1%. Samples for POD activity were evaporated by a vacuum evaporator for 15 min after the centrifugation, and the remaining samples (100 μL) were analyzed.

2.4. HPLC analysis

The determination of respective metabolites was carried out using a Hitachi HPLC system consisting of an L-7100

Table 1
Substrate concentrations, incubation times, contents of CYP, and the internal standards used in the present study

Reaction	Substrate	Incubation time (min)	CYP (pmol)	Internal standards
POD	Phenacetin (10,500 μM)	15	5	Caffeine (0.25 μg)
EROD	7-Ethoxyresorufin (5 μM)	10	5	None
CRH	Coumarin (10 μM)	60	10	7-Hydroxy-4-methylcoumarin (25 pmol)
BROD	7-Benzyloxyresorufin (5 μM)	5	5	None
PROD	7-Pentoxyresorufin (5 μM)	10	5	None
DFH	Diclofenac (25 μM)	20	5	Zaltoprofene (0.1 ng)
SMH	*S*-Mephenytoin (100 μM)	120	5	Cyclobarbital (62.5 ng)
RMH	*R*-Mephenytoin (200 μM)	30	10	Cyclobarbital (62.5 ng)
DMOD	Dextromethorphan (10 μM)	10	5	None
BLH	Bufuralol (10 μM)	15	0.5	None
PNPH	*p*-Nitrophenol (200 μM)	60	5	Phenacetin (0.125 μg)
CZH	Chlorzoxazone (20 μM)	20	5	Bupranolol (2 μg)
MD1H	Midazolam (25 μM)	20	10	Nitrazepam (0.125 μg)
MD4H	Midazolam (25 μM)	20	10	Nitrazepam (0.125 μg)

pump, an L-7400 UV detector, an L-7200 autosampler and a D-7500 integrator, an 821-FP intelligent spectrofluorometer (Jasco) and a CAPCELL PAK C_{18} UG120 column (4.6 mm × 250 mm, 5 μm; Shiseido). The activities of POD, CRH, DFH, SMH, BLH and CZH were determined as described elsewhere [5]. RMH activity was determined by the same method as that used for determination of SMH activity. The mobile phase for EROD, BROD and PROD activities consisted of 20 mM potassium phosphate buffer (pH 7.4) and acetonitrile (45/55, v/v) containing 2.5 mM tetra-*n*-octylammonium bromide, with a flow rate of 1.0 mL/min. The eluent was monitored fluorometrically (excitation: 530 nm; emission: 580 nm). The mobile phase for DMOD activity consisted of 10 mM citrate buffer (pH 3.4)/acetonitrile (80/20, v/v) with a flow rate of 1.0 mL/min. The eluent was monitored fluorometrically (excitation: 270 nm; emission: 312 nm). The mobile phase for PNPH activity consisted of water/acetonitrile (80/20, v/v) adjusted to pH 3.0 by phosphoric acid and was eluted with a flow rate of 0.8 mL/min. The eluent was monitored at 242 nm. The mobile phase for MD1H and MD4H activities consisted of 10 mM potassium phosphate buffer (pH 7.4)/acetonitrile/methanol (5/3/2, v/v/v) with a flow rate of 0.8 mL/min. The eluent was monitored at 220 nm. The mobile phase for 6β-, 7α- and 16α-hydroxylation activities of testosterone was eluted with a linear gradient of water/methanol/acetonitrile (62/36/2 to 46/50/4) for 15 min, followed by a linear gradient to water/methanol/acetonitrile (30/64/6) at 25 min, and then with an isocratic elution for 5 min, followed by a linear gradient to water/methanol/acetonitrile (62/36/2) at 40 min. The flow rate was 0.8 mL/min. The eluent was monitored at 254 nm.

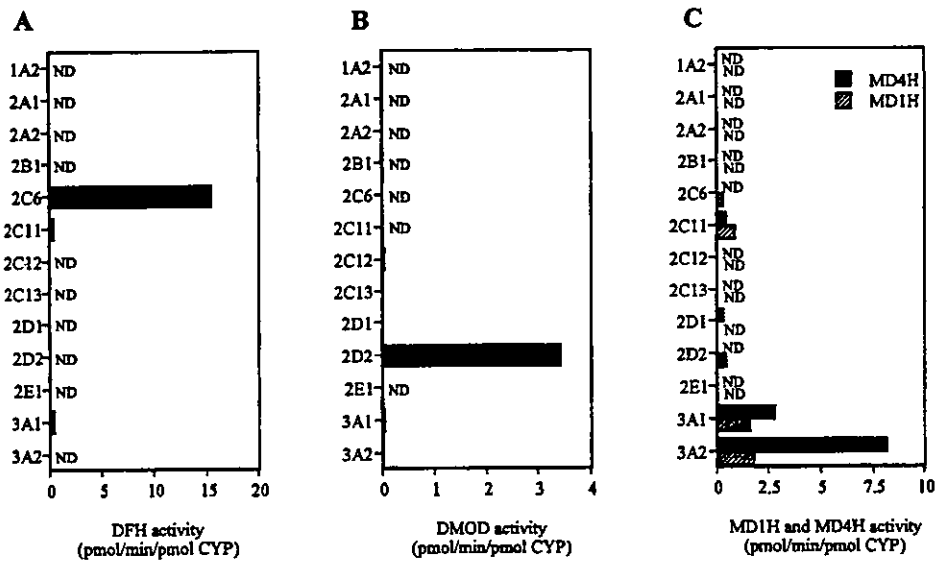

Fig. 1. Activities of DFH (A), DMOD (B), MD1H and MD4H (C) in cDNA-expressed rat CYPs. Substrates (diclofenac, dextromethorphan and midazolam) were incubated at 37° with cDNA-expressed rat CYPs. Substrate concentrations, incubation times and contents of CYP used are shown in Table 1. Each column represents the mean of duplicate experiments. ND < 0.3 pmol/min/pmol CYP for DFH activity, <0.005 pmol/min/pmol CYP for DMOD activity and <0.2 pmol/min/pmol CYP for MD1H and MD4H activities.

3. Results

3.1. Metabolism of diclofenac, dextromethorphan and midazolam

Fig. 1 shows the activities of DFH (A), DMOD (B), MD1H and MD4H (C) in cDNA-expressed rat CYPs. DFH activity, a catalytic marker of human CYP2C9 [6], was selectively catalyzed by CYP2C6 (15.4 pmol/min/pmol-CYP). DMOD activity, a catalytic marker of human CYP2D6 [7], was extensively mediated by CYP2D2 (3.4 pmol/min/pmol CYP). MD1H and MD4H activities, which are known to be catalyzed by human CYP3A4/3A5 [8], were predominantly catalyzed by CYP3A1/3A2. CYP3A2 showed higher MD4H activity than did CYP3A1 (8.2 vs. 2.8 pmol/min/pmol CYP), although CYP3A1 and CYP3A2 showed similar MD1H activities (1.6 and 1.8 pmol/min/pmol CYP, respectively).

3.2. Metabolism of phenacetin, alkoxyresorufins, bufuralol, p-nitrophenol, chlorzoxazone

Fig. 2 shows the activities of POD (A), EROD (B), BROD and PROD (C), BLH (D), PNPH (E) and CZH (F) in cDNA-expressed rat CYPs. Since the kinetics of POD in rat liver microsomes has been reported to be biphasic [9],

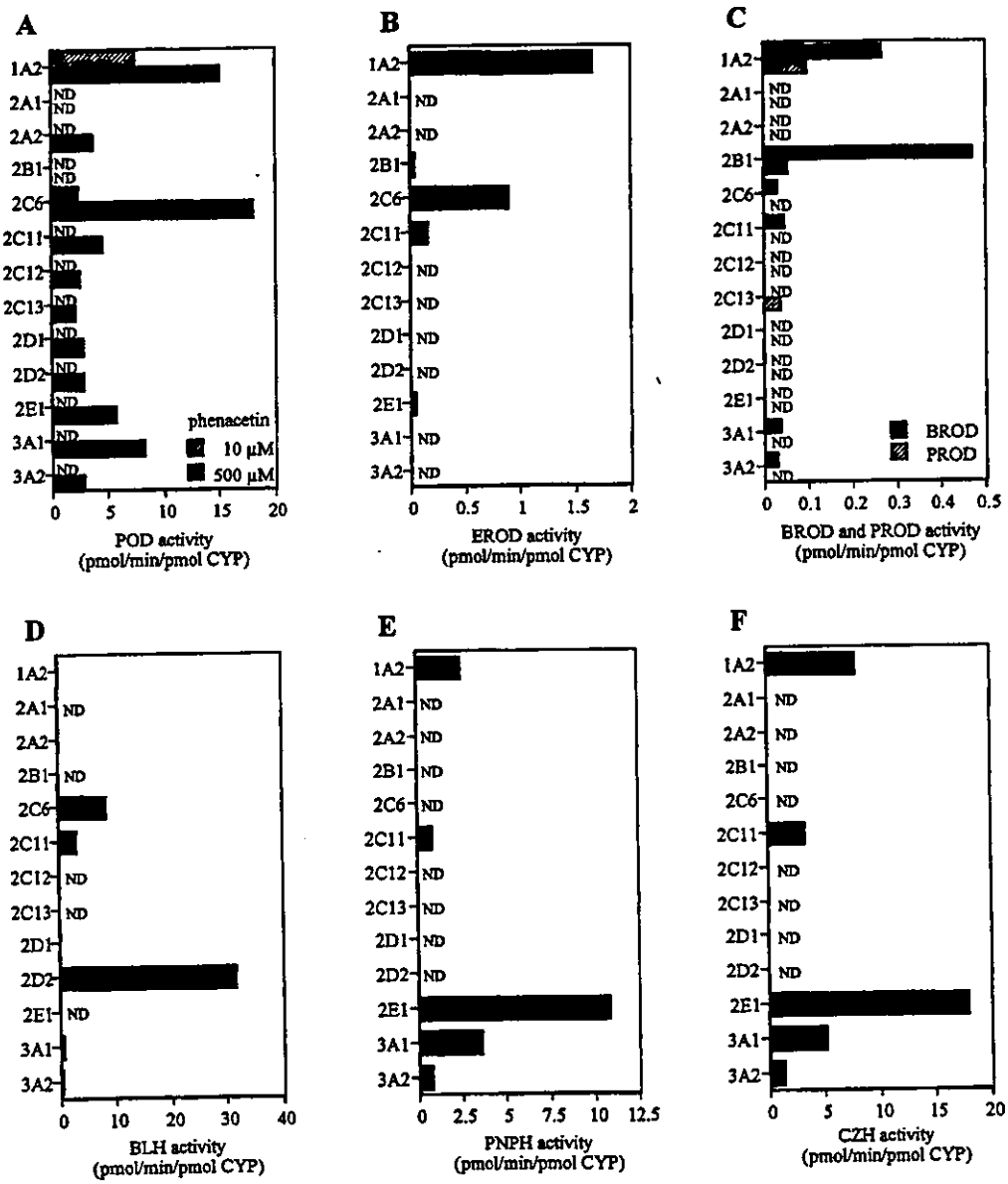

Fig. 2. Activities of POD (A), EROD (B), BROD and PROD (C), BLH (D), PNPH (E) and CZH (F) in cDNA-expressed rat CYPs. Substrates henacetin, 7-ethoxyresorufin, 7-benzyloxyresorufin, 7-pentoxyresorufin, bufuralol, *p*-nitrophenol and chlorzoxazone were incubated at 37° with cDNA-expressed rat CYPs. Substrate concentrations, incubation times and contents of CYP used are shown in Table 1. Each column represents the mean of duplicate experiments. ND < 2 pmol/min/pmol CYP for POD activity, <0.03 pmol/min/pmol CYP for EROD, BROD, PROD activities, <0.02 pmol/min/pmol CYP for BLH activity, <0.2 pmol/min/pmol CYP for PNPH activity and <1.0 pmol/min/pmol CYP for CZH activity.

POD activity was determined at low (10 μM) and high (500 μM) concentrations of the substrate. POD activity at a low substrate concentration, which is well established as a marker activity of human CYP1A2 [10], was observed in CYP1A2 and CYP2C6. CYP1A2 showed a higher POD activity than CYP2C6 did (7.5 vs. 2.5 pmol/min/pmol - CYP). At 500 μM phenacetin, both CYP1A2 and CYP2C6 showed high POD activity (15.1 and 18.0 pmol/min/pmol - CYP, respectively). CYP2A2, CYP2C11 CYP2C12, CYP2C13, CYP2D1, CYP2D2, CYP2E1, CYP3A1 and CYP3A2 also showed POD activity (>2.0 pmol/min/pmol CYP). Similarly, the activity of EROD, an alternative marker of human CYP1A1 and CYP1A2 [11], was also catalyzed by CYP1A2 and CYP2C6 (1.7 and 0.90 pmol/min/pmol CYP, respectively). As for the activity of BROD, which is used as a catalytic marker of human CYP2B6 [12], CYP2B1 showed the highest activity (0.47 pmol/min/pmol CYP), whereas CYP1A2 was also active (0.27 pmol/min/pmol CYP). Although PROD activity was catalyzed by CYP1A2, CYP2B1 and CYP2C13 (0.097, 0.053 and 0.036 pmol/min/pmol CYP, respectively), the activity was much lower than BROD activity in both CYP1A2 and CYP2B1. BLH activity, which is used as a CYP2D6 probe in human liver microsomes [13], was extensively metabolized by CYP2D2 (31.6 pmol/min/pmol CYP), whereas low BLH activity was detected in CYP2C6 and CYP2C11 (8.5 and 3.0 pmol/min/pmol CYP, respectively). PNPH activity and CZH activity, which are used as human CYP2E1 probes [14,15], were extensively catalyzed by CYP2E1 (10.8 and 17.9 pmol/min/pmol CYP, respectively). The activities were also detected in CYP1A2, CYP2C11, CYP3A1 and CYP3A2.

3.3. Metabolism of coumarin, S-mephenytoin and R-mephenytoin

Fig. 3 shows the activities of CRH (A) and RMH (B) in cDNA-expressed rat CYPs. CRH activity, which is specifically catalyzed by human CYP2A6 [16,17], was detected in multiple CYP isoforms, including CYP2A2, CYP2C6, CYP2C12, CYP2D2, CYP3A1 and CYP3A2 (>1.5 fmol/min/pmol CYP), although the activities were very low. Since SMH activity is specifically catalyzed by human CYP2C19 [18], SMH activity was determined using cDNA-expressed rat CYPs. Negligible SMH activity (<0.03 pmol/min/pmol CYP) was detected in all cDNA-expressed rat CYPs studied. On the other hand, RMH activity was detected in CYP2A2, CYP2C6, CYP2C11, CYP2C12 and CYP3A2 (>0.1 pmol/min/pmol CYP).

3.4. Metabolism of testosterone

The regioselective hydroxylation of testosterone has been well used for the identification of a specific isoform of CYP on the basis of results of studies using reconstituted systems consisting of a purified CYP isoform [19–21], although multiple CYP isoforms could catalyze a pathway of testosterone metabolism. In this study, the activities of 7α-, 16α- and 6β-hydroxylation of testosterone, which are mainly catalyzed by CYP2A1, CYP2C11 and CYP3A,

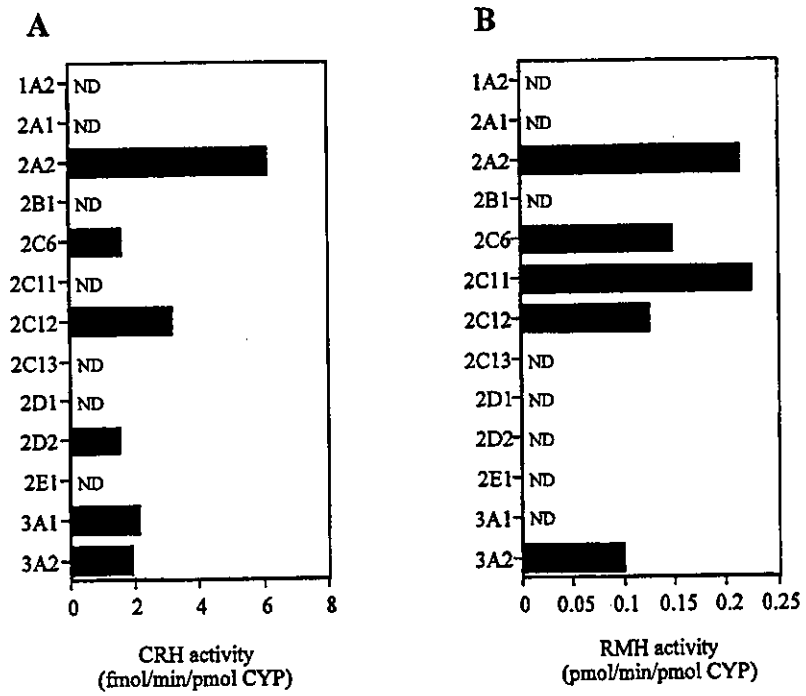

Fig. 3. Activities of CRH (A) and RMH (B) in cDNA-expressed rat CYPs. Substrates (coumarin and *R*-mephenytoin) were incubated at 37° with cDNA-expressed rat CYPs. Substrate concentrations, incubation times and contents of CYP used are shown in Table 1. Each column represents the mean of duplicate experiments. ND < 1.5 fmol/min/pmol CYP for CRH activity and <0.03 pmol/min/pmol CYP for RMH activity.

respectively, were reanalyzed using cDNA-expressed rat CYPs. When testosterone (25 μM) was incubated with each 5 pmol of cDNA-expressed rat CYP for 30 min at 37°, CYP3A2 showed higher 6β-hydroxylase activity than did CYP3A1 (8.8 vs. 1.8 pmol/min/pmol CYP). Testosterone 7α-hydroxylation activity was catalyzed by both CYP2A1 and CYP2A2 (1.7 and 0.48 pmol/min/pmol CYP, respectively). Testosterone 16α-hydroxylation activity was catalyzed by CYP2C11 (11.8 pmol/min/pmol CYP).

4. Discussion

Since the 1990s, the use of cDNA-expressed human CYPs has greatly facilitated the evaluation of metabolic specificities of probe substrates and the identification of individual CYPs involved in the metabolism of many drugs in humans. On the other hand, the specificities of metabolic reactions used as probes of CYP isoforms have not been thoroughly evaluated by the study with cDNA-expressed rat CYP isoforms. Therefore, in the present study, the isoform-specificities of metabolic reactions known as probes of human CYPs were examined by using 13 rat CYPs expressed in baculovirus-infected insect cells or human B-lymphoblastoid cells.

Among the metabolic reactions studied, activities of DFH and DMOD were selectively catalyzed by CYP2C6 and CYP2D2, respectively (Fig. 1A and B). MD1H and MD4H activities were predominantly catalyzed by CYP3A1/3A2, whereas MD1H activity was lower than MD4H activity in both CYP3A1 and CYP3A2 (Fig. 1C). These results suggest that activities of DFH, DMOD and MD4H are useful as catalytic markers of CYP2C6, CYP2D2 and CYP3A1/3A2, respectively.

On the other hand, activities of POD, EROD, BROD, PROD, BLH, PNPH and CZH were catalyzed by more than two isoforms in the present study using cDNA-expressed rat CYPs (Fig. 2). These results suggest that the activities of POD, EROD, BROD, PROD, BLH, PNPH and CZH, reactions known as catalytic markers of human CYPs, were not necessarily specific for rat CYPs. When multiple isoforms of CYP are involved in a metabolic reaction, isoform-selectivity occasionally differs depending on the substrate concentration [22], although the concentrations of probes used in this study were chosen on the basis of the K_m values of human liver microsomes or cDNA-expressed human CYPs. Therefore, additional studies are necessary to clarify the specificity of rat enzymes considering K_m values of rat CYPs for the probes of human CYPs.

In addition, there is a large difference in the levels of individual CYP isoforms in rat liver microsomes [2]. Moreover, levels of CYP isoforms in livers are very different between control rats and rats treated with compounds known to induce certain isoforms of CYP. Therefore, when multiple CYPs are involved in certain metabolic reaction, it is important to predict the contribution of each isoform to the reaction on the basis of the relative abundance of CYPs in rat liver microsomes. However, the turnover number of CYPs in rat liver microsomes is affected by several factors such as NADPH-CYP reductase, cytochrome b_5, membrane lipid composition, and ionic strength of the *in vitro* incubation matrix. Therefore, it is necessary to consider not only relative abundance of a particular CYP isoform in rat liver microsomes but also the differences in turnover number between the cDNA-expressed CYPs and liver microsomes to extrapolate the data obtained from cDNA-expressed CYPs to those of liver microsomes. Additional studies are now underway in our laboratory to clarify the isoform-specificity for activities of POD, EROD, BROD, PROD, BLH, PNPH and CZH in rat liver microsomes. These studies include the use of CYP isoform-selective inhibitors, determination of catalytic activities at different substrate concentrations, and comparison of catalytic activities in liver microsomes of rats treated with inducers of certain CYP isoforms with those of control rats.

Unlike the specificity to human CYPs, activities of CRH and SMH did not show selectivity toward any isoforms of cDNA-expressed rat CYPs (Fig. 3). Although CRH activity was catalyzed by multiple isoforms of cDNA-expressed rat CYP (CYP2A2, CYP2C6, CYP2C12, CYP2D2, CYP3A1 and CYP3A2), the activity was much lower than human CYP2A6-mediated CRH activity [17]. This finding is in agreement with previous results showing that CRH activity in rat liver microsomes was much lower than that in human liver microsomes [23]. In addition, coumarin is extensively metabolized to 7-hydroxycoumarin in human liver microsomes, whereas only one metabolite, *O*-hydroxyphenylacetic acid, was detected in rat liver microsomes [24]. Our results together with these observations suggest that CRH activity is unable to be used as a catalytic probe of a CYP isoform in rat liver microsomes, unlike the specificity of CRH activity to CYP2A6 in humans. Similarly, SMH activity was not substantially discernible in any of the cDNA-expressed CYPs examined. Therefore, SMH activity is also unable to be used as a catalytic probe of a CYP isoform in rat liver microsomes.

It has been reported that the kinetics of POD in rat liver microsomes is biphasic [9], which could indicate the involvement of more than one isoform of CYP. Moreover, Kahn and Rubenfield [25] reported that 3-methylcholanthrene selectively increased the high-affinity component of POD activity in rat liver microsomes, whereas phenobarbital selectively increased the low-affinity component. Since 3-methylcholanthrene specifically induces both CYP1A1 and CYP1A2 [26], the high-affinity component of POD is thought to be catalyzed by CYP1A1/1A2. However, it is not clear which isoform(s) catalyzes the low-affinity reaction of POD in rat liver microsomes because phenobarbital induces multiple CYP isoforms such as CYP2B, CYP2C6 and CYP3A [27]. The present study using cDNA-expressed rat CYPs showed that POD

activity at a high phenacetin concentration (500 μM) was catalyzed by CYP1A2 and CYP2C6, but not by CYP2A1 and CYP2B1 (Fig. 2A). Therefore, the low-affinity reaction of POD in rat liver microsomes might be catalyzed by CYP2C6.

EROD activity and BROD activity in human liver microsomes are widely used as probes for measuring CYP1A1/1A2 and CYP2B6, respectively. On the other hand, *in vitro* study using rat liver microsomes showed that BROD activity was a selective probe for CYP1A1 in 3-methylcholanthrene-induced microsomes and that both BROD activity and PROD activity were selective probes for CYP2B1 in phenobarbital-induced microsomes [28]. However, EROD activity was not a selective probe for CYP1A1 in non-induced rat liver microsomes and was metabolized by purified CYP2C6 [28]. BROD and PROD activity were metabolized by several different CYP isoforms in non-induced microsomes but mainly by both CYP1A1 and CYP1A2 in 3-methylcholanthrene-induced microsomes. The present results obtained from cDNA-expressed rat CYPs showed that EROD activity was not only catalyzed by CYP1A2 but also by CYP2C6 (Fig. 2B) and that BROD and PROD activity were catalyzed by CYP1A2 and CYP2B1 (Fig. 2C). The present results obtained by using cDNA-expressed rat CYPs are in agreement with the results of a previous study using purified rat CYPs [28].

In humans, only one CYP2D isoform, CYP2D6, has been found [1]. On the other hand, five CYP2D isoforms, CYP2D1 through CYP2D5, have been found in the rat liver [29–31]. The present results showed that BLH activity was extensively metabolized by CYP2D2, whereas CYP2D1 barely catalyzed the activity (Fig. 2C). These results are in agreement with previous results [32,33] showing that heterogously expressed CYP2D2 had high affinity for bufuralol compared with the other CYP2D isoforms. In addition to CYP2D2, CYP2C6 and CYP2C11 also showed BLH activity. Therefore, BLH activity might not be a specific probe of CYP2D2 in liver microsomes from rats treated with compounds known to induce CYP2Cs.

The present study showed that activities of PNPH and CZH are extensively catalyzed by CYP2E1 and that CYP1A2 and CYP3A1 are also capable of catalyzing those activities (Fig. 2E and F). These results are consistent with those of previous studies using rat liver microsomes. PNPH activity has been shown to be mainly catalyzed by CYP2E1 in the rat [34]. However, CYP3A inducer treatment increased PNPH activity in rat liver microsomes, and the activity was inhibited by a CYP3A inhibitor [35]. Therefore, not only CYP2E1 but also CYP3A1 appear to be able to catalyze PNPH activity in rat liver microsomes. For CZH activity, kinetic analysis showed a biphasic manner in non-treated rat liver microsomes, and it has been suggested that high- and low-affinity components are responsible for CYP3A and CYP2E1, respectively [36]. In addition, treatment with a CYP1A inducer increased CZH activity in rat liver microsomes and the activity was inhibited by a CYP1A inhibitor. Therefore, CZH activity in rat liver microsomes appears to be catalyzed by several isoforms of CYP, such as CYP1A2, CYP2B1 and CYP3A1.

In humans, 4′-hydroxylation of mephenytoin preferentially occurs with *S*-enantiomer [37]. The SMH activity is mainly catalyzed by CYP2C19. However, negligible SMH activity was detected in all cDNA-expressed rat CYPs studied. Yasumori *et al.* [38] suggested that this is because rats showed a preferential RMH activity in a manner opposite to that in humans. They reported that CYP2C11, CYP3A1 and CYP3A2 were able to efficiently catalyze RMH activity in a reconstituted system. Therefore, RMH activity was determined using cDNA-expressed rat CYPs. The results showed that CYP2A2, CYP2C6 and CYP2C13 were also able to catalyze RMH activity as well as CYP2C11 and CYP3A2, although CYP3A1 showed negligible RMH activity (Fig. 3B). These results suggest that RMH activity is not selective toward any isoforms of cDNA-expressed rat CYPs and that neither RMH activity nor SMH activity appears to be useful as catalytic probes of CYP isoforms in rat liver microsomes. Therefore, it appears that probes for CYP isoforms do not always exhibit the same specificity in human and rat. Caution should be exercised when extrapolating the metabolic data obtained in rat to the human situation.

In the present study, 13 rat CYPs were investigated, however, they might be not all of the possible CYPs involved in the metabolism of the probes studied. Therefore, it should be noted that the other isoforms might contribute to the metabolism of the probes used in the present study.

In conclusion, the present study suggested that activities of DFH, DMOD and MD4H are useful as catalytic markers of CYP2C6, CYP2D2 and CYP3A1/3A2, respectively. In contrast, activities of CRH, SMH and RMH are not able to be used as catalytic probes of CYP isoforms in rat liver microsomes. However, it is necessary to conduct further study to clarify CYP isoform-specificity for these activities in rat liver microsomes, since activities of POD, EROD, BROD, PROD, BLH, PNPH and CZH were found to be catalyzed by more than two isoforms in this study using cDNA-expressed rat CYPs. These results may provide useful information regarding catalytic probes of rat CYP isoforms for studies using rat liver microsomal samples.

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PHARMACOGENETICS AND GENOMICS

Population differences in *S*-warfarin metabolism between *CYP2C9* genotype–matched Caucasian and Japanese patients

Objective: Our objective was to investigate population differences in the metabolic activity of cytochrome P450 (CYP) 2C9 between genotypically matched Caucasian and Japanese patients by using the unbound oral clearance of *S*-warfarin as an in vivo phenotypic trait measure.

Methods: Ninety Japanese and 47 Caucasian patients receiving maintenance warfarin therapy were studied. Steady-state plasma unbound concentrations of *S*-warfarin were measured by a chiral HPLC method coupled with an ultrafiltration technique, and unbound oral clearance for *S*-warfarin was estimated. By combining plasma unbound concentrations of *S*-warfarin with the urinary excretion rates of *S*-7-hydroxywarfarin, the formation clearance of *S*-7-hydroxywarfarin was also determined. Genotyping of CYP2C9 was performed for 6 distinct alleles (*CYP2C9*1*, *CYP2C9*2*, *CYP2C9*3*, *CYP2C9*4*, *CYP2C9*5*, and a *T/C* transition in intron 2).

Results: The frequency distribution of unbound oral clearance for *S*-warfarin obtained from Japanese patients was shifted toward higher values as compared with that in Caucasian patients. Japanese patients had lower allelic frequencies for the 5 variants than Caucasian patients. When interpopulation comparisons of CYP2C9 activity were made for genotype-matched subjects, Japanese patients with the homozygous *CYP2C9*1* (wild-type) genotype (n = 85) had significantly ($P < .01$) greater median values for unbound oral clearance and formation clearance than Caucasian patients with the corresponding genotype (n = 26), 10.4 mL × min^{-1} × kg^{-1} versus 4.25 mL × min^{-1} × kg^{-1} and 0.015 mL × min^{-1} × kg^{-1} versus 0.010 mL × min^{-1} × kg^{-1}, respectively. In addition, Japanese patients heterozygous for the *CYP2C9*3* genotype (n = 4) showed a significantly ($P < .05$) reduced unbound oral clearance for *S*-warfarin, by 63%, as compared with Japanese patients possessing the homozygous *CYP2C9*1* genotype. By contrast, in Caucasian patients, no significant differences were observed in this parameter between *CYP2C9*1* homozygous subjects and those with heterozygous *CYP2C9*2* or *CYP2C9*3* genotypes.

Conclusions: These findings indicate that population differences in the frequencies of known variant *CYP2C9* alleles account only in part for the variability observed in in vivo CYP2C9 activity in different populations. In addition, a gene-dose effect of defective *CYP2C9* alleles on the in vivo CYP2C9 activity is evident in Japanese patients but not in Caucasian patients. Further studies are required to identify currently unknown factor(s) (eg, transcriptional regulation) responsible for the large intrapopulation and interpopulation variability in CYP2C9 activity. (Clin Pharmacol Ther 2003;73:253-63.)

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CYP3A4 Inducible Model for *In Vitro* Analysis of Human Drug Metabolism Using a Bioartificial Liver

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CYP3A is responsible for approximately 50% of the therapeutic drug-metabolizing activity in the liver. The present study was undertaken to establish the CYP3A4 inducible model for analysis of human drug metabolism using a bioartificial liver composed of the functional hepatocellular carcinoma cell (HCC) line FLC-5. A radial-flow bioreactor (RFB), which is a carrier-filled type bioreactor, was used for 3-dimensional perfusion culture of FLC-5 cells. The CYP3A4 messenger RNA (mRNA) expression level 48 hours after rifampicin treatment in the RBF was approximately 100 times higher than that in a monolayer culture. Western blot analysis also demonstrated an increase in expression of the CYP3A protein. When testosterone, a substrate for CYP3A4, was added to the rifampicin-treated cell culture, 6β-hydroxy testosterone as a metabolite was formed. Electrophoretic mobility shift assay (EMSA) with a CYP3A4 ER6 probe demonstrated that relatively high molecular weight complex containing pregnane X receptor (PXR)/retinoid X receptorα(RXRα), compared with that in the monolayer culture, is possibly generated in the RFB culture of FLC-5 treated with rifampicin. Similarly, the assay with a probe of HNF-4α-binding motif indicated the formation of a large protein complex in the RFB culture. Because it is known that PXR transactivates CYP3A4 gene via its response element and expression of PXR is regulated by HNF-4α, the large complexes binding to response elements of PXR or HNF-4α in the RFB culture may contribute to up-regulation of CYP3A4 mRNA. In conclusion, the bioartificial liver composed of human functional HCC cell line was useful in studying drug interactions during induction of human CYP3A4. (HEPATOLOGY 2003;37:665-673.)

Drug-drug interactions can be categorized as mediated by metabolic inhibition and enzyme induction. Numerous studies have been conducted on metabolic inhibition. It is now possible, to some extent, to predict drug-drug interactions *in vivo* based on the results *in vitro*. Enzyme induction has been studied using experimental animals, but, because of the existence of interspecies differences, it is often difficult to extrapolate the results of animal studies directly to humans.[1]

To resolve these problems, enzyme-induction experiments have been performed using human primary cell culture systems, and experimental models established using these cell cultures have been recognized to be useful in the evaluation of enzyme induction.[2] However, long-term primary culture of human hepatocytes can reduce the functions of liver enzymes, as reflected, for example, by a decrease in the level of cytochrome P450 (CYP), an enzyme mainly catalyzing phase-I reactions of drug metabolism, 4 days after isolation, making it difficult to use these cells for prolonged periods of time.[3] Furthermore, because of interracial or sex-related differences and differences in storage periods (cell viability), the drug-metabolizing activity varies greatly among cells, causing problems in reproducibility.[4,5] For these reasons, much has been expected of liver cell lines that have uniform and stable properties, can be used for long-term experiments, are highly differentiated, and retain human liver functions.

Abbreviations: CYP, cytochrome P450; HCC, hepatocellular carcinoma; RFB, radial-flow bioreactor; PXR, pregnane X receptor; RXR, retinoid X receptor; ER, everted repeat; FBS, fetal bovine serum; PB, phosphate buffer; HNF, hepatocyte nuclear factor; FAM, 6-carboxy-fluorescein; JOE, 2,7-dimethoxy-4,5-dichloro-6-carboxy-fluorescein; TAMRA, 6-carboxy-tetramethyl-rhodamine; RT-PCR, reverse transcription-polymerase chain reaction; mRNA, messenger RNA; HPLC, high performance liquid chromatography; EMSA, electrophoretic mobility shift assay.

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Under these circumstances, studies of drug metabolism in cells derived from humans have been conducted using not only primary liver culture systems but also cell lines established from the liver, such as the human hepatoblastoma cell line HepG2, and the expression and induction of drug-metabolizing enzymes have been examined.[6-8] It has also been reported that their cell line differs from human hepatocytes in terms of cell morphology, protein synthesis, and enzymes involved in the metabolism of the drugs. To date, we have established 7 cell lines derived from Japanese hepatocellular carcinoma (HCC) patients. Of these, one that has relatively well-preserved liver functions and can be cultured in serum-free medium has been named *FLC* (functional liver cell).[9] The FLC-5 cell line was selected for this study because CYP3A4 expression can be induced in it, making it very useful for the study of drug metabolism.

The radial-flow bioreactor (RFB), developed in Japan, is a high-function 3-dimensional culture system, which can be used for high-density culture. This system activates a density 10 times that obtained in hollow-fiber culture systems[10,11] and 100 times that in floating cell culture. The cylindrical reactor is filled with porous hydroxy apatite beads. This bioreactor can be characterized as a system in which the medium flows from the periphery toward the center of the reactor. For obtaining a high-density cell culture, it is essential to ensure that biased distribution of oxygen and nutrients at the inlet and outlet of the culture medium is minimized. If the medium flows from the periphery toward the center, the high perfusion rate at the center would allow adequate supply of oxygen and nutrients to the cells at the center even while oxygen and nutrients are consumed at the periphery, thereby allowing the cells to remain viable. When FLC cells were incubated in this RFB system, they could be cultured at high density and maintained viable for long periods of time.[12] It has also been reported that the ability of FLC cells to produce albumin is higher when cultured in the RFB system than in monolayer culture.[13] These results suggest that this RFB system can be used to obtain cultured hepatocytes with improved cell functions.

Regarding drug-metabolizing functions, a model for analysis of drug metabolism in the human liver could be established if the cells were subjected to 3-dimensional high-density culture in the RFB system, and a bioartificial liver will be created. Of all the human CYP isoforms, CYP3A is responsible for approximately 50% of the therapeutic drug-metabolizing activity in the liver.[14] Particularly, CYP3A4 is the most important subtype of CYP3A in humans. Recently, it has been shown that CYP3A4 inductivity in the liver is regulated by the pregnane X receptor (PXR)/retinoid X receptor (RXR)α heterodimer through a response element containing 2 everted repeats separated by 6 nucleotides (ER6) of CYP3A4 promoter region.[15]

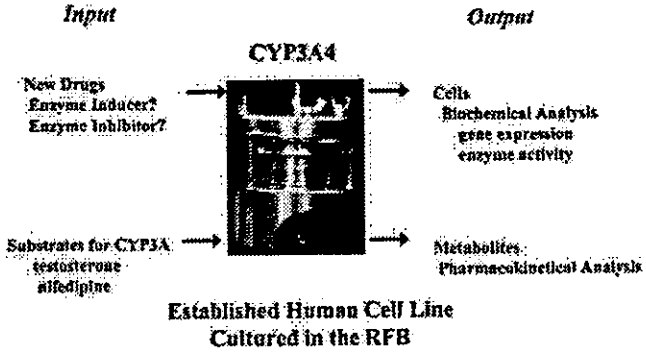

Fig. 1. The simulation system for drug metabolism composed of human functional HCC cell line cultured in RFB. This system is useful in studying drug interactions during induction of human CYP3A4.

In the present study, CYP3A4 inductivity through PXR/RXRα heterodimer in FLC-5 cells cultured in the RFB was compared with that of the cells in monolayer culture, with the goal of establishing a simulation model for the analysis of drug metabolism in the human liver, without recourse to experimental animals (Fig. 1).

Materials and Methods

Human HCC Cell Line. Human HCC cell line FLC-5 is maintained in ASF104 serum-free medium (Ajinomoto Co., Ltd., Tokyo, Japan) in an incubator with a constant temperature of 37°C and a highly humidified atmosphere of 95% air and 5% CO_2. Cell passage was carried out using 25 USP units/mL trypsin (Difco Co., Ltd.) added to 0.02% EDTA in solution to isolate cells. For this experiment, 1 to 2 $\times$ 10^6 cells of FLC-5 were cultured in 4 mL of ASF-104 medium without fetal bovine serum (FBS).

Culture in the Radial Flow Bioreactor. Use of an RFB (RA-15, ABLE Co., Ltd., Tokyo, Japan) and mass flow controller (RAD925, ABLE Co., Ltd.) has previously been reported for the high-density, 3-dimensional mass production of cells, which attach to a matrix.[12,13] The matrix consisted of hydroxy apatite beads (diameter 1-2 mm, pore size <200 μm, Asahi Optical Co., Ltd., Tokyo, Japan), with a high pore density, which gave a wide surface attachment area.

Two to 5 $\times$ 10^7 cells of FLC-5 cell were injected in the reservoir of the RFB system, which was filled in ASF104 medium. Isolated cells were loaded in 15 mL volume of the RFB column using a circulation pump at 25 mL/min. Loading cells became trapped and adhered to the porous culture beads at a rate based on medium flow. Although

FLC-5 cells can grow in serum-free medium, 2% FBS and 3 g/L glucose was added to the inoculation medium to facilitate attachment of cells to the matrix. After attachment of cells, the medium was changed to ASF104 without FBS.

Observation for Fine Morphology. For the scanning electron microscopy (SEM), cultured cells were fixed with 1.2% glutaraldehyde in 0.1 mol/L phosphate buffer (PB), pH 7.4 and postfixed with 1% OsO_4 in 0.1 mol/L PB. The fixed cells were rinsed twice with phosphate-buffered saline, subsequently dehydrated in ascending concentrations of ethanol, critical point dried using carbon dioxide, and coated by vacuum-evaporated carbon and ion-spattered gold. Specimens were observed by JSM-35 (JEOL, Tokyo, Japan) at an accelerated voltage of 10 kV. For transmission electron microscopy (TEM), cultured cells were fixed with 2.0% glutaraldehyde in 0.1 mol/L PB and postfixed with 1% OsO_4 in 0.1 mmol/L PB. Specimens were dehydrated in ethanol and embedded in a mixture of Epon-Araldite. Thin sections were made with a diamond knife mounted on a LKB ultratome and stained with aqueous uranyl acetate. Specimens were examined with a JEOL 1200EX electron microscopy.

Real-Time Polymerase Chain Reaction. Based on the DNA sequences in GenBank, primers and the TaqMan probe for CYP3A4, PXR, RXRα, and hepatocyte nuclear factor 4α (HNF-4α) were designed using the primer design software Primer Express TM (Perkin-Elmer Applied Biosystems, Foster City, CA).

AmpliTaq DNA polymerase extended the primer and displaced the TaqMan probe through its 5′-3′ exonuclease activity. The probes were labeled with a reporter fluorescent dye (6-carboxy-fluorescein [FAM] or 2,7-dimethoxy-4,5-dichloro-6-carboxy-fluorescein [JOE]) at the 5′ end and a quencher fluorescent dye (6-carboxy-tetramethyl-rhodamine [TAMRA]) at the 3′ end.

The primer/probe is as follows: CYP3A4 forward primer: 5′-CTTCATCCAATGGACTGCATAAAT-3′, reverse primer: 5′-TCCCAAGTATAACACTCTACACAGACAA-3′; probe: 5′-(FAM) CCGGGGATTCTGTACATGCATTG (TAMRA)-3′. PXR forward primer: 5′-TCCCCAAATCTGCCGTGTAT-3′, reverse primer: 5′-AGCCCTTGCATCCTTCACAT-3′; probe: 5′-(FAM) ACAAGGCCACTGGCTATCACTTCAATGTCA (TAMRA)-3′. RXRα forward primer: 5′-GCGCTGAGGGAGAAGGTCTAT-3′, reverse primer: 5′-CAGGCGGAGCAAGAGCTTAG-3′; probe: 5′-(FAM) AGGCCTACTGCAAGCACAAGTACCCAGA (TAMRA)-3′. HNF-4α forward primer: 5′-GGTGTCCATACGCATCCTTGA-3′, reverse primer: 5′-TGGCTTTGAGGTAGGCATACTCA-3′; probe: 5′-(FAM) CCTTCCAGGAGCTGCAGATCGATGAC (TAMRA)-3′.

Fifty microliters of reaction mixture were used, containing 10 ng of the extracted total RNA, 0.3 mmol/L forward and 0.9 mmol/L reverse primers, 0.2 mmol/L TaqMan probe, the TaqMan 1-step reverse transcription-polymerase chain reaction (RT-PCR) Master Mix Reagents Kit (4309169; Perkin-Elmer Applied Biosystems Co., Ltd.). The conditions of 1-step RT-PCR were as follows: 30 minutes at 48°C (stage 1, RT), 10 minutes at 95°C (stage 2, RT inactivation and AmpliTaq Gold activation), and 60 cycles of amplification for 15 seconds at 95°C and 1 minute at 60°C (stage 3, PCR). The assay used an instrument capable of measuring fluorescence in real time (ABI Prism 7700 Sequence Detector; Perkin-Elmer Applied Biosystems Co., Ltd.). Signals were detected according to the manufacturer's instructions.

The calibration curve, covering from 1.000 ng total RNA/50 μL reaction system diluted serially at a common ratio of 1:5 to 0.32 ng total RNA/50 μL, was created using the total RNA collected from monolayer FLC-5 cultures.

The specificity was evaluated using GAPDH messenger RNA (mRNA) as the internal control (4310884E; Perkin-Elmer Applied Biosystems Co., Ltd.). Each test was done in triplicate, and averages were obtained.

Microsome Preparation and Western Blotting. Beads (0.5-1.0 g), to which cells stored at −80°C were attached, were mixed with twice the amount of homogenization buffer (0.25 mol/L sucrose, 50 mmol/L Tris Buffer, pH 7.4). The mixture was agitated on a shaker. After sedimentation of the beads spontaneously, the supernatant containing the destroyed cellular components was transferred to a centrifuge tube for centrifugation at 1,000 rpm for 5 minutes. The supernatant was poured into each ultracentrifuge tube (TL-100, Beckman) for centrifugation at 10,000*g* for 10 minutes, and the supernatant was centrifuged at 105,000*g* for 60 minutes. The microsomal fraction, harvested as a pellet, was suspended in homogenization buffer and stored frozen at −80°C.

Protein in the adjusted microsomal suspension was quantified by Protein Assay Rapid Kit (Wako, Osaka, Japan). Subsequently, 2.5 μg of microsomal protein in each lane was subjected to SDS-polyacrylamide gel electrophoresis, in 4% to 20% gradient gel. Upon completion of electrophoresis, the protein was transferred from the gel onto the polyvinylidene difluoride membrane. The polyvinylidene difluoride membrane was immersed in reaction buffer (0.025 mol/L Tris, 0.15 mol/L NaCl, pH 7.4) containing 3% bovine serum albumin and agitated for 30 minutes to effect blocking. The membrane was then exposed to anti-human CYP3A4 antibody, which was made

by Chiba University, at room temperature for 1 hour. Thereafter, after being washed, it was exposed to anti-rabbit-IgG-conjugated alkaline phosphatase (Gentest Corp.) at room temperature for 1 hour. After further washing, it was immersed in alkaline phosphatase buffer (0.1 mol/L NaCl, 0.05 mol/L $MgCl_2$, 0.1 mol/L Tris, pH 9.5) and exposed to substrate solution composed of a mixture of nitroblue tetrazolium (NBT, Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma) for color development.

Investigation of Testosterone Metabolism Using a Bioartificial Liver. To check for CYP3A activity in the FLC-5 cells incubated in the bioreactor, testosterone (50 μmol/L) was added to the reservoir of the bioreactor system, and the bioreactor was perfused with the culture medium. Six hours later, the medium and cell-affixed beads were harvested and stored frozen at −40°C. To examine CYP3A4 induction, 50 μmol/L of rifampicin (Sigma) was added to the medium 48 hours before the addition of testosterone. Testosterone (Wako, Osaka, Japan) was dissolved in DMSO, and the medium was diluted to obtain a final DMSO concentration of 0.25% or lower. Monolayer cultured FLC-5 cells were also treated with 50 μmol/L of rifampicin for 48 hours.

The concentration of testosterone in the frozen samples was measured by high-performance liquid chromatography (HPLC). As an internal standard for HPLC, nitrazepam (Wako) was added to 0.5 mL of the medium at a concentration of 10 μg/mL methanol. Ethyl acetate (5 mL) was added to the mixture, which was then agitated for 10 minutes and thereafter centrifuged at 3,200 rpm for 5 minutes. A 4.5 mL aliquot of the upper layer (organic layer) was transferred to a glass tube, dried in a centrifuge evaporator, and mixed with the mobile phase for HPLC (250 μL). The mobile phase was composed of a 40%:60% deaired mixture of DW and methanol, whose pH was adjusted to 3.4 with H_3PO_4. The column used was a reversed-phase CAPCELLPAK C18 UG120 (Shiseidou Co., Ltd., Tokyo, Japan). The detector was an SPD-10AVP ultraviolet absorption detector (Shimazu Co., Ltd., Tokyo, Japan). The sample was injected at a volume of 70 μL, and the mobile phase flowed at the rate of 0.7 mL/min. The absorbance at 244 nm was then measured.

Electrophoretic Mobility Shift Assay. For electrophoretic mobility shift assay (EMSA), 20 μL of each sample contained 10 mmol/L Tris (pH 8.0), 40 mmol/L KCl, 0.05% NP-40, 6% glycerol, 1 mmol/L DTT, 0.2 μg of poly (dI-dC), and 20 μg each of FLC-5 nuclear extracts. Competitor oligonucleotides were included at a 5-fold or 10-fold excess. After a 10-minute incubation on ice, 10 ng of [^{32}P]-labeled oligonucleotide was added, and the incubation continued for an additional 30 minutes. DNA-protein complexes were resolved on a 5% polyacrylamide gel in 0.5 × TBE (1 × TBE = 90 mmol/L Tris, 90 mmol/L boric acid, 2 mmol/L EDTA). Gels were dried and determined by a Fujix bioimage analyser BAS2000 (Fuji Photo Film Co. Ltd., Tokyo, Japan). The following oligonucleotides were used as either radiolabeled probes or competitors (sense strand is shown): CYP3A4ER6 binding site: 5′-GATCAATATGAACTCAAAGGAG-GTCAGTG-3′; HNF4α-binding site: 5′-CTCAGCTT-GTACTTTGGTACAACTA-3′; NF-κB binding site: 5′-AGTTGAGGGGACTTTCCCAGGC-3′. Anti-PXR (N-16, s.c. 9,690 X; Santa Cruz Biotechnology), anti-HNF-4α (S-20, s.c. 6,557 X; Santa Cruz Biotechnology), and control goat IgG (s.c. 2,028; Santa Cruz Biotechnology) were used in super-shift assays.

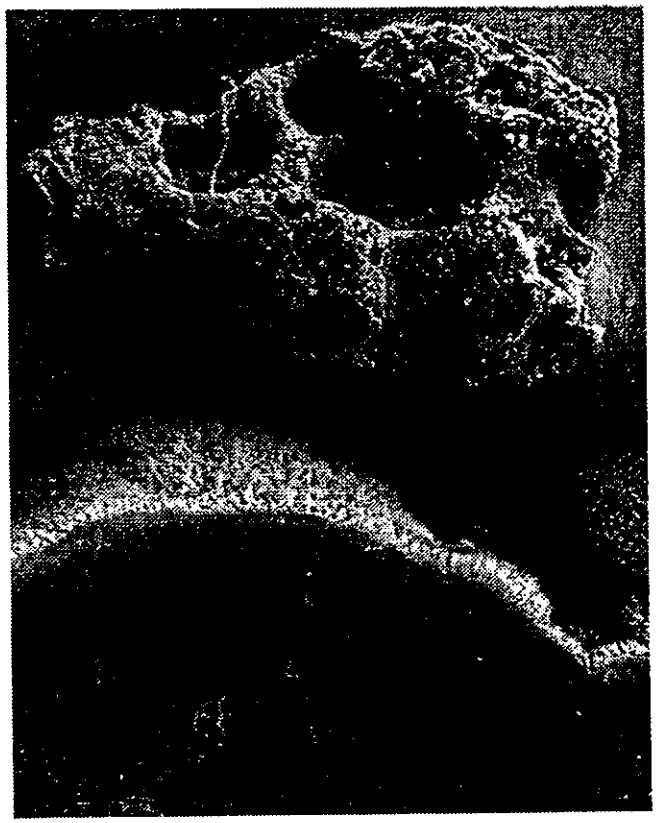

Fig. 2. Fine structural observation of FLC-5 cells cultured in a radial-flow bioreactor under an SEM. (A) The FLC-5 cells remain viable and have formed layers on the hydroxyapatite beads. (B) On cross-section, microvilli are distributed densely on the side facing the culture medium flow tract.

Results

Observation With Electron Microscope. FLC-5 cells remain viable and have formed layers on the surface of the hydroxy apatite beads to be present within the pores (Fig. 2A). On cross-section, microvilli had developed

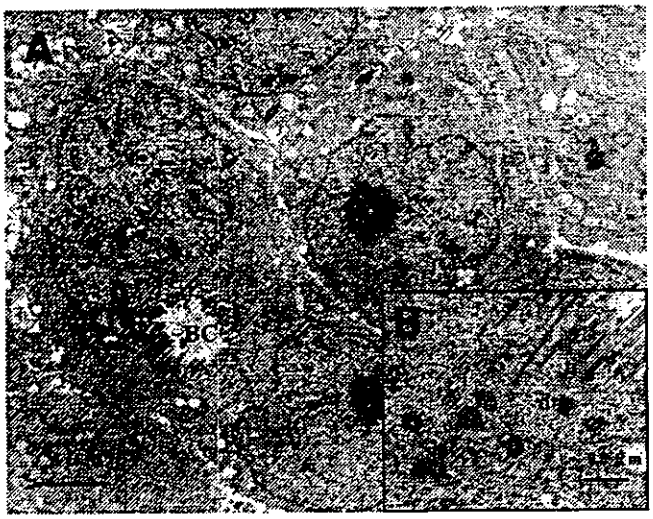

Fig. 3. Fine structural observation of FLC-5 cells incubated in the radial-flow bioreactor under TEM. (A) The cultured cells assume a spherical or cubical form. BC, Bile canaliculi-like structures. Tight junctions (tj) and desmosome (d) are visible in the intercellular spaces.

densely on the side of the viable cells facing the medium flow tract (Fig. 2B). Under a transmission electron microscope, the cells were distributed at a high density, and bile-canaliculi-like structures and desmosome and tight junction were visible in the intercellular spaces (Fig. 3). The cells were spherical or cubical in shape, indicating that a 3-dimensional culture had been obtained.

mRNA and Protein Expression of CYP3A4 in FLC-5 Cultured in the RFB. We compared mRNA expression of CYP3A4 in FLC-5 cultured in the RFB with that in FLC-5 cultured in monolayer. In the absence of rifampicin, the amount of CYP3A4 expressed in the RFB system was 7.7 times higher than that in monolayer culture. The amount expressed in a monolayer culture with rifampicin was 2.4 times that in a monolayer culture without rifampicin. In the RFB system, the amount of CYP3A4 expressed with rifampicin was 34 times higher than that without rifampicin. The amount expressed in the RFB system with rifampicin was 108 times higher than that in a monolayer culture with rifampicin (Fig. 4A). The microsomal fraction of the rifampicin-treated cells exhibited a markedly increased CYP3A expression level, even at the protein level (Fig. 4B). These results demonstrated that FLC-5 cultured in the RFB with rifampicin increased mRNA and protein expression of CYP3A4.

Metabolism of Testosterone in FLC-5 Cultured in the RFB With or Without Rifampicin. Next, we investigated whether a testosterone as one of the substrates for CYP3A4 might be metabolized obviously or not. FLC-5 cells incubated in a 15-mL volume RFB were combined with 50 μmol/L of testosterone and subjected to a 6-hour testosterone metabolism experiment in a closed system. In the control group, which was not pretreated with rifampicin, the amount of 6β-hydroxy testosterone released in the medium was significantly smaller than that in the rifampicin-pretreated condition (Fig. 5A). In cases in which CYP3A4 had been induced by pretreatment with rifampicin, the culture medium showed peaks of 6β-hydroxy testosterone (Fig. 5B). These results demonstrated that the RFB was useful for the *in vitro* metabolic system of substrate of CYP3A4.

Induction of CYP3A4 by PXR and Regulation of PXR by HNF-4α. We hypothesized that PXR, transcriptional factor for CYP3A4, and HNF-4α, transcriptional factor for PXR, effectively regulated the induction of CYP3A4 in RFB culture. To investigate, we first estimated the mRNA of PXR and HNF-4α by the TaqMan real-time PCR. In the absence of rifampicin induction, the amount of PXR mRNA expressed in the RFB system was 2 times higher than that in the monolayer culture but RXRα was almost of the same level. In the RFB system, the amount of PXR mRNA expressed with rifampicin was 15.5 times higher than that without rifampicin but RXRα was half of the lower level. The amount of PXR mRNA expressed in the RFB system with rifampicin was 22 times higher than that in a monolayer culture with rifampicin but of RXRα was 0.4 times the lower level (Fig. 6A).

On the other hand, in the absence of rifampicin, the amount of HNF-4α expressed in the RFB system was 1.23 times higher than that in the monolayer culture. The amount expressed in a monolayer culture with rifampicin was 1.25 times that in a monolayer culture without rifampicin. In the RFB system, the amount of HNF-4α mRNA expressed with rifampicin was 1.7 times higher than that without rifampicin. The amount expressed in the RFB

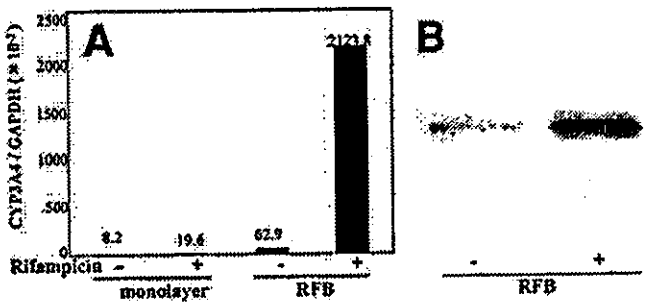

Fig. 4. (A) Comparison of the expression of human CYP3A4 mRNA in FLC-5 cells incubated under different conditions, as assessed by TaqMan 1-step RT-PCR. Each RNA concentration was calculated from the average of triplicate measurements and divided by the average of the corresponding GAPDH values obtained in the same way to yield a corrected RNA concentration. (B) Comparison of protein expression of human CYP3A4 in FLC-5 cells cultured in RFB with or without rifampicin, as analyzed by Western blotting. A 57-kd-specific band was observed corresponding to CYP3A4.

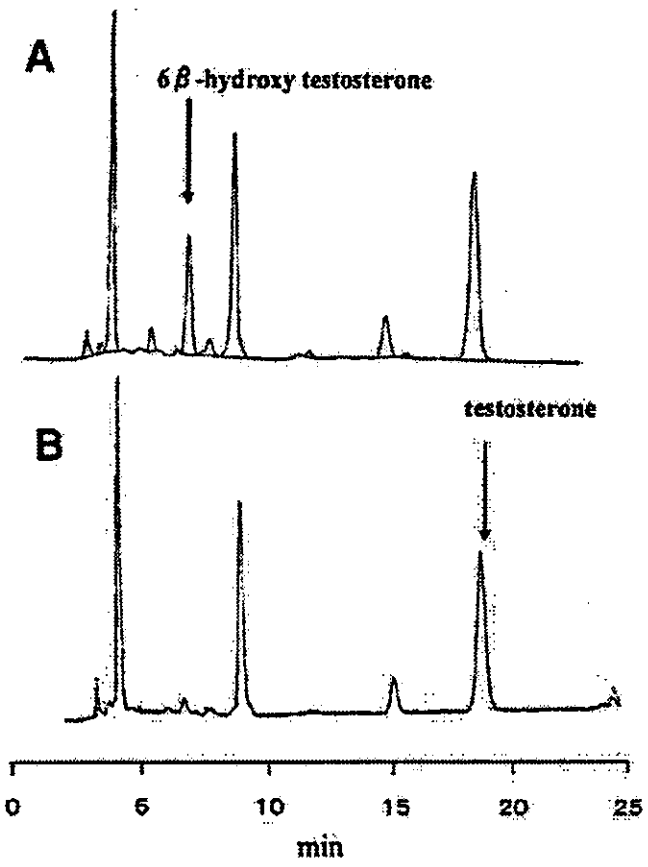

Fig. 5. Formation of 6β-hydroxy testosterone in FLC-5 cells. Testosterone was added to the medium used for the incubation of FLC-5 cells pretreated without and with rifampicin in the radial-flow bioreactor. (A) In the medium under rifampicin pretreatment condition, 6β-hydroxy testosterone was clearly detected. (B) 6β-hydroxy testosterone (a metabolite of CYP3A4) was also detectable but traced level in the medium without pretreatment of rifampicin.

system with rifampicin was 1.67 times higher than that in a monolayer culture with rifampicin (Fig. 6B).

Furthermore, to investigate the functional activity of PXR/RXRα proteins in our culture systems, we performed EMSA, with nuclear extracts prepared from FLC-5 cultured by monolayer or by RFB and radiolabeled oligonucleotide containing CYP3A4 ER6. As shown in Fig. 7A, specific protein-DNA complexes were observed both in nuclear extracts from monolayer- and in RFB-cultured FLC-5. However, the complex in RFB culture (shown as LC) exhibited a retarded mobility compared with that in monolayer culture (shown as C). This result further suggests that nuclear factor(s) was interacted with either PXR/RXRα heterodimer or ER6 motif in the RFB-cultured cells. Competition experiments with unlabeled oligonucleotides of wild-type CYP3A4 ER6-binding and NF-κB-binding site further demonstrated the specificity of the binding as exemplified in Fig. 7A. In addition, the complex in RFB culture was retarded further with anti-PXR antibodies (Fig. 7A, lanes 7 and 8, shown as SS).

To characterize the nature of protein-ER6 complex in the nuclear extracts from the RFB culture treated with rifampicin, we performed a long-time electrophoresis in the EMSA. As shown in Fig. 7B, lanes 5 and 6, we found a further retarded mobility of the protein-ER6 complex present in rifampicin-treated RFB culture (shown as SLC), which was hardly recognized in the standard condition of polyacrylamide gel electrophoresis (Fig. 7B, lanes 1-4). Such a super-shifted band was not observed in the monolayer culture in the presence of rifampicin (Fig. 7B, lane 3). Thus, it seemed possible that rifampicin induced formation of nuclear protein-DNA complex with higher molecular weight in the RFB culture.

On the other hand, we investigated the functional activity of HNF-4α proteins by the same method with nuclear extracts prepared from FLC-5 cultured by monolayer or by the RFB and radiolabeled oligonucleotide containing HNF-4α-binding site. As shown in Fig.

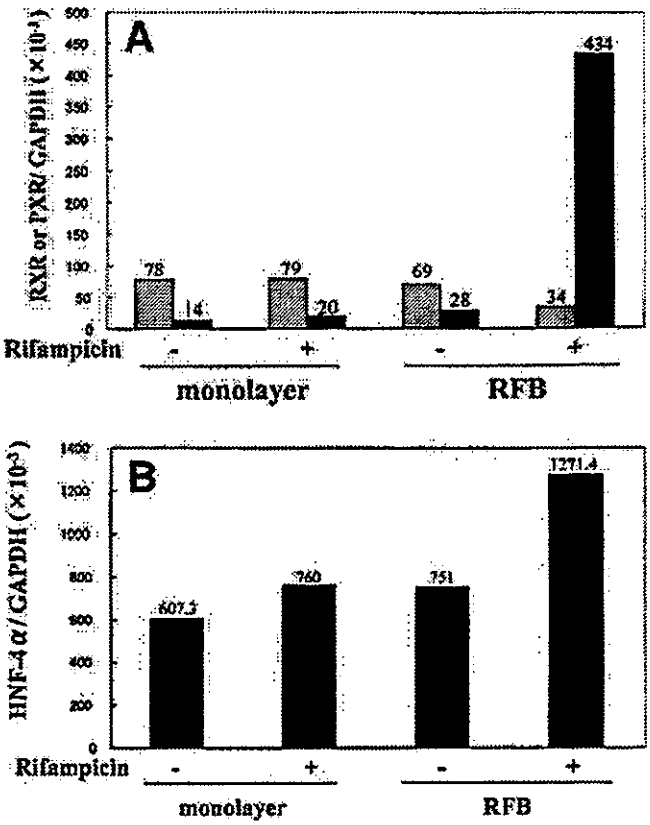

Fig. 6. Comparison of the expression of RXRα, PXR, and HNF-4α mRNA in FLC-5 incubated under different conditions, as assessed by TaqMan 1-step RT-PCR. Each RNA concentration was calculated from the average of triplicate measurements and divided by the average of the corresponding GAPDH values obtained in the same way to yield a corrected RNA concentration. (A) Data represent mean PXR (**solid bars**), and RXRα (**grey bars**). (B) Data represent mean HNF-4α.

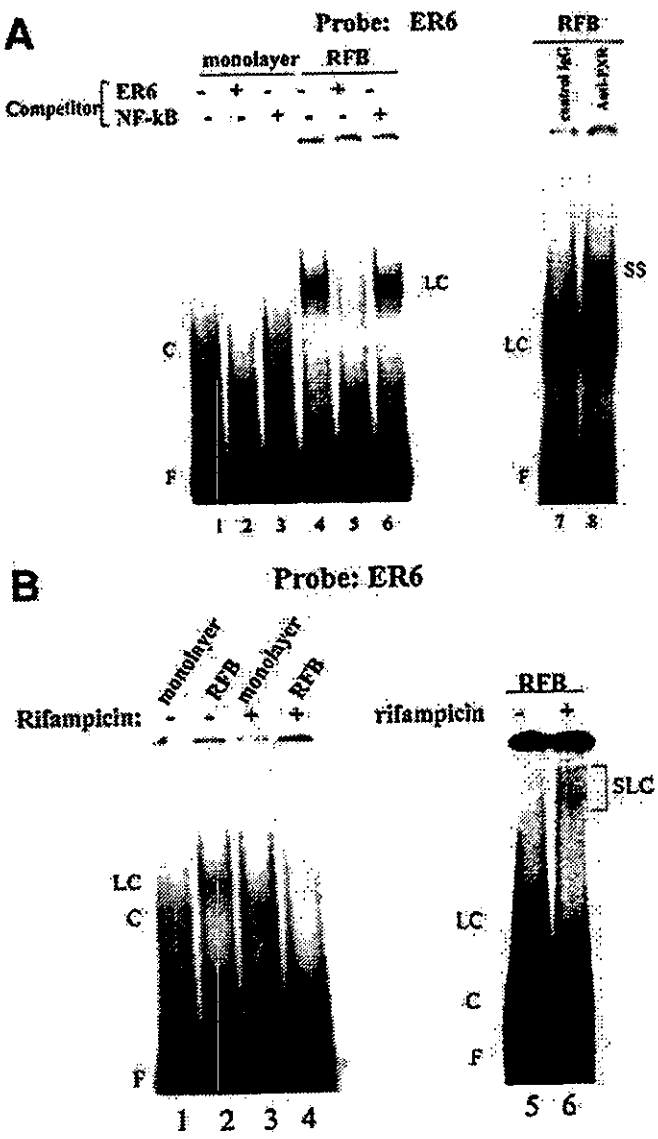

Fig. 7. Three-dimensional culture forms the large DNA nuclear factor complex, including RXRα/PXR heterodimer, their binding site, and the other factors. Ten micrograms of nuclear extracts from FLC-5 cultured by monolayer or RFB were used for EMSA with a ^{32}P-labeled CYP3A4 ER6 oligonucleotide in the presence of either a 50-fold excess of unlabeled oligonucleotide containing the CYP3A4 ER6 or an NF-κB-binding site as indicated. (A) **Lanes 1-8**, ER6 PXRE probe with FLC-5 nuclear extracts. **Lanes 1-3**, monolayer culture. **Lanes 4-8**, RFB culture. **Lanes 1 and 4**, no unlabeled oligonucleotide. **Lanes 2 and 5**, 50-fold excess of an unlabeled ER6 PXRE oligonucleotide. **Lanes 3 and 6**, 50-fold excess of an unlabeled NF-κB-binding site oligonucleotide. **Lane 7**, with the addition of a control goat IgG. **Lane 8**, with the addition of an antibody against PXR. (B) **Lanes 1-6**, ER6 PXRE probe with FLC-5 nuclear extracts. **Lane 1**, monolayer culture without rifampicin. **Lane 2**, RFB culture without rifampicin. **Lane 3**, monolayer culture with rifampicin. **Lane 4**, RFB culture with rifampicin. **Lanes 5 and 6**, long-time EMSA assay. **Lane 5**, no rifampicin. **Lane 6**, containing of rifampicin. c, lc, slc, and ss indicate specific, large, super-large protein-DNA complexes, and antibody supershift, respectively, and f represents unbound probe.

8A, specific protein-DNA complexes were observed both in nuclear extracts from monolayer- and RFB-cultured FLC-5. However, the complex in the RFB culture (shown as LC) exhibited the retarded mobility compared with that in monolayer culture (shown as C). This result suggests that nuclear factor(s) was interacted with either HNF-4α or HNF-4α-binding motif in the RFB-cultured cells. Competition experiments with unlabeled oligonu-

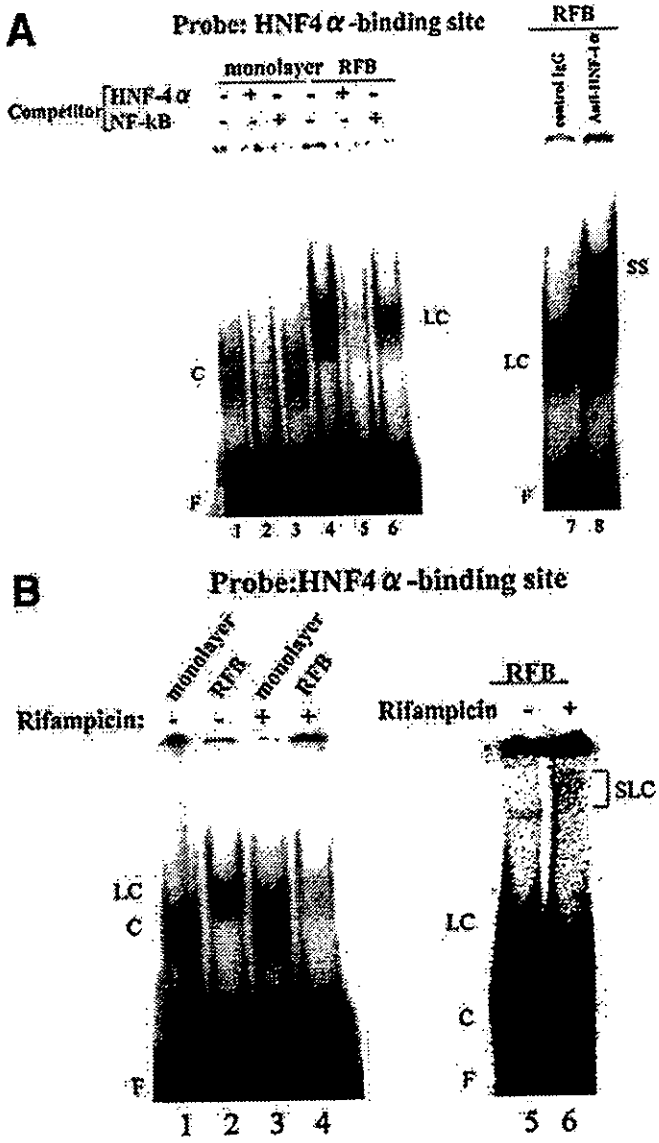

Fig. 8. Three-dimensional culture forms the large DNA-nuclear factor complex including HNF-4α, their binding site, and the other factors. Ten micrograms of nuclear extracts from FLC-5 cultured by monolayer or RFB were used for EMSA with a ^{32}P-labeled HNF-4α oligonucleotide in the presence of either a 50-fold excess of unlabeled oligonucleotide containing the HNF-4α or an NF-κB-binding site as indicated. (A) **Lanes 1-8**, HNF-4α probe with FLC-5 nuclear extracts. **Lanes 1-3**, monolayer culture. **Lanes 4-8**, RFB culture. **Lanes 1 and 4**, no unlabeled oligonucleotide. **Lanes 2 and 5**, 50-fold excess of an unlabeled HNF-4α-binding oligonucleotide. **Lanes 3 and 6**, 50-fold excess of an unlabeled NF-κB-binding site oligonucleotide. **Lane 7**, with the addition of a control goat IgG. **Lane 8**, with the addition of an antibody against HNF-4α. (B) **Lanes 1-6**, HNF-4α probe with FLC-5 nuclear extracts. **Lane 1**, monolayer culture without rifampicin. **Lane 2**, RFB culture without rifampicin. **Lane 3**, monolayer culture with rifampicin. **Lane 4**, RFB culture with rifampicin. **Lanes 5 and 6**, long-time EMSA assay. **Lane 5**, no rifampicin. **Lane 6**, containing of rifampicin. c, lc, slc, and ss indicate specific, large, super-large protein-DNA complexes, and antibody supershift, respectively, and f represents unbound probe.