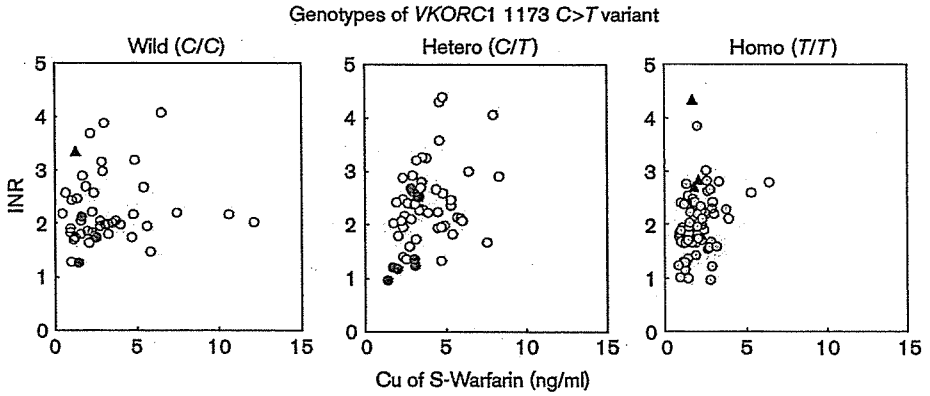
Table 2 Allelic frequencies of *VKORC1* and *CYP2C9* variants

	African-American (n=64)	Caucasian (n=115)	Japanese (n=64)
VKORC1 129 C>T (Cys43Cys, exon 1)	0	0.009	0
VKORC 497T>G (intron 1)	0.039§	0.288	0†
VKORC1 1173C>T (intron 1)	0.086§	0.422	0.891†‡
VKORC1 1196G>A* (intron 1)	0	0.017	0
VKORC1 1331G>A (Val66Met, exon 2)	0.016	0	0
VKORC1 3462C>T (Leu120Leu, exon 3)	0.227§	0.004	0‡
VKORC1 3730G>A 3′-downstream)	0.523§	0.374	0.167†‡
*CYP2C9*1 (wild-type) (Arg_{144}/ Arg_{335}/Ile_{359})	0.953§	0.743	0.984†
*CYP2C9*2 (exon 3) (Arg/Cys_{144})	0§	0.143	0†
*CYP2C9*3 (exon 7) (Ile/Leu_{359})	0.008§	0.109	0.016†
*CYP2C9*4 (exon 7) (Ile/Thr_{359})	0	0	0
*CYP2C9*5 (exon 7) (Asp/Glu_{360})	0.008	0	0
*CYP2C9*6 (exon 5) (818delA)	0.008	0	0
*CYP2C9*11 (exon 7) (Arg/Trp_{335})	0.023	0.004	0

African-American DNA samples were obtained from healthy subjects.
*a novel polymorphism.
†$P<0.01$ between the Caucasian and Japanese groups.
‡$P<0.01$ between the Japanese and African-American groups.
§$P<0.05$ between the Caucasian and African-American groups.

Fig. 2

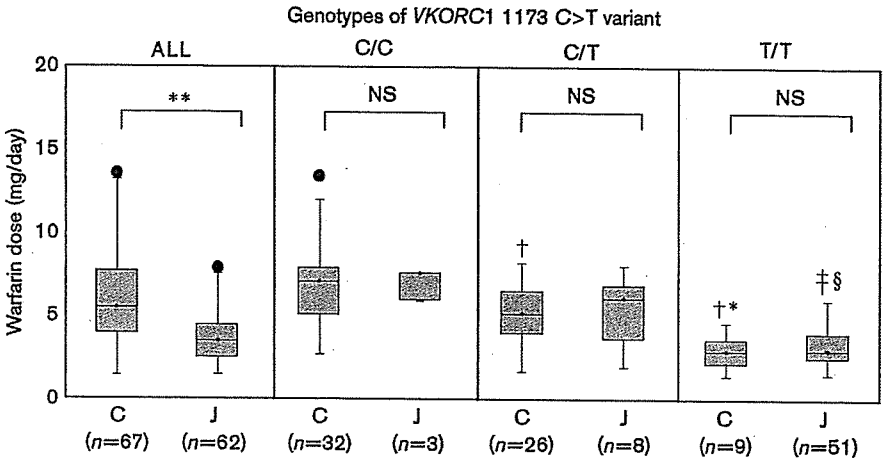


Relationships between plasma unbound concentrations (Cu) of S-warfarin and INR in Caucasian (open circles) and Japanese (grey or halftone circles) patients with three different genotypes of *VKORC1* 1173C>T: those with the wild-type (C/C), heterozygote (C/T) and homozygote (T/T) are shown separately. Four Caucasian patients carrying *VKORC1* 1196G>A are presented by black triangles. Significant ($P<0.05$) and apparently steeper correlations between the two parameters were observed in the C/T ($r=0.35$) and T/T genotypes ($r=0.36$), respectively.

the *VKORC1* 1173 homozygous mutant allele (T/T), but one had the 1173 wild-type genotype. No differences in metabolizing ability, as measured by the oral clearance of unbound S-warfarin, were observed between the three *VKORC1* 1173 C > T genotype groups in Caucasians and Japanese. However, reduced maintenance doses of warfarin in patients carrying *CYP2C9*2* and/or *CYP2C9*3* were observed in the Caucasians and Japanese patients (5.5 ± 2.6, 4.0 ± 1.8, 3.2 ± 1.5, 2.0 ± 1.3 mg/day in Caucasians with *CYP2C9*1/*1*, **1/*2*, **1/*3* versus **2/*3* or *versus *2/*2* or versus **3/*3*, respectively, and 3.6 ± 1.7 and 1.8 ± 0.5 mg/day in Japanese with *CYP2C9*1/*1* and **1/*3* genotypes, respectively). In order to perform further genotype: phenotype analysis (Fig. 3), patients homozygous for the wild-type *CYP2C9* gene (67 Caucasian and 62 Japanese patients) were selected to exclude the influence of population differences in the frequencies of defective *CYP2C9*2* and *CYP2C9*3* alleles on the maintenance doses.

The median daily warfarin dose in Caucasians was significantly greater ($P < 0.01$) than that in Japanese (5.5 versus 3.5 mg/day, respectively), when the two such populations were compared irrespective of *VKORC1* genotype (ALL in Fig. 3). There was a significant ($P < 0.05$) *VKORC1* 1173C > T gene–dose effect present in each population, e.g., a lower dose was observed in patients carrying homozygous mutations (T/T) compared with those with wild-type (C/C) and heterozygous mutations (C/T) except for Japanese patients with C/C

Fig. 3



Comparisons of the median maintenance doses of warfarin between Caucasian (C) and Japanese (J) patients carrying the wild-type *CYP2C9* genotype. Comparisons were made irrespective of *VKORC1* 1173C>T genotypes (ALL) and with regard to the *VKORC1* 1173C>T genotype (C/C, C/T and T/T, respectively) between Caucasian and Japanese patients. Data are shown by box-and-whisker plots. Subdivisions of the boxes and the top and bottom lines on the boxes represent median values and the upper and lower quartiles, respectively. The closed circles (●) are outlying values beyond the maximum length in terms of the interquartile range. Numbers of patients in each group are shown in the parentheses. There was a significant difference in warfarin doses between Caucasian and Japanese patients when compared irrespective of *VKORC1* genotype (ALL). There were also significant differences in warfarin doses between Caucasian patients having different *VKORC1* genotypes and between Japanese patients having 1173 C/C and T/T genotypes and between patients with 1173 C/T and T/T genotypes. $^{**}P<0.01$ between the Caucasian and Japanese groups; $^{\dagger}P<0.01$ between Caucasian patients with 1173 C/C and those with C/T or T/T genotypes; $^{*}P<0.05$ between Caucasian patients with 1173 C/T and those with T/T genotypes; $^{\ddagger}P<0.01$ between Japanese patients with 1173 C/C and those with T/T genotypes; $^{\S}P<0.01$ between Japanese patients with 1173 C/T and those with T/T genotypes.

and C/T genotypes: the mean maintenance doses obtained from Caucasian patients carrying C/C, C/T and T/T genotypes were 6.9 versus 5.2 versus 3.0 mg/day, respectively, and the corresponding values obtained from Japanese patients were 7.0 versus 5.4 versus 3.3 mg/day. In contrast, no significant differences were observed between these two populations in the daily dose within each 1173C > T genotype (Fig. 3).

Haplotype frequencies were 0.156 and 0.847 for H1, 0.256 and 0 for H2, 0.363 and 0.109 for H7/H8 and 0.200 and 0 for H9 in Caucasian and Japanese patients, respectively. Haplotype analysis revealed no significant differences in warfarin doses adjusted for age, sex, body weight and *CYP2C9* genotype and 'warfarin sensitivity index' for S-warfarin between patients in Group A, i.e., with the H1 versus H2 haplotype (3.4 versus 3.5 mg/day, and 1.0 versus 1.0 ml/ng, respectively). No significant differences were observed in the corresponding values in Group B patients with the H7/H8 haplotype and those with the H9 haplotype (5.8 versus 5.2 mg/day, and 0.66 versus 0.58 ml/ng). Haplotype groups of A/A, A/B and B/B completely corresponded to the genotype groups of *VKORC1* 1173 T/T, T /C and C/C.

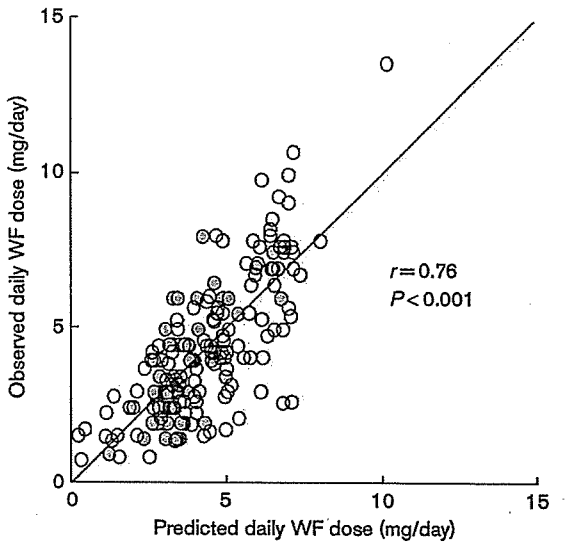
Univariate analysis to identify patient covariates associated with the interindividual variability in daily warfarin dose showed that age ($r = -0.22$), body weight ($r = 0.29$), *CYP2C9* variant ($r = -0.32$), *VKORC1* 1173C > T ($r = -0.58$) and Japanese ancestry ($r = -0.20$) were all significantly ($r < 0.05$) correlated. Further multivariate analysis with these covariates in 115 Caucasian and 64 Japanese patients revealed that *CYP2C9* and *VKORC1* genotypes, age and body weight had independent and statistically significant contributions to the overall variability in warfarin dose (Table 3). The final regression equation for estimating maintenance doses (MD) of warfarin was as follows: for patients with homozygous wild-type genotype for both *CYP2C9* and *VKORC1*: MD (mg) $= 6.6 - 0.035 \times$ (age, years) $+ 0.031 \times$ (body weight, kg); for those with either heterozygous or homozygous variant of *CYP2C9*, the MD was reduced by 1.7 and 2.8 mg, respectively, and for those with either heterozygous or homozygous variant of *VKORC1* 1173C > T, the MD was further reduced by 1.3 and 2.9 mg, respectively, from those predicted by the respective equations. Based on the standardized partial regression coefficients, genotypes of *CYP2C9* and *VKORC1* were the principal covariates contributing equally to interpatient variability in warfarin requirements. Collectively, the identified covariates accounted for 57% of the overall variability in the daily dose of warfarin. Also, a significant correlation ($r = 0.76$, $P < 0.001$) without systematic bias was observed between the actual maintenance doses taken by the Caucasian and Japanese patients and those predicted from the multiple regression model (Fig. 4).

Table 3 Multivariate analysis for patients' covariates that are associated with interindividual variability of warfarin doses

Covariates	Partial regression coefficient ± SE	Standardized partial regression coefficient	*P*-value
Constant	6.656 ± 0.973		
Age (years)	−0.035 ± 0.010	−0.252	0.000808
Body weight (kg)	0.031 ± 0.007	0.298	0.000059
*CYP2C9*2/*3/*11* (Heterozygous)	−1.706 ± 0.290	−0.408	<0.0000005
(Homozygous variant)	−2.815 ± 0.473	−0.413	<0.0000005
VKORC1 1173 *C>T* (Heterozygous)	−1.316 ± 0.309	−0.310	0.000034
(Homozygous variant)	−2.941 ± 0.310	−0.590	<0.0000005

SE, standard error of mean.

Fig. 4



Relationship between maintenance doses of warfarin predicted from the multiple regression model and those actually observed in the 115 Caucasian (○) and 64 Japanese (●) patients. There is a significant correlation between the predicted and observed doses ($y=x+0.0008$, $r=0.76$, $P<0.001$). The solid line represents the line of identity.

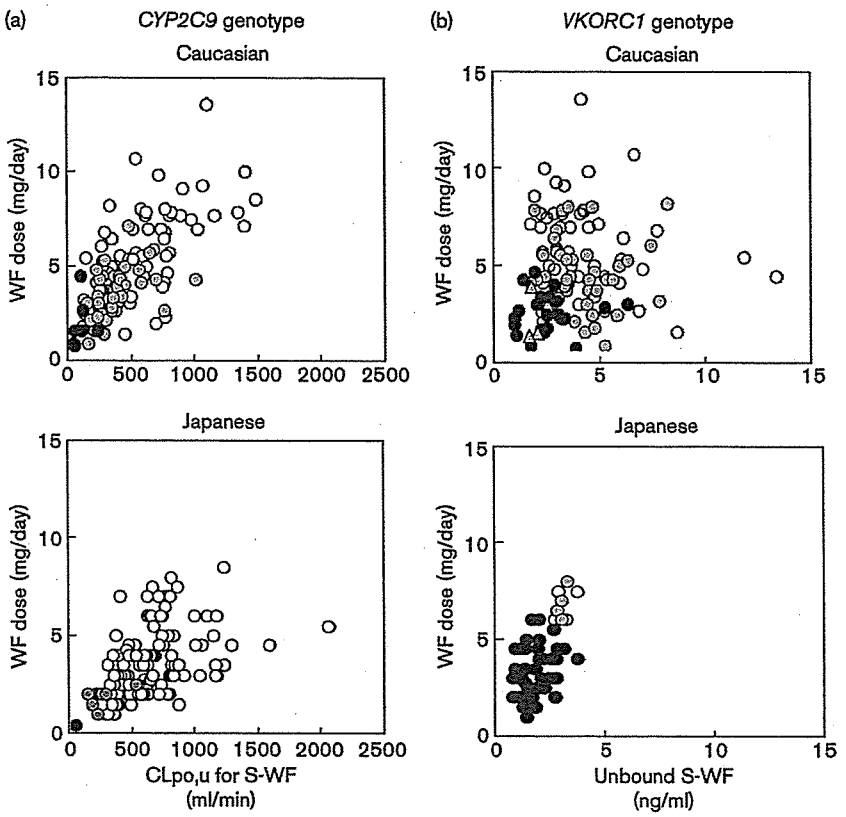
Caucasian and Japanese patients who carried *CYP2C9* variants possessed a lower unbound oral clearance for S-warfarin (decreased metabolic activity), thereby required a smaller daily dose of the drug (Fig. 5a). In addition, those carrying the *VKORC1* 1173C/C wild-type allele needed higher unbound concentrations of S-warfarin to achieve a therapeutic anticoagulation response (reduced sensitivity), and a greater daily dose was required regardless of race (Fig. 5b). Forty-seven percent of Caucasian patients possessed one of the *CYP2C9* variant alleles (*CYP2C9*2*, *CYP2C9*3* or *CYP2C9*11*) and 48% the *VKORC1* 1173 C/C wild-type allele, respectively. The corresponding values for African-Americans were 11% and 83%, and those for Japanese were 3% and 17%, respectively. These genetic polymorphisms in *CYP2C9* and *VKORC1* were independent to each other and allelic frequencies of these genetic variants differed among the three populations (Table 2). As a result, 70% of Caucasian, 83% of African-American and 20% of Japanese patients were found to carry pharmacokinetic (*CYP2C9*) and pharmacodynamic (*VKORC1*) genetic factors which are associated with a lower and a higher requirement, respectively, resulting in the wide interindividual variation in warfarin doses.

Discussion

Warfarin therapy is complicated by large interpatient variability in maintenance dose requirement and the associated risk of under- and over-anticoagulation. This is the first study demonstrating that there are population differences not only in pharmacokinetics but also in pharmacodynamics of warfarin based upon the dose-plasma concentration and plasma concentration–INR relationships. The pharmacodynamics of S-warfarin evaluated by its 'warfarin sensitivity index' showed significant differences between African-Americans, Caucasians and Japanese patients, although the number of African-American patients ($n=36$) participating in the study was smaller than the Caucasians and Japanese groups (Table 1). In addition, the sensitivity of S-warfarin to inhibit normal or fully carboxylated prothrombin (NPT) production was found to differ between populations and this may play a pivotal role in the population differences of warfarin dose requirement.

Readily determinable demographic factors such as age and body weight have been considered as contributing covariates [1–3], and this is confirmed in the present study. The age factor may be related to a reduced ability to metabolize warfarin with aging [1]. A similar mechanistic explanation may also account for the body weight covariate although a pharmacodynamic factor may also be involved, since obese subjects have been found to have elevated plasma levels of fibrinogen and factor VII compared to lean individuals [28]. Nonetheless, such demographic factors only have limited utility for optimizing the warfarin maintenance dose and it has become increasingly appreciated that genetic factors may have an important role. Recent focus has been upon drug metabolizing enzymes involved in warfarin's metabolism that influence its plasma concentration.

Fig. 5



Relationships between unbound oral clearance (CLpo,u) for S-warfarin and daily doses of warfarin (left column, a) and those between plasma unbound concentration (Cu) for S-warfarin and daily doses of warfarin (right column, b) in Caucasian and Japanese patients with different genotypes of *CYP2C9* (a) and *VKORC1* (b). Symbols (a): *CYP2C9*1/*1* (open circles), *CYP2C9*1/*2* or **1/*3* or **1/*11* (grey circles) and *CYP2C9*2/*2*, or **3/*3* or **2/*3* (black circles); symbols in (b): *VKORC1* 1173 C/C and 1196 G/G (open circles), *VKORC1* 1173 C/C and 1196 G/A (open triangle), *VKORC1* 1173 C/T and 1196 G/G (grey circles), *VKORC1* 1173 T/T and 1196 G/G (black circles) and *VKORC1* 1173T/T and 1196 G/A (grey triangles).

Clinically available warfarin is a racemic mixture of R- and S-enantiomers. However, S-warfarin has been shown to be three to five times more potent than R-warfarin based upon the anticoagulation responses elicited after the administration of the respective enantiomers separately in healthy subjects [11]. While plasma concentrations of R-warfarin are, on average, approximately twice those of S-warfarin following oral administration of the racemate, pharmacokinetic-pharmacodynamic analysis concluded that the anticoagulant effect is attributable almost entirely to S-warfarin concentrations [29]. Moreover, as noted in the present study, there was a significant correlation between the oral clearance of unbound S-warfarin and that for R-warfarin ($P < 0.0001$), indicating that demographic factors (e.g., body weight and age), nutritional and certain environmental factors linked with variability in both of these parameters may also be associated. Accordingly, it is likely that interindividual variability in the plasma concentration of S-warfarin is more important than that of R-warfarin when considering the variability of anticoagulant activity following the administration of racemic warfarin.

CYP2C9 and its allelic variants have been investigated since the encoded enzyme is largely responsible for the metabolism of S-warfarin. Several relatively large retrospective clinical studies in several different populations have now demonstrated associations between warfarin's maintenance dose and adverse events, i.e., increased bleeding complications, and the presence of *CYP2C9* variants leading to markedly reduced catalytic activity of the resulting enzyme such as CYP2C9.2 and CYP2C9.3 [1–3,9,12–14]. Collectively, the present data confirm these previous observations that lower doses are required in patients carrying these variant alleles especially *CYP2C9*3*. Despite such associations, however, the contribution of such genetic variability to the overall variability in warfarin's maintenance dose is relatively low – less than 20% of the variance [1–3]. The present findings based on the presence of *CYP2C9*2*, *CYP2C9*3*

and *CYP2C9*11* variants, all of which are associated with reduced enzyme activity, also confirm this small contribution even when variant homozygosity is present. Moreover, the difference in warfarin dosage requirement between Japanese and Caucasians cannot be explained by a greater frequency of *CYP2C9* variants with reduced catalytic activity in Caucasians (Table 2), and the former population have higher unbound oral clearances of S-warfarin than the latter when matched for the wild-type genotype in the 5′-flanking (up to −2 kb) and coding regions of *CYP2C9* [9,27]. Therefore, the present results strongly suggest the involvement of other factors.

The molecular target of warfarin is vitamin K epoxide reductase, which is critically involved in the production of functionally active vitamin K-dependent coagulation factors [e.g., factors II (prothrombin), VII, IX and X)] through γ-glutamyl carboxylation [30]. Subunit 1 of this lipoprotein complex has recently been shown to exhibit genetic polymorphisms, and several such allelic variants have been shown to have reduced catalytic activity that is associated with 'warfarin-resistance', i.e., require substantially higher doses to achieve satisfactory anticoagulation [15,17]. However, only two such heterozygous *VKORC1* 1331G > A, Val66Met, African-American individuals were found in the present study. Other variants reported to be associated with 'warfarin-resistance' [15] were not detected. A number of other nucleotide transitions including a novel *VKORC1* 1196G > A were, however, identified and appeared to have selective distribution according to racial ancestry, but their rarity made it impossible to assess whether they have functional consequences. On the other hand, a haplotype combination including a *VKORC1* 1173C > T transition, previously reported to be present in 40% of European-Caucasians, was found to be common with higher and lower frequencies in Japanese and African-Americans, respectively [16–21]. This variant was also found to be associated with a gene–dose effect and a lower warfarin maintenance dose [16–21]. The present findings confirm this observation in Caucasians and extend the relationship to Japanese. Interestingly, this *VKORC1* variant appeared to affect the relationship between the unbound concentrations of S-warfarin and the resulting INR value – the slopes of the regression curves of the relationship being steeper in heterozygous and homozygous variant patients than in those homozygous for the wild-type allele. Importantly, the different population frequency of the *VKORC1* 1173T variant allele in Japanese compared to Caucasians, appeared to account for the increased 'warfarin sensitivity' of the former group of patients, matched according to *CYP2C9* genotype, i.e., *CYP2C9*1* homozygous, since no differences in dosage requirement was observed between the populations when stratified according to *VKORC1* genotype. Furthermore, multiple regression analysis showed that the *VKORC1* 1173C > T variant was an important covariate with respect to the interindividual variability in warfarin dosage. Patients carrying the T allele at the position of 1173 of *VKORC1* gene are classified into the Group A haplotype associated with a lower dose requirement [21]. However, this haplotype system is no more informative than a single segregating SNPs among those at positions 381, 3673, 6484, 6853 and 7566 of the reference sequence (GenBank accession number AY587020) as shown previously by others [16], when the influence of *VKORC1* genotype on the interindividual variability in warfarin doses is considered. Overall, these results also suggest that the higher dose requirements in African-Americans [6,7] may possibly reflect the higher frequency of the *VKORC1* 1173C allele (91%) compared to Japanese (11%) and Caucasians (58%) (Table 2).

The 1173C > T transition in intron 1 of *VKORC1* was recently reported to be in complete disequilibrium with −1639G > A at a putative NF1 binding site [18], −4931T > C, 1542G > C and 2255C > T [21]. While there is a controversy regarding the influence of this *VKORC1* haplotype on the transcriptional activity of this gene [16,18,19], a recent report indicates that this haplotype was associated with lower mRNA levels in human liver [21]. This finding suggests that the 1173C > T variant may be associated with the lower levels of reduced form of vitamin K, thereby making patients with this variant more susceptible to the anticoagulation effect of warfarin. In addition to the conventional measure of anticoagulation, namely, the INR value, the concentration of NPT was also determined in the patients. No population differences could be discerned in the relationship between these two biomarkers, indicating comparable functionality of the involved fully carboxylated vitamin K-dependent factors and fibrinogen. However, Japanese patients appeared to be more sensitive to γ-carboxylation of prothrombin in that a comparable NPT response was achievable at lower plasma concentrations of unbound S-warfarin compared to Caucasians and African-Americans. The reason for this difference is unknown but may involve population differences in NPT's baseline level (preliminary unreported data), and further studies are required to explore this possibility. In addition, the question of whether the *VKORC1* haplotypes may influence the baseline levels of VKOR and NPT remains to be clarified. Regarding functionally related genes, multiple variants in several vitamin K-dependent proteins have been identified including factor II, factor VII and γ-glutamyl carboxylase [20,31]. Moreover, some of these are associated with altered 'warfarin sensitivity' [20,31] and preliminary data (not shown) indicates that their allelic frequencies differ between Caucasian and Japanese populations. Therefore, influences of these polymorphisms on the overall variability in warfarin responses are also to be clarified.

In summary, the present study shows that interindividual variability and population differences in the maintenance dose of warfarin required to achieve anticoagulation involves demographic, pharmacokinetic, and pharmacodynamic factors. Furthermore, genetic variability in CYP2C9-mediated metabolism of S-warfarin and the drug's molecular target, VKOR, are specific determinants. The present study shows that 70% Caucasian and 83% African-American patients carried either *CYP2C9* or/and *VKORC1* genotype(s) which leads to either reduced metabolic activity or attenuated sensitivity of warfarin. In contrast, only 20% of Japanese population possesses these genotypes. Thus, the relative contribution of the *VKORC1* and *CYP2C9* genotypes to the overall interpatient variability in warfarin doses differs between the three populations according to racial ancestry. Moreover, it should be of note that the identified demographic and genetic covariates of warfarin doses only account for 57% of interindividual variability. Accordingly, other currently unknown determinants remain to be identified, and populations other than those currently studied need to be investigated.

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◆特集：血栓症治療の最前線◆

個別化されたワルファリン療法確立への道

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A quest for individualized warfarin therapy

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Key words: warfarin, cytochrome P450 (CYP), *CYP2C9*, *VKORC1*, genetic polymorphism

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1978年北海道大学医学部卒業，医学博士（東京大学，1986年）．国立病院医療センター内科，コロラド大学臨床薬理，ドイツ共和国ボン大学内科，国立健康栄養研究所成人栄養部，北里大学医学部薬理学を経て1995年から現職．

1. はじめに

ワルファリンカリウム（ワーファリン®，以下ワルファリンと略）は国際的に最も広く使用されている経口抗凝固薬であるが[1]，比較的狭い治療域INRを得るために必要とされるワルファリン投与量は0.5～7.0mg/日と個人の間で10倍以上の個人差がある．そこで，ワルファリン療法の個別化を目指す研究が精力的に行われ，ワルファリン投与量の個人差を説明するワルファリンの薬物動態（pharmacokinetics: PK）とワルファリンの標的分子であるビタミンKエポキシド還元酵素複合体（VKORC）および関連分子における薬力学（pharmacodynamics: PD）の個人間変動要因の検討がなされている．

2. ワルファリンPKの個人間変動要因

市販のワルファリン製剤は1対の（S体とR体）光学異性体からなるラセミ体である．S体-ワルファリンの抗凝固活性はR-体よりも3～5倍高いため，PK上の個人差要因としてはS体ワルファリンの体内動態の個人差が重要である．S体ワルファリンの不活化には肝薬物代謝酵素チトクロームP450（*CYP*）*2C9*分子種がほぼ選択的に関与するため，このCYP分子種の活性を支配する要因が検討された[2]．歴史的には，まずCYP2C9活性の個人差解明に向けて*CYP2C9*遺伝子の発現領域の変異探索が行われ，その結果はデータベースにまとめられている[3]．現時点でアレル頻度の比較的高いCYP2C9*2およびCYP2C9*3の1塩基置換（SNP）変異以下*CYP2C9*24*まで変異型アレルと野生型*CYP2C9*1*の亜系（*CYP2C9*1A, B, C, D*）が登録されている．*CYP2C9*6*（フレームシフト変異）を除いては全て1塩基置換（SNP）である．*CYP2C9*2*，*CYP2C9*3*，*CYP2C9*6*，*CYP2C9*11*変異は，*in vitro*で発現された酵素蛋白の活性低下のみならず，*in vivo*でのS体ワルファリン代謝活性の低下と投与量の低下にも反映される変異であるが，他の変異アレルについては*in vivo*ワルファリン代謝活性との関連は不明確なものが多い[4]．更に，これらの変異アレル保有者を除外し，現時点で野生型*CYP2C9*を有すると推定される患者間においても極めて大きなワルファリンPKの個人差が存在するため，ワルファリン投与量の個人差に関わる遺伝要因の探求は*CYP2C9*近傍の非翻訳

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領域のゲノム情報を考慮したより広範なゲノム探索へと向かった．特に*CYP2C9*の5'上流領域には，CARやPXRなどの発現調節因子の結合サイトが複数存在するため[4]，この領域のユニークな変異がワルファリンPKの個人差を説明する期待がもたれた．

最も詳細なCYP2C9ゲノム近傍の解析はVeenstraら[6]による192名の欧州起源の白人を対象とした研究で，彼らは*CYP2C9*ゲノムおよびその近傍の上・下流およびイントロン領域を総計60kbに渡り詳細に解析し，121個の非翻訳領域変異と従来の*exon*領域変異を併合解析することで23種のハプロタイプを見いだした．更に，これらのハプロタイプはクラスター解析により6種のグループ（1A, 1B, 1C, 1D, 2, 3）に分類された．しかし，これほど詳細な*CYP2C9*ゲノム解析によっても非翻訳領域には*CYP2C9*の発現量の調節を介してワルファリンPKの個人差を説明できるような変異は発見されなかった．ワルファリン投与量の低下と関係する*CYP2C9*ハプロタイプはグループ2とグループ3であるが，こられのグループはいずれもexon領域に従来から知られた構造領域のloss of function（LOF）変異である*CYP2C9*2*と*CYP2C9*3*と強い連鎖不平衡を持っていたのであった．

臨床的観点からは，これらの*CYP2C9*変異アレルの検出頻度が重要である．*CYP2C9*変異アレルの出現頻度には人種差が認められ，白人はCYP2C9*2（13％），*3（7％），*11（1.3％）と比較的変異アレルの頻度が高いので遺伝子診断の有用性はあるが，黒人では変異アレルの頻度が*CYP2C9*2*（3.4％），**3*（1.5％），**5*（1.8％），**6*（<0.5％），**11*（2.3％）と白人より低く，アジア人（日本人を含む）では*CYP2C9*3*（1.8％），とさらに低い[2)4)]．従って，アジア人においてはワルファリン療法の個別化において*CYP2C9*の遺伝子診断をする意義は白人よりも少ないと考えられる．*CYP2C9*のLOF変異アレルを有する患者を1人発見するために遺伝子スクリーニングを受ける患者数は，白人，黒人，アジア人の順に，それぞれ5人，11人，56人である．従って，現時点での対費用効果関係では白人以外に日常的な臨床検査としては医療経済的に実施困難と思われる．白人患者における*CYP2C9*のジェノタイピングの臨床的有用性を検討した約50名の小規模な臨床試験[7]によれば，整形外科手術に先立って*CYP2C9*遺伝子診断を行いワルファリン投与量の個別化を行うと，*CYP2C9*の変異アレルを保有する患者でも治療域のINRを得るまでの時間は野生型アレル保有患者と同等とすることができた．しかし，出血副作用の発現は依然として*CYP2C9*変異アレル保有者に多く遺伝子診断の意義はより大規模な臨床試験における検討が必要とされている．

3. ワルファリンPDの個人間変動因子の探求

2004年にRostら[8]は永年に渡って難攻不落であったワルファリンの標的分子であるビタミンKエポキシド還元酵素複合体（VKORC）遺伝子の一部と想定されるVKORCサブユニット1（*VKORC1*）を同定した．彼らは同時に遺伝的な血液凝固異常症家系（combined deficiency of vitamin K-dependent clotting factors）から6種類のVKORC1 SNPを発見した．但し，これらの変異は健常人対照群では発見されないほど頻度は低かった．しかし，*VKORC1*遺伝子が同定されると，ワルファリン応答性の高い患者集団のゲノム探索から，ついにD'Andrea Gら[9]が*VKORC1*のイントロン1部位のSNP（1173C>T）がワルファリンに対する応答性増大に関係することを明らかにした．その後堰を切ったように多くの報告がなされ，*VKORC1*には連鎖不平衡の関係にある多くの変異が存在し，なかでも上記の変異（1173C>T）を含むハプロタイプは*VKORC1*のmRNA発現量の低下と関係し，ワルファリン応答性の増加（即ち投与量の低下）と関連することが判明した[10]．*VKORC1*の

1173C>T変異には，人種差があり，日本人を含むアジア人では1173 Cアレル頻度がは0.9前後と高い（ワルファリン高応答性）が，白人では0.4前後，アフリカ系アメリカ人では0.1前後と大きな差異があった[11]．また，*VKORC1* 1173C>T変異のワルファリン応答性への影響は人種を越えて保存されており[11]，従来から経験的に知られていたアジア人の平均的ワルファリン投与量が白人やアフリカ系アメリカ人よりも低いことのよい説明となっていた．

肝細胞小胞体（ER）のビタミンK redoxサイクルに関係するVKORCは複数の機能サブユニット複合体から構成される膜蛋白である．VKORCのサブユニットモデルとしては，グルタチオンS-トランスフェラーゼ（GST），ミクロゾームエポキシドヒドロラーゼ（mEH）が想定されている[12]．但し，mEHについてはmEH-nullマウスにビタミンK欠乏を示唆する表現系上の所見がないため[13]，VKORCのサブユニット構成員ではないとする説もある．GSTには多数の分子種が存在するが，VKORCに関係するGSTの分子種は精製されたVKOR活性酵素のGST配列のホモロジー検索からその配列が*GSTA1*分子種のαクラスのサブユニットに類似しているため*GSTA*分子種と想定されている[12]．*GSTA*と*mEH*にはそれぞれ複数の機能変化に関係する遺伝多型が報告されており，特に*mEH*については*mEH 612T>C*変異がワルファリン抵抗性（高用量投与）患者に多い可能性が示唆されている[14]．γ-グルタミルカルボキシラーゼ（GGCまたはGGCX）はビタミンK依存性の凝固因子のグルタミン残基のγ-カルボキシル化を担う酵素であるが，還元型ビタミンKを受け取るためにVKORCの近傍に存在すると考えられている．*GGC*の遺伝多型，特にイントロン6のCAA繰り返し配列についてはくり返し数が多いほどワルファリン抵抗性を生じるとの報告がある[15)-17)]が，一致した見解に達していない．更に，ヒトのワルファリン抵抗性の動物モデルとして古くから研究されているビタミンK拮抗作用を持つ殺鼠剤抵抗性ラットの研究から，カルメニン（calumenin；CALU）の過剰発現とワルファリン耐性の関係が注目されている．CALUはERに存在するCa^{2+}結合能を有するシャペロン蛋白で，VKORあるいはGGCに結合し還元型ビタミンKのVKORからGGCへの受け渡しを阻害するとされる．Vecslerら[17]は，*VKORC1*と*CYP2C9*の多型とは独立して*CALU* 11G>A変異がワルファリン抵抗性と関連すると報告している．また，ビタミンK依存性蛋白である第X因子と第VII因子の挿入（I）および欠失（D）がそれぞれワルファリン応答性に関係するとの報告がなされている[18]．

本稿で論じたワルファリンのPKおよびPD上の変動要因の遺伝多型と年齢，喫煙，体重，ビタミンK摂取量などの非遺伝的あるいは環境因子を変動要因としてワルファリン投与量の個人差に対する多変量解析をおこなうと，全ての変動因子の貢献はこれまで60％を超えたことはない．この事実の意味するところは，未知の変動因子の存在を示唆するのか，それともワルファリン応答性機構においては遺伝因子に束縛されない本来ランダムなエピジェネティクな変動因子が存在する事を暗示しているのかの解明は今後の検討に委ねられている．

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INVOLVEMENT OF HEPATOCYTE NUCLEAR FACTOR 4α IN THE DIFFERENT EXPRESSION LEVEL BETWEEN *CYP2C9* AND *CYP2C19* IN THE HUMAN LIVER

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ABSTRACT:

CYP2C9 and CYP2C19 are clinically important drug-metabolizing enzymes. The expression level of CYP2C9 is much higher than that of CYP2C19, although the factor(s) responsible for the difference between the expression levels of these genes is still unclear. It has been reported that hepatocyte nuclear factor 4α (HNF4α) plays an important role in regulation of the expression of liver-enriched genes, including *P450* genes. Thus, we hypothesized that HNF4α contributes to the difference between the expression levels of these genes. Two direct repeat 1 (DR1) elements were located in both the *CYP2C9* and *CYP2C19* promoters. The upstream and downstream elements in these promoters had the same sequences, and HNF4α could bind to both elements in vitro. The transactivation levels of constructs containing two DR1 elements of the *CYP2C9* promoter were increased by HNF4α, whereas those of the *CYP2C19* promoter were not increased. The introduction of mutations into either the upstream or downstream element in the *CYP2C9* gene abolished the responsiveness to HNF4α. We also examined whether HNF4α could bind to the promoter regions of the *CYP2C9* and the *CYP2C19* genes in vivo. The results of chromatin immunoprecipitation assays showed that HNF4α could bind to the promoter region of the *CYP2C9* gene but not to that of the *CYP2C19* promoter in the human liver. Taken together, our results suggest that HNF4α is a factor responsible for the difference between the expression levels of CYP2C9 and CYP2C19 in the human liver.

Cytochromes P450 (P450s) comprise a superfamily of metabolic enzymes that play important roles in the oxidative metabolism of xenobiotics and endogenous substrates (Gonzalez and Gelboin, 1994). The human CYP2C subfamily is composed of four isoforms (CYP2C8, CYP2C9, CYP2C18, and CYP2C19) that account for about 20% of the total human adult liver P450 contents (Shimada et al., 1994). Among the CYP2C subfamily isoforms, CYP2C9 and CYP2C19 play critical roles in the metabolism of clinically used drugs (Goldstein and de Morais, 1994). It has been reported that the expression level of the *CYP2C9* gene in the human liver is about 20 times higher than that of the *CYP2C19* gene (Furuya et al., 1991; Romkes et al., 1991; Inoue et al., 1997), indicating that there are some differences between the regulatory mechanisms of *CYP2C9* and *CYP2C19* gene transcriptions. It has been reported that pregnane X receptor, constitutive androstane receptor, glucocorticoid receptor, and hepatocyte nuclear factor 3γ participate in the basal expression of *CYP2C9* and *CYP2C19* genes (Ferguson et al., 2002; Gerbal-Chaloin et al., 2002; Chen et al., 2003; Bort et al., 2004). However, the factor(s) responsible for the difference between the expression levels of *CYP2C9* and *CYP2C19* genes is still unclear.

Hepatocyte nuclear factor 4α (HNF4α) is a member of the nuclear receptor superfamily (Sladek et al., 1990) and is expressed at high levels in the liver, kidney, pancreas, and small intestine (Sladek et al., 1990; Thomas et al., 2001). HNF4α appears to be an important factor for liver differentiation and function because it is involved in regulation of the expression of numerous liver-enriched genes, such as those related to glucose or lipid metabolism (Watt et al., 2003), those related to synthesis of blood coagulation factors (Sladek and Seidel, 2001), and drug-metabolizing enzymes, including CYP3A4, CYP2A6, CYP2C9, and CYP2D6 (Jover et al., 2001). It is thought that HNF4α binds to a specific DNA sequence called a direct repeat 1 (DR1) element as a homodimer to stimulate transcription of these genes (Cairns et al., 1996; Tirona et al., 2003; Pitarque et al., 2005). However, HNF4α does not always transactivate all the genes that have a DR1 element. For example, it has been reported that a DR1 element exists in the *CYP2C18* promoter but that HNF4α does not bind to the DR1 element of the *CYP2C18* gene and does not transactivate this promoter (Ibeanu and Goldstein, 1995).

It has been reported that there are two DR1 elements in the promoter region of the *CYP2C9* gene, and HNF4α can activate the transcription of this gene via the DR1 element (Ibeanu and Goldstein, 1995; Chen et al., 2005). We also identified two DR1 elements in the *CYP2C19* promoter, but it is not clear whether these elements are functional. Therefore, to clarify the mechanism determining the dif-

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ABBREVIATIONS: P450, cytochrome P450; HNF4α, hepatocyte nuclear factor 4α; DR1, direct repeat 1; kb, kilobase(s); kbp, kilobase pair(s); EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; PCR, polymerase chain reaction; WT, wild-type; MT, mutated.

ference between the expression levels of *CYP2C9* and *CYP2C19* genes, we hypothesized that HNF4α contributes to the difference between the expression levels of CYP2C9 and CYP2C19 in the human liver. The 5′-flanking regions from −2 kilobase pairs (kbp) to the translation start site of these genes were analyzed by electrophoretic mobility shift assays (EMSAs), cotransfection assays, mutagenesis, and chromatin immunoprecipitation (ChIP) assays. Our results suggest that HNF4α participates in the regulation of *CYP2C9* gene transcription but not in that of the *CYP2C19* gene despite the fact that the same DR1 elements exist in both gene promoters.

Materials and Methods

EMSAs and Supershift Assays. EMSAs were performed using double-stranded DNA labeled with [γ-^{32}P]dATP (GE Healthcare Bio-Sciences, Piscataway, NJ) and 10 μg of the nuclear extracts as described previously (Furihata et al., 2004). The following is the sequence of the oligonucleotides used as probes, wild-type, or mutated specific cold competitors: 5′-ACAAGACCAAAGGACATTT-3′ for the DR1-A WT, 5′-ACACCCCCAAAGGACATTT-3′ for the DR1-A MT, 5′-AGTGGGTCAAAGTCCTTTC-3′ for the DR1-B WT, 5′-AGTCCCTCAAAGTCCTTTC-3′ for the DR1-B MT, 5′-TCGAGCGCTGGGCAAAGGTCACCTGC-3′ for the HNF4 WT, and 5′-TCGAGCGCTAGGCACCGGTCACCTGC-3′ for the HNF4 MT. Only the sequences of the sense strands are displayed above, and mutated nucleotides are underlined. Nuclear extracts were prepared from HepG2 cells by using a CelLytic Nuclear Extraction Kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocol. After extracting nuclear contents, the protein concentration was determined by using a Bio-Rad *Dc* Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). The nuclear extracts were stored at −80°C until used.

For competition experiments, unlabeled competitive double-stranded DNA was added to the binding reaction mixture at a 50-fold excess of the probe amount before addition of the probe. For supershift assays, either 2 μg of IgG against HNF4α (2ZK9218H; Perseus Proteomics, Tokyo, Japan) or control mouse IgG (sc-2025; Santa Cruz Biotech, Santa Cruz, CA) was added to the binding reaction mixture at room temperature for 30 min before addition of the probe.

Plasmids. The 5′-flanking regions of the *CYP2C9* and *CYP2C19* genes were isolated by polymerase chain reaction (PCR) with the common sense primer 5′-ACCTCTAGATTGCTTTTCTTTGCCCTGTAT-3′ (for *CYP2C9* and *CYP2C19*) and the antisense primer 5′-GAGGACCTGAAGCCTTCTCT-TCTTGTTA-3′ (for *CYP2C9*) or 5′-GGGGACCTGAAGCCTTCTCCTCTT-GTTA-3′ (for *CYP2C19*) using human genomic DNA as a template. The amplicons were subcloned into a pGEM-T-easy vector (Promega, Madison, WI). After XbaI and BamHI digestion, the fragment was ligated into a pGL3-basic vector (Promega). These constructs are hereafter referred to as 2C9 −2k and 2C19 −2k, respectively. The nucleotide sequences were determined using a Dye Terminator Cycle Sequencing-Quick Start Kit (Beckman Coulter, Fullerton, CA) and a CEQ 2000 DNA Analysis System (Beckman Coulter). Fourteen deletion constructs were generated by nested PCR of the primary clone using the following sense primers: 5′-TCTCTAGAGGTTAA-TCTAAATCTAAGAATTCA-3′ (2C9 −380 and 2C19 −380), 5′-ATTTCT-AGAGCATCAGATTATTTACTTCA-3′ (2C9 −340), 5′-ATTACGCGTGC-ATCAGATTGTTTACTTCA-3′ (2C19 −340), 5′-TCTAGAGTGCTCTCAA-TTATGATGGTG-3′ (2C9 −320), 5′-TCTAGACAGTGCTCTCAATTATG-AC-3′ (2C19 −320), 5′-TTTTCTAGAAATACCTAGGCTCCAACCAAG-T-3′ (2C9 −255), 5′-TCTAGAATTACCAATACCTAGGCTTCAA-3′ (2C19 −255), 5′-ATACGCGTAAGGAGAACAAGACCAAAGGAC-3′ (2C9 −195 and 2C19 −195), 5′-TTTCTAGATATCAGTGGGTCAAAGTCCT-3′ (2C9 −160 and 2C19 −160), and 5′-ATCTAGATTTCAGAAGGAGCATATAG-T-3′ (2C9 −140 and 2C19 −140). The antisense primer used was the same as that used in genome cloning. The obtained 5′-deletion fragments except for 2C9 −195, 2C19 −340, and 2C19 −195 were transferred into the pGL3-basic vector as described above. 2C9 −195, 2C19 −340, and 2C19 −195 were inserted into the pGL3-basic vector by MluI and BamHI digestion. All the constructs are named as shown in parentheses.

The cDNA clone of mouse HNF4α2 was isolated from mouse liver cDNA by PCR amplification and was subcloned into pTARGET mammalian expression vector (Promega) by EcoRI digestion, resulting in pHNF4α2 as described elsewhere (Furihata et al., 2006).

Site-Directed Mutagenesis. Site-directed mutagenesis was carried out as described elsewhere (Furihata et al., 2004). To introduce mutations into the reporter plasmids, complementary primers harboring a few mutations were designed for each target site as follows: 5′-GGAGAACAAGACCT__GGA-CATTTTATTTTTATCTGTATCAGTGGG-3′ and 5′-CCCACTGATACAG-ATAAAAATAAAATGTCCA__GGTCTTGTTCTCC-3′ for the *CYP2C9* DR1-Amt; 5′-CTGTATCAGTGGGTCT__GTCCTTTCAGAAGGAGCAT-ATAGTGG-3′ and 5′-CCACTATATGCTCCTTCTGAAAGGACA__GAC-CCACTGATACAG-3′ for the *CYP2C9* DR1-Bmt; 5′-CGAAGGAGAACA-AGACCT__GGACATTTTATTTTTATCTCTATCAGTGG-3′ and 5′-CCA-CTGATAGAGATAAAAATAAAATGTCCA__GGTCTTGTTCTCCTTC-G-3′ for the *CYP2C19* DR1-Amt; 5′-CTCTATCAGTGGGTCT__GTCCTT-TCAGAAGGAGCATATAGTGGG-3′ and 5′-CCCACTATATGCTCCTTC-TGAAAGGACA__GACCCACTGATAGAG-3′ for the *CYP2C19* DR1-Bmt. The mutagenic sites are underlined, and spaces indicate deletions of 2-bp nucleotides.

Cell Culture, Transient Transfection, and Dual Luciferase Assay. FLC7 cells (Kawada et al., 1998), a human hepatocellular carcinoma cell line, were provided by Dr. S. Nagamori (Kyorin University, Tokyo, Japan). FLC7 cells were maintained at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium/F-12 (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum and 50 U/ml penicillin and 50 μg/ml streptomycin.

FLC7 cells were plated at a density of 1.8×10^5 cells/well in 24-well plates 1 day before transfection. The reporter plasmids (200 ng/well) were cotransfected with pHNF4α (100 ng/well) and phRL-TK vector (Promega, 4 ng/well) into FLC7 cells by *Trans*IT-LT1 (Mirus, Madison, WI). Twenty-four hours after transfection, luciferase reporter activities were measured as described previously (Kobayashi et al., 2004). The *Renilla* luciferase activity derived from the control plasmid phRL-TK was used to normalize the results of the firefly luciferase activity of reporter plasmids. Experiments were performed in triplicate, and each value is the mean ± S.D. from three or four separate assays.

ChIP Assays. ChIP assays were performed by using a ChIP-IT kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. Human liver (from a 56-year-old Caucasian male) was supplied by the National Disease Research Interchange (Philadelphia, PA) through HAB Research Organization (Tokyo, Japan), and this study was approved by the Ethics Committee of Chiba University (Chiba, Japan). The human liver tissue (2.4 g) was isolated and chopped on ice and then cross-linked by 1% formaldehyde for 12 min. Cross-linking was stopped by the addition of glycine solution. The chromatin was sheared by using an ultrasonic disruptor UD-201 (TOMY SEIKO, Tokyo, Japan) at 25% power with 14 pulses. Nine micrograms of the sheared chromatin was immunoprecipitated with either control mouse IgG or anti-HNF4α IgG (2ZH1415H). After incubating for 4 h at 4°C with gentle rotation, salmon sperm DNA/protein G agarose was added to the mixture, and it was further incubated for 1.5 h under the same conditions. The DNA fragment was purified and used as a template for PCR. The DNA sequences around DR1 elements of the *CYP2C9* and *CYP2C19* genes were amplified by using the sense primers 5′-CAACCAAGTACAGTGAAACTG-3′ (for *CYP2C9*) and 5′-CAGAATG-TACAGAGTGGGCAC-3′ (for *CYP2C19*) and the antisense primers 5′-TAA-CACTCCATGCTAATTCGG-3′ (for *CYP2C9*) and 5′-AACACTCCAT-GCTAATTAAGT-3′ (for *CYP2C19*). The specificity of the CYP2C9 and CYP2C19 primers was verified by the lack of amplification from sheared genomic DNA than the intended target. PCR conditions were as follows: 94°C for 2 min, followed by 94°C for 30 s, 47°C (for *CYP2C9*) or 50°C (for *CYP2C19*) for 30 s, and 72°C for 30 s, 40 cycles. The amplicons were visualized by ethidium bromide staining, and the sequence of each amplicon was confirmed by direct DNA sequence.

Determination of mRNA Levels. To measure the CYP2C9 and CYP2C19 mRNA levels, cDNA prepared from total RNA of the same human liver used for ChIP assays was subjected to quantitative real-time PCR with an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The mRNA levels of CYP2C9 and CYP2C19 were determined by using Gene Expression Assays (Applied Biosystems) gene expression products for CYP2C9 and CYP2C19, respectively. The mRNA levels were normalized against glyceraldehyde-3-phosphate dehydrogenase mRNA determined by

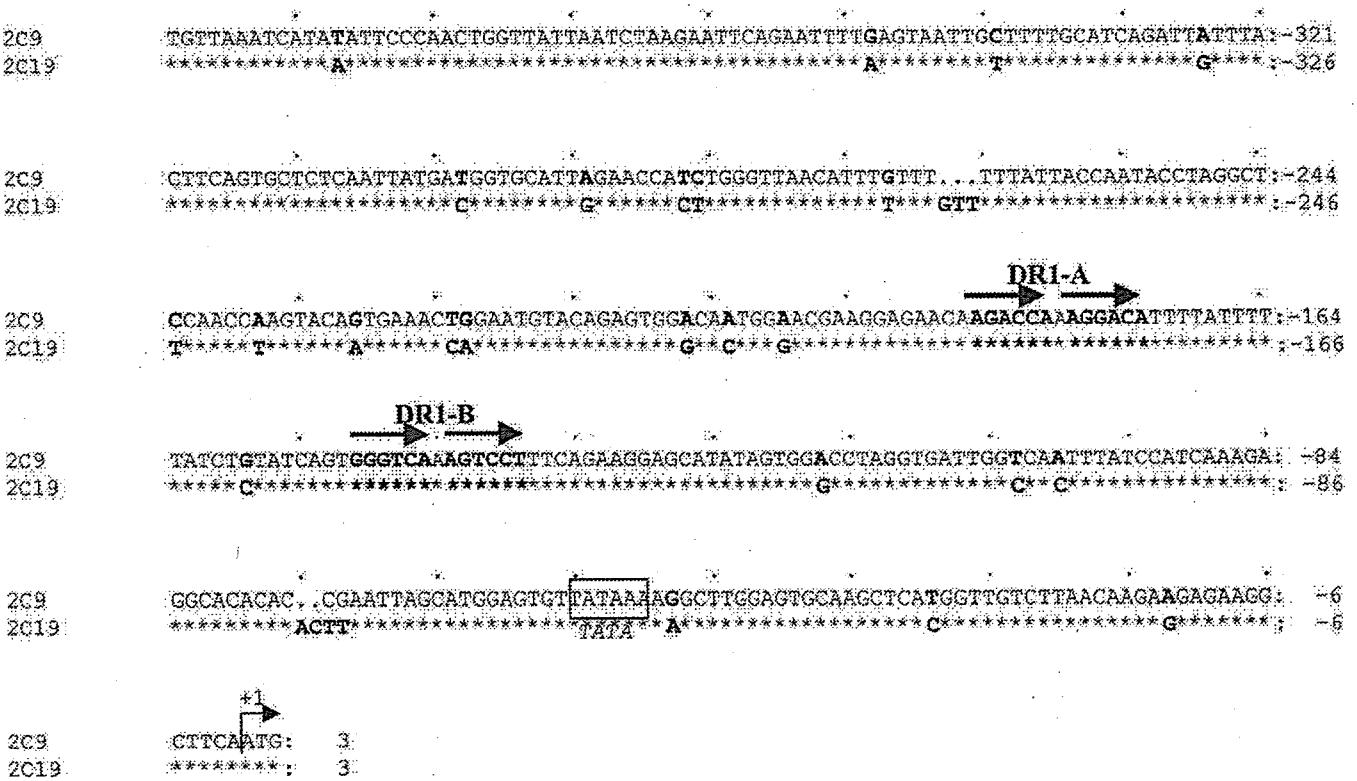

FIG. 1. Nucleotide sequences of the promoter regions of the *CYP2C9* and *CYP2C19* genes. Nucleotides are arbitrarily numbered in negative numbers from the ATG coding for the initiation codon (+1). Differences in nucleotide sequence are highlighted in bold letters, and putative HNF4α binding sites (DR1-A and DR1-B) are indicated by arrows.

PreDeveloped TaqMan Assay Reagents for glyceraldehyde-3-phosphate dehydrogenase (Applied Biosystems).

Statistical Analyses. Data are presented as mean ± S.D. The *p* values for each experimental comparison were determined using Student's *t* test.

Results

Identification of Two DR1 Elements in the *CYP2C19* Gene. A comparison of the 5′-flanking regions of the *CYP2C9* and *CYP2C19* genes is shown in Fig. 1. The 5′-flanking region from −2 kbp to the translation start site of the *CYP2C9* gene was 88.8% identical to that of the *CYP2C19* gene. We searched for the DR1 element in the *CYP2C19* promoter by using a searching program for nuclear receptor binding sites (http://www.nubiscan.unibas.ch/; Podvinec et al., 2002) and found two putative DR1 elements (score, ≥0.75). No other DR1 element was identified with this score in this region. The upstream and downstream elements identified in the *CYP2C19* promoter had the same sequences as those of two DR1 elements of the *CYP2C9* promoter to which it has been reported that HNF4α can bind (Ibeanu and Goldstein, 1995; Chen et al., 2005). The upstream elements and the downstream elements in both genes are hereafter referred to as the DR1-A element and the DR1-B element, respectively.

Binding of HNF4α to the DR1-A and DR1-B Elements of the *CYP2C9* and *CYP2C19* Promoters in Vitro. EMSAs were performed to examine whether HNF4α could bind to the DR1-A and DR1-B elements in the *CYP2C9* and *CYP2C19* promoters. We used a nuclear extract prepared from HepG2 cells because it has been reported that this cell line endogenously expressed HNF4α (Ihara et al., 2005; Furihata et al., 2006). As shown in Fig. 2A, specific protein-DNA complexes were formed when the radiolabeled probe containing either the DR1-A element (DR1-A WT) or the DR1-B element (DR1-B WT) was incubated with HepG2 nuclear extracts (lanes 3 and 9, respectively). These complexes migrated at the same position as that of the one formed with the radiolabeled probe of HNF4α consensus (HNF4 WT, lane 1). The formation of the complexes was eliminated by the addition of self-competitors (DR1-A WT, lane 4; DR1-B WT, lane 10) or unlabeled HNF4 WT (lanes 6 and 12). However, complex formation was not inhibited in the presence of mutated competitors (DR1-A MT, lane 5; DR1-B MT, lane 11; HNF4 MT, lanes 7 and 13).

To determine the proteins forming these complexes, supershift assays were performed using IgG against HNF4α. The addition of anti-HNF4α IgG to the mixture resulted in generation of a supershifted band when either DR1-A WT or DR1-B WT was used as a probe (Fig. 2B, lanes 3 and 7, respectively). Control mouse IgG, used as a negative control, did not affect the formation of any complexes (lanes 4 and 8). These results indicate that HNF4α can bind to both the DR1-A and DR1-B elements of the *CYP2C9* and *CYP2C19* promoters.

Effects of HNF4α on the Transcriptional Activity of the *CYP2C9* and *CYP2C19* Promoters in FLC7 Cells. Cotransfection analyses were performed by using human hepatocarcinoma FLC7 cells to examine whether HNF4α played different roles in the transactivation of the *CYP2C9* and *CYP2C19* promoters. We have determined that this cell line does not express endogenous HNF4α (Furihata et al., 2006). Several constructs containing various lengths of the *CYP2C9* promoter region and the HNF4α expression vector were cotransfected into FLC7 cells (Fig. 3, left). The levels of the transcriptional activities of the five constructs containing two DR1 elements (2C9 −2k, 2C9 −380, 2C9 −340, 2C9 −320, and 2C9 −255) in the presence of HNF4α were increased to 4.9-, 2.4-, 4.2-, 4.0-, and 3.5-fold, respectively, compared with those in the absence of HNF4α.

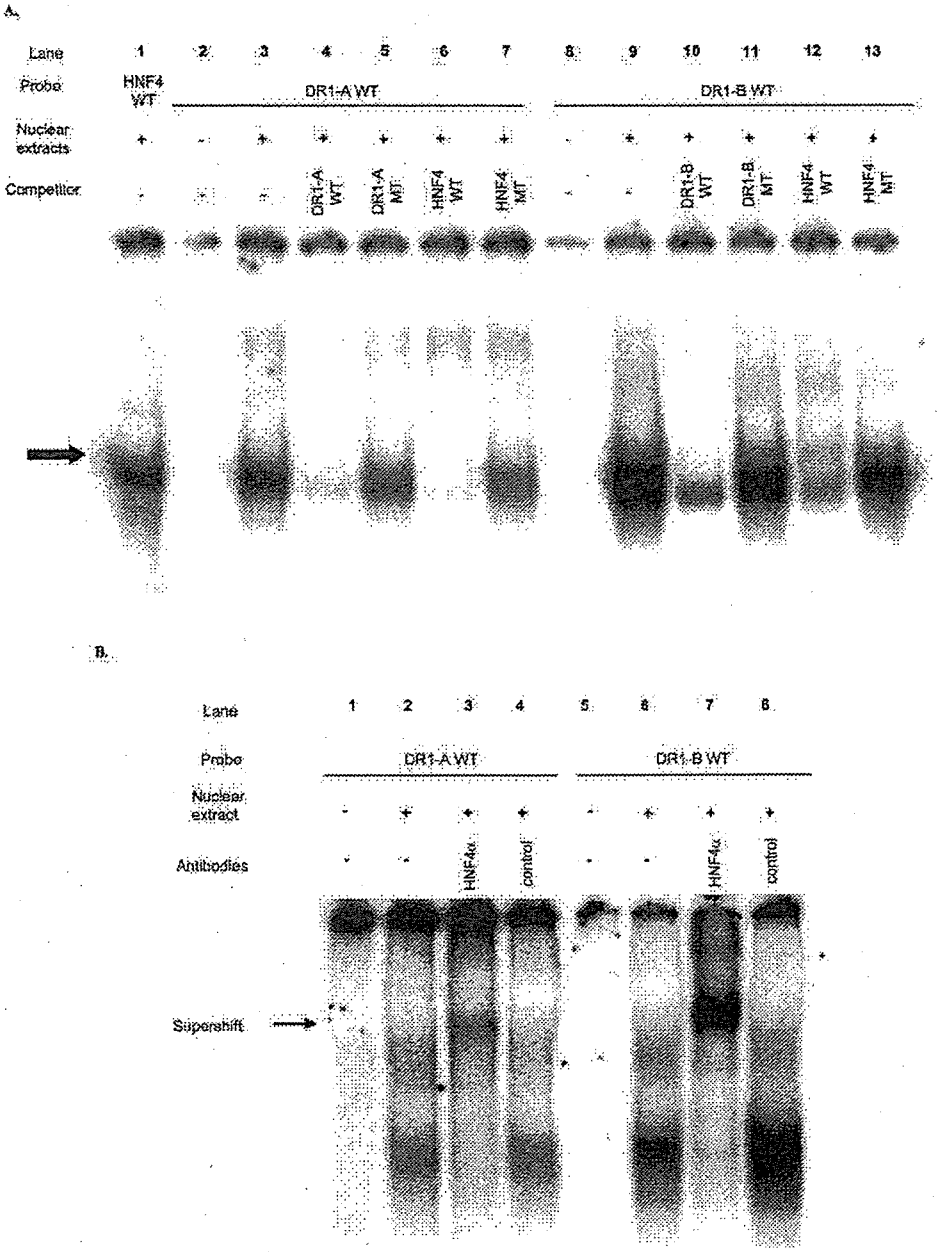

FIG. 2. Binding of HNF4α to the DR1-A and DR1-B elements of the *CYP2C9* and *CYP2C19* promoters in vitro. A, EMSAs were performed using nuclear extracts prepared from HepG2 cells with the following probes: HNF4 WT in lane 1, DR1-A WT in lanes 2 through 7, and DR1-B WT in lanes 8 through 13. Oligonucleotide competitors were added with 50-fold excess amounts of the following probes: DR1-A WT in lane 4, DR1-A MT in lane 5, DR1-B WT in lane 10, DR1-B MT in lane 11, HNF4 WT in lanes 6 and 12, and HNF4 MT in lanes 7 and 13. Symbols (+) and (−) indicate the presence and absence of the nuclear extracts or competitors, respectively. B, supershift assays were performed using antibodies specific for HNF4α. Anti-HNF4α IgG (2 μg) was added to the reaction mixtures in lanes 3 and 7. Control IgG (2 μg) was used as a negative control (lanes 4 and 8). Symbols (+) and (−) indicate the presence and absence of the nuclear extracts or IgG, respectively. The arrow indicates supershifted bands.

However, the transcriptional activity of 2C9 −195 was not increased by HNF4α despite the fact that this construct contained two DR1 elements. Deletion of the DR1-A element (2C9 −160) or both elements (2C9 −140) from the promoter region abolished its response for transactivation by HNF4α. The same experiments were also performed using eight different deletion constructs of the *CYP2C19* promoter (Fig. 3, right). In contrast to the results obtained from the CYP2C9 constructs, the levels of the transcriptional activities of CYP2C19 constructs were not increased in the presence of HNF4α. Deletion of the DR1-B elements of CYP2C9 (2C9 −140) and CYP2C19 (2C19 −140) from the promoter regions abolished the transcriptional activities in the presence and absence of HNF4α.

Mutation analyses were performed to examine whether HNF4α required two DR1 elements for its transactivation ability (Fig. 4). As for the CYP2C9 constructs, HNF4α could stimulate the level of the promoter activity of the wild-type construct (2C9 −2k) to approximately 4-fold, but the introduction of mutation of each DR1 element resulted in complete loss of transactivation of the mutated *CYP2C9* promoter (2C9 DR1-Amt, 2C9 DR1-Bmt) by HNF4α (Fig. 4, left). On the other hand, the levels of the transcriptional activities of constructs

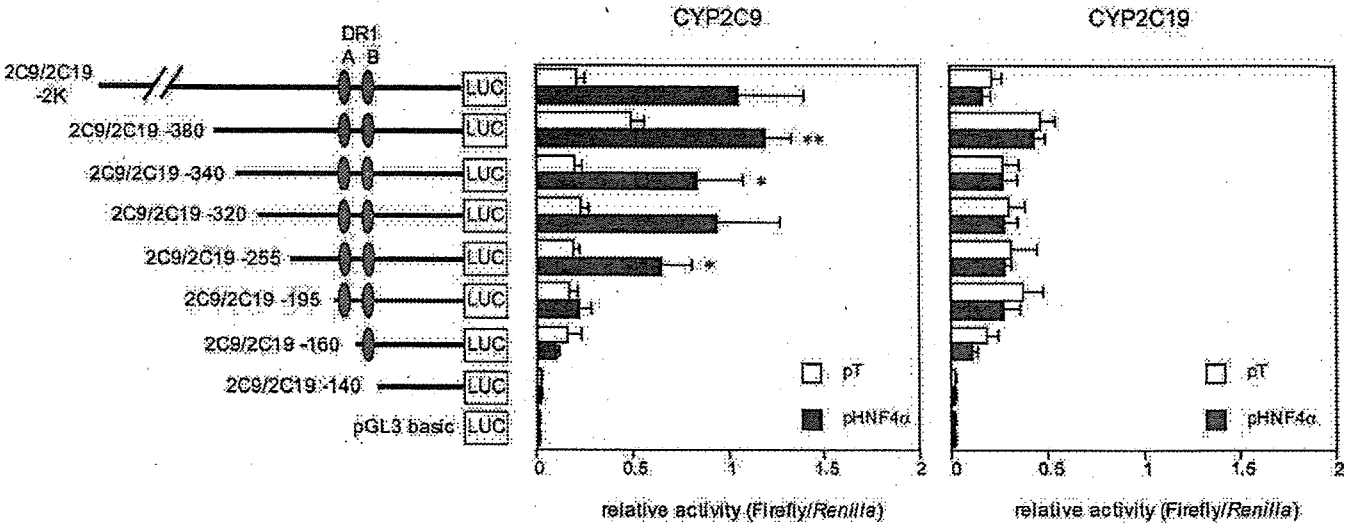

FIG. 3. Effects of HNF4α on transcriptional activity of the *CYP2C9* and *CYP2C19* promoters in FLC7 cells. Deletion constructs (200 ng) of the *CYP2C9* or *CYP2C19* promoter were cotransfected with 100 ng of HNF4α expression vector (pHNF4α, open bars) or 100 ng of an empty vector (pT, closed bars). Two HNF4α binding sites are shown in circles. Each value is the mean ± S.D. of relative activity (firefly/*Renilla*) for four separate experiments, each performed in triplicate. *, $p < 0.05$ and **, $p < 0.01$ compared with the empty vector. Luc, luciferase.

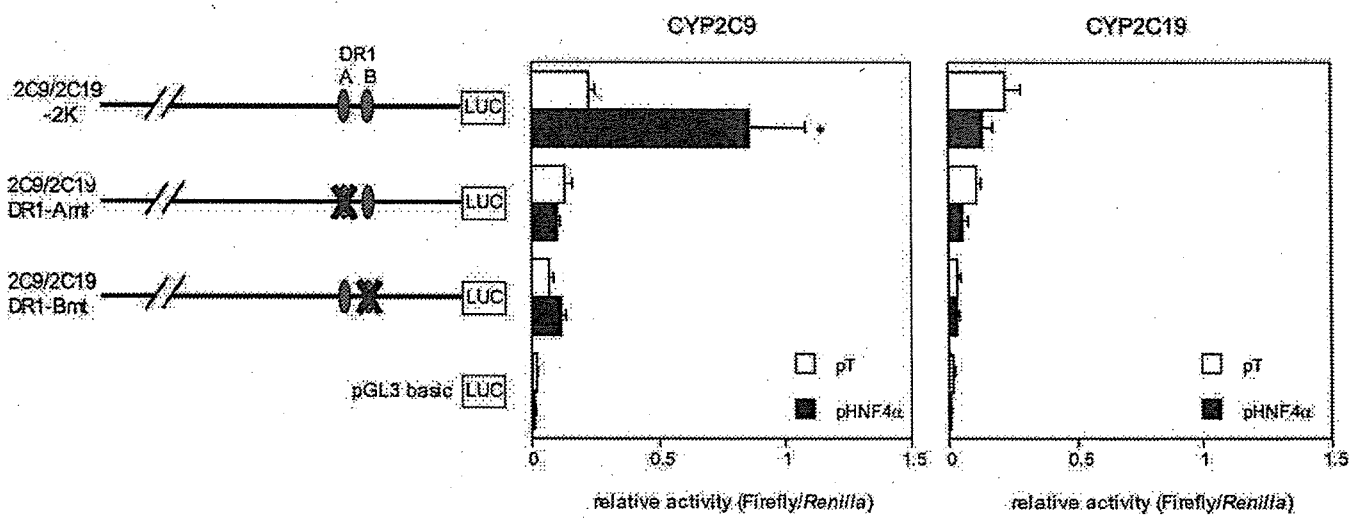

FIG. 4. Mutation analysis for two HNF4α binding sites of the *CYP2C9* and *CYP2C19* promoters in FLC7 cells. Reporter constructs (200 ng) of the *CYP2C9* or *CYP2C19* promoter were cotransfected with 100 ng of HNF4α expression vector (pHNF4α, open bars) or 100 ng of an empty vector (pT, closed bars). Two HNF4α binding sites are shown in circles, and mutations are indicated by crosses. Each value is the mean ± S.D. of relative activity (firefly/*Renilla*) for three separate experiments, each performed in triplicate. *, $p < 0.05$ and **, $p < 0.01$ compared with the empty vector. Luc, luciferase.

of the *CYP2C19* promoter (2C19 −2k, 2C19 DR1-Amt, and 2C19 DR1-Bmt) were not increased by HNF4α (Fig. 4, right). These results indicate that HNF4α can increase the level of transcriptional activity of the *CYP2C9* promoter but not that of the *CYP2C19* promoter and that this activation occurred only when two DR1 elements of the *CYP2C9* promoter were simultaneously functional. Introduction of mutation of DR1-B elements decreased transcriptional activities of the *CYP2C9* and *CYP2C19* promoters in the presence and absence of HNF4α.

Binding of HNF4α to the *CYP2C9* Promoter but Not to the *CYP2C19* Promoter in Vivo. ChIP assays were performed using human liver to examine whether HNF4α could bind to the *CYP2C9* and *CYP2C19* gene promoters in vivo (Fig. 5). After DNA extraction of the immunoprecipitated chromatin, PCR was performed to detect the occupancy of DR1 elements of the *CYP2C9* and *CYP2C19* genes by HNF4α. As for *CYP2C9*, the DR1 elements were much more abundant in DNA extracted from chromatin immunoprecipitated with anti-HNF4α IgG than in that with control mouse IgG (Fig. 5, top). On the other hand, no DNA fragment around the DR1 elements of the *CYP2C19* gene was detected in both extracted DNA samples (Fig. 5, bottom). We also determined the expression levels of CYP2C9 and CYP2C19 mRNA in the same liver used for ChIP assays by using quantitative real-time PCR. The expression level of CYP2C9 mRNA was 82.5 times higher than that of CYP2C19 mRNA.

Discussion

The present study showed that two DR1 elements were located in the *CYP2C9* promoter (Fig. 1) and that the transcriptional activities of the *CYP2C9* promoter were increased by exogenous HNF4α (Fig. 3). The introduction of mutation to each DR1 element resulted in complete loss of transactivation (Fig. 4). These results are consistent with the results presented in a recent report (Chen et al., 2005). We also

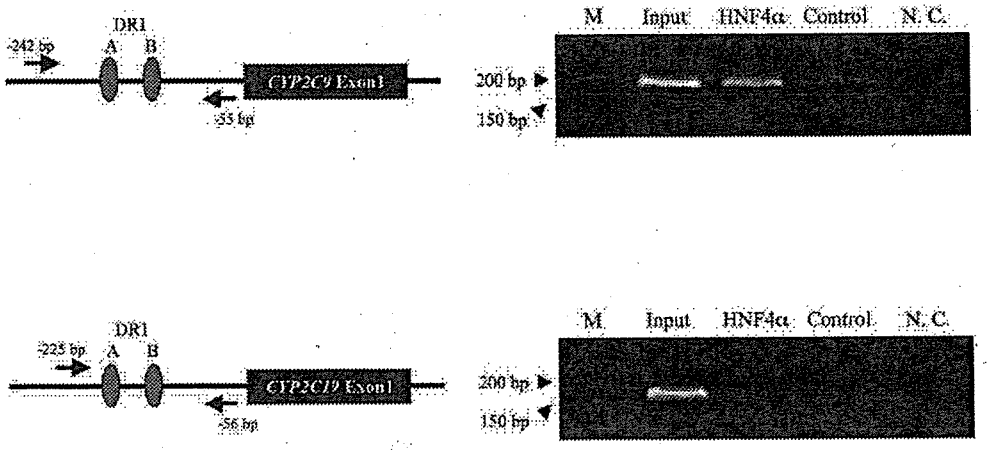

FIG. 5. Binding of HNF4α to the DR1-A and DR1-B elements of the *CYP2C9* and *CYP2C19* promoters in vivo. ChIP assays were performed using the sheared genomic DNA extracted from human liver (9 μg), control mouse IgG (3 μg), and anti-HNF4α IgG (3 μg). M, DNA size marker; input, control sheared genomic DNA; HNF4α, sheared genomic DNA immunoprecipitated with anti-HNF4α IgG; control, sheared genomic DNA immunoprecipitated with control mouse IgG; N.C., nontemplate control.

performed the same experiments for the *CYP2C19* promoter. In contrast to the case of the *CYP2C9* promoter, transactivation by HNF4α was not observed in the *CYP2C19* promoter despite the existence of two DR1 elements (Fig. 3). In addition, HNF4α could bind to the DR1 elements located in the *CYP2C9* promoter but not to those in the *CYP2C19* promoter in vivo (Fig. 5). These results suggest that HNF4α participated in the transactivation of at least -2 kbp of the *CYP2C9* promoter but not that of the *CYP2C19* promoter.

Significant decreases in the levels of CYP3A4, CYP3A5, CYP2A6, CYP2B6, CYP2C9, and CYP2D6 mRNA have been observed in HNF4α-deficient human hepatocytes (Jover et al., 2001), and several studies have shown that transcription of *CYP3A4*, *CYP2A6*, and *CYP2D6* genes are regulated by HNF4α via DR1 elements located in their promoters (Cairns et al., 1996; Tirona et al., 2003; Pitarque et al., 2005). Our results and the results of a recent study by Chen et al. (2005) showed that HNF4α was involved in the expression of the *CYP2C9* gene. Therefore, these findings suggest that HNF4α plays important roles in regulation of the expression of these *P450* genes in the human liver. On the other hand, it has been reported that HNF4α is not involved in transactivation of the *CYP2C18* promoter, although a DR1 element is located in this promoter (Ibeanu and Goldstein, 1995). Considering the fact that the expression level of CYP2C18 mRNA in the human liver is very low compared with the expression levels of other genes of the *CYP2C* subfamily (Goldstein and de Morais, 1994), it is possible that the lack of a functional DR1 element in the *CYP2C18* promoter contributed to this low level of expression of CYP2C18 mRNA in the human liver. Accordingly, the same idea would also explain why the expression level of CYP2C19 is lower than that of CYP2C9. That is, the existence of "functional" DR1 elements in the regulatory region of the *CYP2C9* gene would be crucial factors for its higher level of expression than that of the *CYP2C19* gene in the human liver.

The reason for the different effects of HNF4α on transactivation of the *CYP2C9* and *CYP2C19* genes is currently unknown. However, the results obtained from our study provided some clues for understanding this difference. HNF4α could not transactivate the *CYP2C9* promoter in the absence of the region from -255 to -195 bp (-255/-195 bp), although two DR1 elements were still present in the promoter (Fig. 3), suggesting that the region $-255/-195$ bp of the *CYP2C9* promoter is necessary for HNF4α to up-regulate the transcription of the *CYP2C9* gene. One possible explanation for these results is that other HNF4α binding sites exist in the region $-255/-195$ bp of the *CYP2C9* gene, and they can help the action of HNF4α that is recruited to the downstream elements. However, no DR1 elements were found in this region of the *CYP2C9* promoter by a searching program for nuclear receptor binding sites (http://www.nubiscan.unibas.ch/; Podvinec et al., 2002), and HNF4α could not bind to this region in EMSA by using an oligonucleotide probe ranging from -255 to -195 bp (data not shown). Therefore, effects of the region $-255/-195$ bp on transcription of the *CYP2C9* gene are unlikely to be mediated by the direct binding of HNF4α to this region. Another possibility is that a certain factor, which assists with HNF4α-mediated transactivation of the *CYP2C9* promoter, specifically binds to the region $-255/-195$ bp of the *CYP2C9* gene but not to the *CYP2C19* gene. Actually, there are 8-bp differences between the region $-255/-195$ bp of the *CYP2C9* promoter and the region $-257/-197$ bp of the *CYP2C19* promoter. The factor that binds to the region $-255/-195$ bp of the *CYP2C9* promoter may stabilize the binding of HNF4α to the DR1 element of the *CYP2C9* promoter, or it may recruit cofactors that are required for function of HNF4α. However, no complexes were formed in EMSAs using HepG2 nuclear extracts and an oligonucleotide probe ranging from -255 to -195 bp (data not shown). A searching program for transcriptional factors could not identify any factors that fulfill these requirements. Thus, further detailed study is needed to elucidate the role of the region $-255/-195$ bp of the *CYP2C9* promoter in HNF4α function.

Deletion and mutation of DR1-B elements decreased transcriptional activities of the *CYP2C9* and *CYP2C19* promoters in the presence and absence of HNF4α (Figs. 3 and 4). A putative binding site of CCAAT enhancer-binding protein was found in the downstream of DR1-B elements partly overlapped. Therefore, the binding of CCAAT enhancer-binding protein to the *CYP2C9* and *CYP2C19* promoters may be inhibited by deletion and mutation of DR1-B elements, resulting in the decrease of basal activities of *CYP2C9* and *CYP2C19* promoters.

In conclusion, we showed that HNF4α is one of the important factors regulating promoter activity of the *CYP2C9* gene but not that of the *CYP2C19* gene in the human liver. The direct binding of HNF4α to two DR1 elements of the *CYP2C9* promoter is essential for HNF4α-mediated transactivation of the *CYP2C9* promoter. In addition, this transactivation requires certain factors that facilitate the function of HNF4α via the region from $-255/-195$ bp of the *CYP2C9* promoter. The results of the present study suggest that HNF4α is one of the determinants for the difference between expression levels of CYP2C9 and CYP2C19 in the human liver.

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AUTOINDUCTION OF MKC-963 [(*R*)-1-(1-CYCLOHEXYLETHYLAMINO)-4-PHENYLPHTHALAZINE] METABOLISM IN HEALTHY VOLUNTEERS AND ITS RETROSPECTIVE EVALUATION USING PRIMARY HUMAN HEPATOCYTES AND CDNA-EXPRESSED ENZYMES

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ABSTRACT:

MKC-963, (*R*)-1-(1-cyclohexylethylamino)-4-phenylphthalazine, a potent inhibitor of platelet aggregation, was synthesized and used in clinical trials in the 1990s. In the process of clinical study, it was found that urinary excretion ratios for 6β-hydroxycortisol and free cortisol increased significantly in parallel with decreases in the plasma concentrations of MKC-963 after repeated oral administration of the compound to healthy volunteers. These findings suggested that MKC-963 caused autoinduction (defined as the ability of a drug to induce enzymes that enhance its own metabolism, resulting in dispositional tolerance) in humans, and clinical studies using the compound were stopped. This experience prompted us to reevaluate the effects of this compound on CYP3A4 using primary human hepatocytes and cDNA-expressed human cytochrome P450 (P450) enzymes to determine whether the autoinduction of MKC-963 metabolism in humans could have been predicted if these in vitro systems had been used for the evaluation of MKC-963 in the preclinical study. The results of in vitro study showed that MKC-963 increased CYP3A4 mRNA expression level and activity of testosterone 6β-hydroxylation to extents similar to those observed with rifampicin in primary human hepatocytes. In addition, approximately 90% of the MKC-963 metabolism in human liver microsomes was estimated to be attributable to CYP3A4. These in vitro findings are in good agreement with the results of clinical study, suggesting that studies using human hepatocytes and cDNA-expressed human P450s are useful for assessing the autoinductive nature of compounds under development before starting clinical studies.

MKC-963, (*R*)-1-(1-cyclohexylethylamino)-4-phenylphthalazine (Fig. 1), a potent inhibitor of platelet aggregation, was synthesized and used in clinical trials by Mitsubishi Chemical Corp. (Tokyo, Japan) in the 1990s. In the process of clinical trials, the urinary excretion of 6β-hydroxycortisol (6β-OHF) and free cortisol (F) was studied after repeated oral administration of MKC-963 in human volunteers to determine whether this compound induces CYP3A4 or not. This was because the compound would be used for treatment of circulatory disorders together with drugs such as antihypertensives, antihyperlipidemics or antidiabetes. Many of these drugs are metabolized by CYP3A4 (Li et al., 1995; Lehmann et al., 1998; Prueksaritanont et al., 2003; Jerling et al., 2005), a predominant P450 enzyme found in the adult human liver that catalyzes the oxidation of a wide variety of exogenous compounds (Guengerich et al., 1986). In addition, CYP3A4 had been reported to be induced by several drugs, including rifampicin, phenytoin, and phenobarbital, that caused clinical drug-drug interactions (Holtbecker et al., 1996; Anderson 1998; Ridtitid et al., 2002). Moreover, measurement of the urinary ratio of 6β-OHF and F (6β-OHF/F) had been regarded as a safe and simple method for evaluating induction of CYP3A4 because it is noninvasive and does not require administration of a probe drug to volunteers (Galteau and Shamsa, 2003).

In this clinical study on MKC-963, we found that 6β-OHF/F increased significantly in parallel with decreases in the plasma concentrations of MKC-963 after repeated oral administration of the compound to healthy volunteers. This finding suggested that CYP3A4 is induced by MKC-963 and that the compound itself is an autoinducer in humans. Because autoinduction[1] was thought to reduce the therapeutic response of MKC-963 and might cause clinical problems,

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[1] Autoinduction is defined as the ability of a drug to induce enzymes that enhance its own metabolism, resulting in dispositional tolerance.

ABBREVIATIONS: MKC-963, (*R*)-1-(1-cyclohexylethylamino)-4-phenylphthalazine; d5MKC-963, MKC-963 with 5 hydrogen substituted by deuterium in the phenyl ring; MGB, minor groove binder; AUC, area under the plasma concentration-time curve; C_{max}, maximum plasma concentration; t_{max}, time to reach C_{max}; $t_{1/2}$, terminal half-life; F, cortisol; 6β-OHF, 6β-hydroxycortisol; P450, cytochrome P450; CL, in vitro clearance.

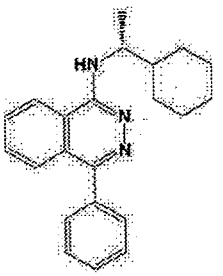

FIG. 1. Chemical structure of MKC-963.

the clinical study on MKC-963 was abandoned at that time. Thus, we have recently decided to reevaluate the effects of this compound on CYP3A4 using primary human hepatocytes and cDNA-expressing human P450 enzymes to determine whether we could have predicted the autoinduction of MKC-963 metabolism if we had used these in vitro systems for the preclinical evaluation of MKC-963.

In this paper, we describe the results of the clinical study on the pharmacokinetics of MKC-963 and its effects on the urinary excretion ratio of 6β-OHF and F after repeated oral administration of the compound to healthy volunteers, and the results of in vitro studies on the effects of MKC-963 on the expression and activities of CYP3A4 and identification of the P450 enzyme(s) responsible for the metabolism of MKC-963 using primary human hepatocytes and cDNA-expressed human P450 enzymes, respectively. The results suggest that these in vitro systems would have been useful for the prediction of the autoinductive nature of MKC-963 in the preclinical study.

Materials and Methods

Materials. MKC-963 was provided by Mitsubishi Chemical Corp. (Tokyo, Japan), and its chemical purity was 99.8%. Rifampicin, testosterone and 6β-hydroxytestosterone were purchased from Sigma-Aldrich (St. Louis, MO), Tokyo Kasei Kogyo Co. (Tokyo, Japan), and Sumika Chemical Analysis Service, Ltd. (Osaka, Japan), respectively. All other chemicals were of analytical reagent grade.

In Vivo Study. *Subjects.* Six healthy male volunteers aged between 20 and 35 years were recruited for the study. They were within ±20% of their ideal body weight and in good general health according to routine medical history and laboratory data. They did not use any medications for at least 2 weeks before and were not using any concurrent medications during the study. All of them agreed to refrain from consumption of alcohol and grapefruit or grapefruit juice during the study. Subjects who had clinically significant abnormalities on preliminary examination, those who had a history of drug or food allergies or a history of drug or alcohol abuse, and those who had donated blood or received an investigational drug within 4 months before the start of this study were excluded from this study.

Study Protocol. The subjects received a single oral dose of MKC-963 on day 1 and on day 14, and two oral doses per day with a 12-h interval for 12 days (from day 2 to day 13). Each dose was 120 mg, and the drug was supplied as tablets (40 mg). The oral doses were administered with 100 ml of water at 9:00 AM after breakfast or at 9:00 PM after dinner. Breakfast and dinner were standardized for all the subjects. Blood samples (each 4 ml) were collected by venipuncture at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, and 8 h after the first administration of MKC-963 on days 1 and 14 and at 0, 1, and 2 h on days 2, 5, 8, and 11. The blood samples collected were centrifuged at 1500*g* for 10 min at 4°C, and plasma samples were separated and stored at −20°C until analyses. Urine samples were pooled over a period of 24 h and collected at the end of designated days: the day before and 1, 2, 5, 8, 11, and 14 days after starting drug administration. The urine was kept cool during collection, and then the total volume was recorded and a 10-ml aliquot was stored at −20°C until analyses.

The study was conducted at Hohsen Clinic, Research Center for Clinical Pharmacology, The Kitasato Institute (Tokyo, Japan), and the protocol was approved by the institutional review board. The study was conducted in accordance with the guidelines on good clinical practice and the ethical standards for human experimentation established by the Declaration of Helsinki. Each subject gave written informed consent.

Determination of MKC-963 Concentrations in Plasma. Plasma concentrations of MKC-963 were determined by liquid chromatography-tandem mass spectrometry. d5MKC-963 was used as an internal standard. The plasma (0.5 ml) was mixed with 0.4 ml of Titrisol buffer (pH 9) and applied on a solid-phase extraction column (Extrelut-1; Merck KGaA, Darmstadt, Germany). The MKC-963 and internal standard were isolated from the column with 5 ml of diethyl ether. The organic extract was dried under nitrogen and reconstituted in 1 ml of acetonitrile. The sample was separated by a Waters HPLC system (Waters, Milford, MA) equipped with a Capcell Pak CN column (5 μm, 35 × 4.6 mm in internal diameter; Shiseido, Tokyo, Japan). The mobile phase consisted of acetonitrile/water/acetic acid (90:10:1, v/v/v) and the flow rate was maintained at 0.2 ml/min. MKC-963 and the internal standard were detected by tandem mass spectrometry using a Finnigan TSQ7000 mass spectrometer (Thermo Electron Corp., Waltham, MA). For mass spectral detection, the following precursors to product ion reactions were monitored: m/z 332.1 > m/z 222.1 for MKC-963 and m/z 337.0 > m/z 227.1 for d5MKC-963. The standard curves were linear from 0.1 ng/ml to 50 ng/ml. The interassay precision (% CV) assessed from the blank plasma to which known concentrations of the analytes was added (final concentrations of 0.1 ng/ml to 50 ng/ml) ranged from 2.0% to 7.7%. The limit of sensitivity of the assay was 0.01 ng/ml.

Pharmacokinetic Parameters. The pharmacokinetic parameters of MKC-963 were estimated by noncompartmental methods with the use of WinNonlin V4.1 (Pharsight Corporation, Mountain View, CA). The values of C_{max} and t_{max} were determined directly from the plasma concentration-time profiles. The area under the plasma concentration-time curve (AUC) from 0 to 24 h was determined by the linear trapezoidal rule from the beginning of drug administration to the last quantifiable data point. The value of $t_{1/2}$ was calculated by linear regression analysis of the last elimination phase after log transformation of the data.

Determination of Urinary 6β-OHF and F. Determination of 6β-OHF and F in urine samples was performed by using enzyme immunoassay kits for urinary 6β-OHF (Stabiligen, Villers-Les-Nancy, France) and F (Biométreux, Marcy l'Etoile, France), respectively, according to the manufacturer's instructions. The cross-reactivity of these kits for urinary F and 6β-OHF were 4.4 and 1.1%, respectively.

In Vitro Study. *Human Primary Hepatocytes and Treatment with MKC-963.* Cryopreserved human hepatocytes (lot 100, white female, 74 years old) were obtained from In Vitro Technologies, Inc. (Baltimore, MD). Hepatocytes were suspended in Hepatocyte Culture Medium (Cambrex, Walkersville, MD), centrifuged at 50*g* for 3 min, and resuspended in the same medium. The cells were plated onto Matrigel-coated 24-well plates at a density of 1.5×10^5 cells/well and were maintained in an atmosphere of 95% air and 5% CO_2 at 37°C. The cell viability was more than 80% assessed by a trypan blue exclusion test. Stock solutions of MKC-963 and rifampicin were prepared in dimethyl sulfoxide and were diluted before each use. Treatments of hepatocytes with chemicals were begun on the fourth day after seeding and continued for 4 days. The hepatocytes were treated with dimethyl sulfoxide (final concentration of 0.2%), rifampicin (10 μM), a positive control, or MKC-963 (0.25 μM). The concentration of MKC-963 used in the present study was determined considering that C_{max} of MKC-963 was 0.29 μM when 120 mg of MKC-963 was administered orally to human volunteers (Fig. 2). The concentration of rifampicin used in the present study also corresponded nearly to C_{max} of rifampicin after an oral administration of 450 to 600 mg in patients with tuberculosis (Smith, 2000).

RNA Extraction and Real-Time PCR. Total RNA was extracted using TRIzol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. All samples were stored at −80°C until used for cDNA preparation. One microgram of total RNA was reverse-transcribed into cDNA with random hexamers using a SuperScript II Transcription system (Invitrogen Corp) according to the manufacturer's instructions. The expression levels of specific mRNAs were determined by using a quantitative real-time PCR method. The primer and TaqMan minor groove binder (MGB) probe sets were designed by using Primer Express software (Applied Biosystems, Foster City, CA). The sequences (5′ to 3′) for the primers and probes are as follows: CYP3A4, forward primer (GCAGGAGGAAATTGATGCAGTT), TaqMan MGB probe [FAM (Applied Biosystems)-ATAAGGCACCACCCACCTA-MGB], and reverse primer (CTGAGCGTTTCATTCACCACC); β-actin, forward primer (CCTGGCACCCAGCACAAT), fluorogenic probe [VIC

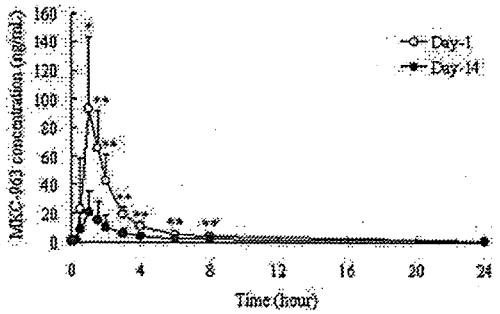

FIG. 2. Plasma concentration-time profiles of MKC-963 on day 1 (open circles) and day 14 (closed circles) after oral administration of 120 mg to six healthy subjects. Data are expressed as means ± S.D. *, $p < 0.05$; **, $p < 0.01$.

(Applied Biosystems)-ATCATTGCTCCTCCTGAG-MGB], and reverse primer (CCGATCCACACGGAGTACTTG). The sequence of fluorogenic probe for CYP3A4 was one base different from that of CYP3A5, which is recognized by MGB-probe according to the supplier's manuals. Cycling conditions of the PRISM 7900 Sequence Detection system (Applied Biosystems) were 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. CYP3A4 mRNA levels in cultured human hepatocytes were expressed as ratio against β-actin mRNA levels.

Determination of CYP3A4 Activities in Human Hepatocyte Culture. CYP3A4 activities were determined by the measurement of 6β-hydroxylation activities for testosterone in intact hepatocytes cultured on 24-well plates (Donato et al., 1995). After treatment with chemicals, monolayers were incubated with testosterone (250 μM) for 30 min. Quantification of 6β-hydroxytestosterone was performed by high-performance liquid chromatography (Donato et al., 1993).

Identification of P450 Enzyme(s) Contributing to the Metabolism of MKC-963. Recombinant P450 enzymes expressed in insect cells infected with baculovirus containing human P450 and human NADPH-P450 reductase cDNA inserts were obtained from BD Gentest (Woburn, MA). Incubation mixtures contained cDNA-expressed P450s (50 pmol/ml) in potassium phosphate buffer (pH 7.4), an NADPH-generating system, and MKC-963. Substrate (2 μM MKC-963) was incubated at 37°C for 0, 5, 15, and 30 min with microsomes expressing CYP1A2, CYP2C9, CYP2C19, CYP2D6, or CYP3A4, and determined by liquid chromatography/mass spectrometry. The remaining percentage of MKC-963 was calculated using the $t = 0$ value as 100%. Then, in vitro clearance of each P450 enzyme (CL) was estimated from the following equation: CL (μl/min/pmol P450) = −slope (1/min)/P450 concentration (pmol P450/ml) × 1000, where slope was determined from linear regression analysis between log percentage of MKC-963 and incubation time (Obach, 1999), and P450 concentration was the concentration of recombinant P450 enzyme in the incubation mixture. CL was corrected with the P450 contents in native human liver microsomes (Rodrigues, 1999) as follows: Corrected CL = CL × enzyme content of each P450. Therefore, the contribution of each P450 enzyme to overall clearance was estimated from the following equation: Contribution of each P450 enzyme (%) = corrected CL for each P450 enzyme/sum of corrected CL × 100.

Statistics. Statistical analysis was performed with SAS software (version 8.2; SAS Institute, Cary, NC). A P value of <0.05 was considered statistically significant.

Results

In Vivo Study. *Pharmacokinetics of MKC-963.* Plasma concentration-time profiles of MKC-963 showed a dramatic change after repeated oral administration of the compound (120 mg) to healthy subjects. As shown in Fig. 2, the mean (±S.D.) plasma concentrations of MKC-963 on day 14 at 1 to 8 h after administration were significantly lower than those on day 1. As a result, C_{max} and AUC values on day 14 had decreased by 77% and 69%, respectively, compared with the values on day 1 (Table 1). There were no notable differences between t_{max} and $t_{1/2}$ values on day 1 and those on day 14 (Table 1).

Figure 3 shows the changes in mean plasma concentrations of MKC-963 at 1 and 2 h after administration from day 1 to day 14. As shown in this figure, the plasma concentrations of MKC-963 at 1 h decreased significantly ($p < 0.05$) from day 2 to day 14, and those at 2 h also showed significant ($p < 0.05$) decreases from day 5 to day 14.

TABLE 1

Pharmacokinetic parameters of MKC-963 on day 1 and day 14 after repeated oral administration of 120 mg to six healthy subjects

Data are expressed as means ± S.D. except for t_{max} data, which are given as median with range.

	Day 1	Day 14
C_{max} (ng/ml)	96.2 ± 46.7	22.6 ± 14.8**
t_{max} (h)	1 (1–1.5)	1 (0.5–1)
AUC (h · ng/ml)	206.0 ± 76.5	64.8 ± 31.8**
$t_{1/2}$ (h)	7.2 ± 2.1	10.9 ± 5.0

** $p < 0.01$.

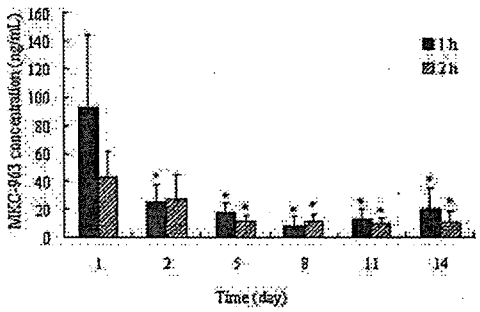

FIG. 3. Plasma concentrations of MKC-963 at 1 h and 2 h after oral administration of the compound (120 mg) to six healthy subjects on days 1, 2, 5, 8, 11, and 14. Data are expressed as means ± S.D. *, $p < 0.05$.

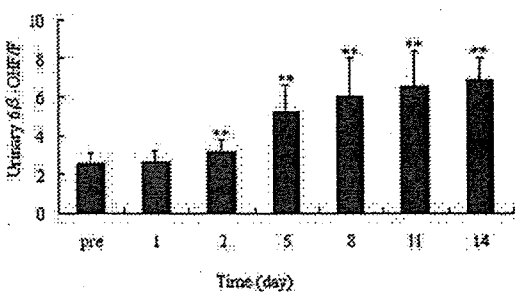

FIG. 4. Twenty-four-hour urinary excretion ratios of 6β-hydroxycortisol and free cortisol in six healthy subjects on the day before the start of administration and on days 1, 2, 5, 8, 11, and 14. Results are expressed as means ± S.D. **, $p < 0.01$.

Urinary 6β-OHF/F. Figure 4 shows the mean 24-h urinary excretion ratios of 6β-OHF and F on the day before the start of administration and from day 1 to day 14. The mean value of 6β-OHF/F increased significantly ($p < 0.05$) from day 2 to day 14 compared with the value on the day before the start of administration, and all subjects showed increases in the urinary excretion ratios of 6β-OHF from day 2 to day 14.

In Vitro Study. *Primary Human Hepatocytes.* The effects of MKC-963 (0.25 μM) on the expression of CYP3A4 mRNA and on the activity for testosterone 6β-hydroxylation were investigated using human primary hepatocytes. The effect of rifampicin (10 μM) was also investigated as a positive control. As shown in Fig. 5A, MKC-963 increased the expression level of CYP3A4 mRNA by 6-fold, comparable to the effect of rifampicin (increase of approximately 11-fold). Testosterone 6β-hydroxylation activity was also increased by 9-fold in the presence of MKC-963, which is also comparable to the effect of rifampicin (14-fold increase, Fig. 5B).

Identification of P450 Enzyme(s) Involved in the Metabolism of MKC-963. cDNA-expressed human P450s were used to estimate the