意 思 表 示 書

××××病院倫理委員会 殿

私は説明者によって、別紙の点について、説明を受けました。

提供の内容は、手術終了後の切除片の一部を研究用として厚生労働省細胞バンク（JCRB、責任者：○○○）へ提供すること、細胞バンクは医学生物学研究用に培養されたヒト細胞を一般研究者に提供すること、という趣旨を理解いたしました。尚、提供の業務はヒューマンサイエンス財団研究資源バンク（HSRRB、責任者：○○○○）が担当をする。

そこで手術後に切除片の一部を医学生物学研究のために提供することを、

□拒否いたします。　□承諾いたします。

（チェックボックスに印をしてください。）

（表）

年　月　日

提供者　氏 名 _____ 印

生年月日 ____年__月__日（男・女）

住 所 _____

説明者　氏 名 _____ 印

所 属 _____

住 所 _____

電 話 _____

年　月　日

ヒト子宮頚部組織由来の培養上皮細胞利用誓約書

厚生労働省・細胞バンク（JCRB、HSRRB）は研究に理解のある患者の皆様から人体組織の一部を提供いただき、研究用に提供する事業を行っております。公共性の高いヒト試料バンクとしてこれらのヒト試料を入手・保存・分譲するにあたって倫理的な側面を十分に配慮し、非倫理的な取り扱いを最大限に防止する義務があると考えます。そのため、分譲申請者の研究機関に倫理委員会が設置されており、申請研究計画が承認を得ているか、もしくは承認手続きに入っていることを条件として上記ヒト試料の配布をするよう配布規定を定めております。

これに基づいて、上記ヒト試料の分譲を申請されるにあたって、本誓約書に必要事項を正確に記載し、申請者ご署名捺印してください。虚偽の記載に対しては断固たる処置をとることもありますので、十分注意し正しく記載してください。

（案）

厚生労働省細胞バンク
国立医薬品食品衛生研究所、細胞バンク（JCRB）殿
ヒューマンサイエンス振興財団、研究資源バンク（HSRRB）殿

分譲依頼細胞：ヒト子宮頚部組織由来の培養上皮細胞
研究目的：＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿

□ 申請者の所属する研究機関には倫理委員会が存在している。
倫理委員会代表者氏名：　　年　　月　　日　現在（申請日）

□ 申請書に記載した研究は倫理委員会で承認を受けた。
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□ 申請書に記載した研究は倫理委員会で承認を受ける予定である。
承認予定日：　　年　　月　　日

上記の申請内容が虚偽でないことを誓約します。

申請者氏名：＿＿＿＿＿＿＿＿　印
申請者所属機関名：＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿
所属機関住所：＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿

臨床薬理 Jpn J Clin Pharmacol Ther 35(2) Mar 2004

〈抄録〉第24回 日本臨床薬理学会年会 2003年12月11～12日 横浜
シンポジウム13：ヘルスサービスシステムの中のファーマコジェネティクス

5．ファーマコジェネティクスとインセンティブ

津 谷 喜一郎*

【Scope is wide】

ファーマコジェネティクスは，医薬品の研究・開発から合理的使用まで，幅広い領域に関係する．2002年にThe Council for International Organizations of Medical Sciences（CIOMS，国際医科学協議会）が設立したWorking Group for Pharmacogenetics and Pharmacoeconomicsが討議するtopicsは，開発から，教育・倫理・経済まで幅広いものである[1]．

この領域は，医療技術政策からみると，推進・評価・規制と，基礎研究・臨床研究・採用・普及標準化の2次元に整理できる[2]．また，市販前を「創薬」，市販後を「育薬」と呼ぶ流儀にならえば，「ゲノム創薬」から「ゲノム育薬」までと称することができる．

【Possible questions】

各playerにとってのインセンティブを意識しながら，いくつかのquestionの形で考えてみよう．

Q1：Pharmacogenetic（PGx）testを用いると臨床試験の症例数は減るのだろうか？

このquestionは，安全性ではどうか？ どのphaseについてか？ 市販後の臨床試験・臨床研究ではどうか？ 非臨床研究でゲノム情報を用いて開発された薬ではどうか？ などに分けられる．

Q2：effect sizeはどうなるか？

1990年代後半からのエビデンスに基づく医療（evidence-base medicine: EBM）のなかで，effect sizeはnumber needed to treat（NNT）を用いて表すことも多い．PGx testを用いたうえで治療すると，NNTはどの程度小さくなるのであろうか？ ここでは，responderを見出すためのnumber needed to screen（NNS）と，薬物治療の反応率との双方からなる．いずれも確率的なものでありバラツキを考慮する必要がある．

Q3：結果にバイアスはないか？

PGx testを用いて，どの程度，正確（accurate）な結果が得られるのであろうか？これには研究デザイン（study design）に関係する[3]，いくつかのタイプがある．(1) PGx testを行う群vs行わない群．PGx test＋，すなわちresponderにのみ投薬．(2) 同じく2群で，PGx testを行った群で，extensive metabolizer（EM），poor metabolizer（PM）に応じて最適の投与量，行わない群では従来の投与量を用いる．(3) 安全性をメインにして，同じく2群で，PGx testを行った群で，副作用が予想されるresponderには非投与．(4) 長期の効果を見ようとする場合，まず全員にPGx testを行い，responderを2群にわけ，投薬群と非投薬群で，数年後のアウトカムを比較する．(5) PGx testそのものの比較．PGx test AとPGx test Bの2群でそれぞれのresponderに投薬して比較する．

これらの研究デザインは，tree structureに書くと分かりやすく，各コスト情報を収集し臨床アウトカムを含めて解析すればpharmacoeconomics studyができる．ここで2群を構成する時，ランダム割付（random allocation）を行えばエビデンスのgradeは高くなるが，その方法の受容性（acceptability）は，有効性・安全性・コストに依存する．

2002年10月の大阪での第23回日本臨床薬理学会年会での発表を研究デザインで分類するとTableになる．前向き（prospective study）がほとんどないことが読み取れる．

研究デザインとは別に，臨床研究の質も問題になる．Quality control（QC）/quality assurance（QA）がここでも重要である．さらに，研究結果の如何によるpublication biasは，他の領域よりも重大な問題となりうる．臨床試験の登録制度が望ましい．

Q4：誰がPGx testのコストを払うべきか？

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Table　日本臨床薬理学会年会でのPGx study

2002年	study数
1. Case Report	0
2. PK Study	9
3. PK-PD Study	3
4. Cross-sectional Clinical Analysis	4
5. Cohort Study	0
6. Retrospective Clinical Study	0
7. Prospective Clinical trial	0
8. Pharmacoeconomics Assessment	0
9. Development of PGx diagnosis/test	0

税金か，公的保険か，個人か，また私的保険の場合もあり得，ヘルスサービスシステムの中で，混合診療の是非を含めた論議が必要である．PGx testは発病した患者のみならず，予防にも用いられる．日本の保険制度は原則的に予防給付はしないが，この議論も必要となろう．

Q5：何に対するコストか？

この領域では，便益（benefit）とともにコストも多様なものとなる[4]．直接コストは薬コストやPGx testコストだけではない．そこで得られた情報を管理するコストも必要である．一方ではPGx testによってコストは低減される．不必要や不適切な投薬が減る．副作用に伴うコストが減る．従来の“try and see”スタイルが変わり受診の回数が減る．また間接コストとしての労働生産性も考慮すべきである．

【Decision making】

エビデンスのgradeは，retrospective designで得られた情報をそのまま添付文書（labeling）に入れるか，あるいはprospective studyでconfirmすべきかという，行政当局の意思決定にも関わる．

より広く医療における意思決定には，古典的には，効果（effectiveness），効率（efficiency），公平（equity）の“3E”が知られているが，開発の視点も含めると「ダイナミズム」（dynamism）も必要になる．

高価な高度医療技術に伴うコストをコントロールするには2通りの方法がある．一つは強制的（coercitive）方法である．これには需要サイドの医師などに対するnegative listやpositive listによる使用制限，予算制，などが含まれる．また治療ガイドラインの作成も適正使用に加えてコスト抑制に貢献するかもしれない．供給サイドの医薬品企業に対しては開発の抑制である．

もう一つは，刺激的（incitative）方法である．コスト低減にインセンティブを与えるもので，医師などに対しては，限られた患者のみに処方したり，低価格の医薬品を用いることに対するインセンティブなどがある．

ゲノム創薬・育薬では，PGx testを用いた処方により，一般に対象となる患者の数は減ることが予想される．ここでは公的資金によるインセンティブが必要になると思われる．2002年9月−10月に，ヒューマンサイエンス振興財団（HS財団）の賛助会員企業91社に対し，全36問のアンケート調査が行われた．その中で「Non-responderへの無用な投薬が激減する場合，承認・上市にあたり，行政に求めるもの（インセンティブ）はどのようなものか」，「Non-responderの存在が明らかになったとき，non-responderを対象とした薬物の開発にあたり，行政に求めるものは何でしょうか」などが問われた[1]．多様な要素をもつこの領域に対処するレギュラトリーサイエンスの進展[5]が望まれる．

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ORIGINAL ARTICLE

CYP3A5 genotype did not impact on nifedipine disposition in healthy volunteers

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ABSTRACT

CYP3A5 expression is regulated by single-nucleotide polymorphisms (SNPs). The CYP3A5 genotype might contribute to a marked interindividual variation in CYP3A-mediated metabolism of drugs. Nifedipine is a typical substrate of CYP3A4 and CYP3A5 *in vitro*. The aim of this study was to elucidate the influence of the CYP3A5 genotype on nifedipine disposition in healthy subjects. A single capsule containing 10 mg of nifedipine was administered to 16 healthy male Japanese subjects (eight subjects: *CYP3A5*1/*3*; eight subjects: *CYP3A5*3/*3*). Blood samples were collected to analyze the pharmacokinetics of serum nifedipine and nitropyridine metabolite (M-I). The area under the plasma concentration–time curve (AUC), the peak plasma concentration (C_{max}) and the terminal half-life ($t_{1/2}$) of nifedipine, and the ratio of the nifedipine AUC to M-I AUC showed large intragroup variations, but no significant differences between the two genotypes. Based on the present findings, the functional relevance of CYP3A5 polymorphism should be re-evaluated in clinical trials.
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Keywords: CYP3A5; nifedipine; polymorphism; clinical; pharmacokinetics

INTRODUCTION

Cytochrome *P*450 3A (CYP3A) is abundantly expressed in human liver and small intestine, [1,2] and contributes to the metabolism of 50% of prescribed drugs. The activities of CYP3A in the general population show interindividual variations in CYP3A-mediated metabolism of drugs.[2] Recently, single-nucleotide polymorphisms (SNPs) were identified in intron 3 (*A–G*: *CYP3A5*3*) and exon 7 (*G–A*: *CYP3A5*6*) of the *CYP3A5* gene.[3] In addition, *CYP3A5*5* and *CYP3A5*7* were reported as a defective allele of CYP3A5, which gave a substantial impact on CYP3A5 expression.[4,5] These SNPs cause a frame-shift mutation or alternative splicing and protein truncation, and result in the absence of CYP3A5, suggesting that only people with at least one *CYP3A5*1* allele express large amounts of CYP3A5 protein. Therefore, these findings suggest that polymorphic CYP3A5 expression might be one factor contributing to the marked interindividual variation observed in CYP3A-mediated metabolism of drugs.

We previously reported the frequencies of CYP3A5-related SNPs in 200 healthy Japanese subjects.[6] As a result, the allele frequency of *CYP3A5*3* was approximately 70%, but *CYP3A5*6* was not detected in the Japanese population. Accordingly, these findings suggested that about 40% of Japanese express relatively high levels of metabolically active CYP3A5 protein.

Table 1 Enzyme kinetic analyses of the conversion of M-I using baculovirus-expressed human = P450s

	Km (μM)	*V_{max} (pmol/min/pmol P450)*	*V_{max}/Km (μl/min/pmol P450)*
CYP3A4	3.0	3.1	1.0
CYP3A5	6.5	3.3	0.5

To obtain clinical evidence of CYP3A5 polymorphism, we focused on nifedipine, a typical substrate of CYP3A4 and CYP3A5,[7–9] because a large individual difference was observed in nifedipine disposition,[10] which was thought to be regulated by a genetic background rather than environment.[11] Consistently, using baculovirus-expressed human CYP3A5 and CYP3A4, we confirmed their contribution to the metabolism of nifedipine (Table 1). These findings suggested that CYP3A5 contributes to the metabolism of nifedipine with kinetics similar to CYP3A4, implying that the interindividual differences in nifedipine disposition might be explained in part by CYP3A5 polymorphism. Thus, in the present study, we evaluated the influence of the CYP3A5 genotype on nifedipine disposition in healthy subjects to examine the polymorphic activities of CYP3A5 *in vivo*.

RESULTS

The subjects were genotyped and divided into two groups, *CYP3A5*1/*3* and *CYP3A5*3/*3* (Table 2). No subject had the other CYP3A5 alleles, *CYP3A5*5*, *CYP3A5*6* and *CYP3A5*7*. First, the plasma concentration profiles of nifedipine and M-I were compared between **1/*3* and **3/*3* groups. Unexpectedly, the time profiles of both plasma nifedipine and M-I were not significantly different between the two genotypes (Figure 1). Moreover, plasma nifedipine and M-I showed a large intragroup variation. Next, the typical pharmacokinetic parameters of nifedipine, such as the area under the plasma concentration–time curve from 0 to 12 h after administration (AUC_{0-12h}), the peak plasma concentration (C_{max}), terminal half-life ($t_{1/2}$) and clearance (CL/F) were calculated (Table 3). The AUC_{0-12h} values showed large intragroup variations without significant differences between the two genotypes (218.8±80.9 ng h/ml in *CYP3A5*1/*3* subjects, 178.7±92.8 ng h/ml in *CYP3A5*3/*3* subjects; mean±SD). Furthermore, the ratio of the nifedipine AUC_{0-12h} to the M-I AUC(4.77 in *CYP3A5*1/*3* subjects, 3.62 in *CYP3A5*3/*3* subjects; mean) also showed large intragroup variations with no significant differences between the two genotypes (Figure 2). The differences in the C_{max}, $t_{1/2}$ and CL/F of nifedipine between the two groups were not significant.

Finally, we measured systolic and diastolic blood pressure and pulse rate to estimate the significance of CYP3A5 polymorphism in the pharmacodynamics of nifedipine. Consistent with the pharmacokinetics, there were no significant differences in the pharmacodynamics between the two genotypes (Figure 3 and Table 4).

Table 2 Characteristics of the subjects in the study

Subject no.	*Age (years)*	*Height (cm)*	*Weight (kg)*	*BMI (%)*
*CYP3A5*1/*3 (n=8)*				
1	23	174.2	65.0	97.3
2	21	171.2	65.4	102.1
3	22	169.0	57.5	92.6
4	23	177.7	58.9	84.2
9	22	185.3	64.9	84.5
10	21	180.1	72.3	100.3
11	23	177.7	62.6	89.5
12	23	163.3	49.8	87.4
Mean	22.3	174.8	62.1	92.2
SD	0.9	6.9	6.7	7.0
*CYP3A5*3/*3 (n=8)*				
5	22	170.8	56.1	88.0
6	32	182.4	70.9	95.6
7	21	166.7	60.9	101.4
8	20	179.5	66.1	92.4
13	22	179.4	61.2	85.6
14	21	175.0	62.9	93.2
15	22	173.5	57.7	87.2
16	25	171.8	71.6	110.8
Mean	23.1	174.9	63.4	94.3
SD	3.9	5.3	5.7	8.4

DISCUSSION

In the present study, we examined the effects of CYP3A5 genotype on nifedipine pharmacokinetics, and demonstrated that an interindividual variation of plasma nifedipine concentration was not over-ridden by the CYP3A5 genotype. The interindividual variation was not beyond our conception and was almost similar to that described in the previous report following the administration of a 10-mg capsule.[12] The present finding suggests that CYP3A5 polymorphism is unlikely to be responsible for interindividual variation in the plasma level of nifedipine because the remaining CYP3A5 alleles, *CYP3A5*5*, *CYP3A5*6* and *CYP3A5*7*, were not found in the present subjects.

With respect to nifedipine metabolism, nifedipine disposition is slightly affected by the expression of intestinal CYP3As because grapefruit juice influences nifedipine disposition significantly but to a lesser extent than felodipine or nisoldipine,[10,13,14] suggesting that nifedipine is mainly metabolized not in the intestine but in the liver. In addition, it is hypothesized that P-glycoprotein (P-gp) is responsible for the large interindividual difference in

CYP3A-mediated drug disposition, since P-gp exists in the similar tissue to CYP3A4. However, this hypothesis is not the case with nifedipine disposition because nifedipine is not a substrate of P-gp.[15,16] Therefore, nifedipine pharmacokinetics must be crucially determined by the total liver CYP3A activities.

It was reported that nifedipine, as well as midazolam, were not only metabolized by CYP3A4 but also by CYP3A5 *in vitro*.[7–9,17] Prior to clinical study, we conducted kinetic study on the formation of M-I using recombinant microsomes (CYP3A4 and CYP3A5) because previous reports provided the oxidation activity at a single high concentration of nifedipine. We confirmed the contribution of CYP3A5 toward the metabolism of nifedipine at relatively low concentrations.

We, however, observed a discrepancy between the *in vitro* and *in vivo* contribution of CYP3A5 to nifedipine metabolism in the present study. Interestingly, a similar result has been obtained in the case of midazolam, a typical CYP3A5 substrate. Namely, midazolam pharmacokinetics was also hardly influenced *in vivo* by the genotype of CYP3A5,[18] although midazolam is metabolized *in vitro* by CYP3A5 rather than CYP3A4.[3,17,19] Several possibilities can be proposed to explain these discrepancies between the *in vitro* and *in vivo* data.

It was previously reported that total CYP3A activity showed an interindividual variation,[2] and the ratio of CYP3A4 to CYP3A5 might also vary interindividually in the liver.[20] Recently, Westlind-Johnsson *et al*[21] reported that CYP3A5 did not contribute to total CYP3A activity using

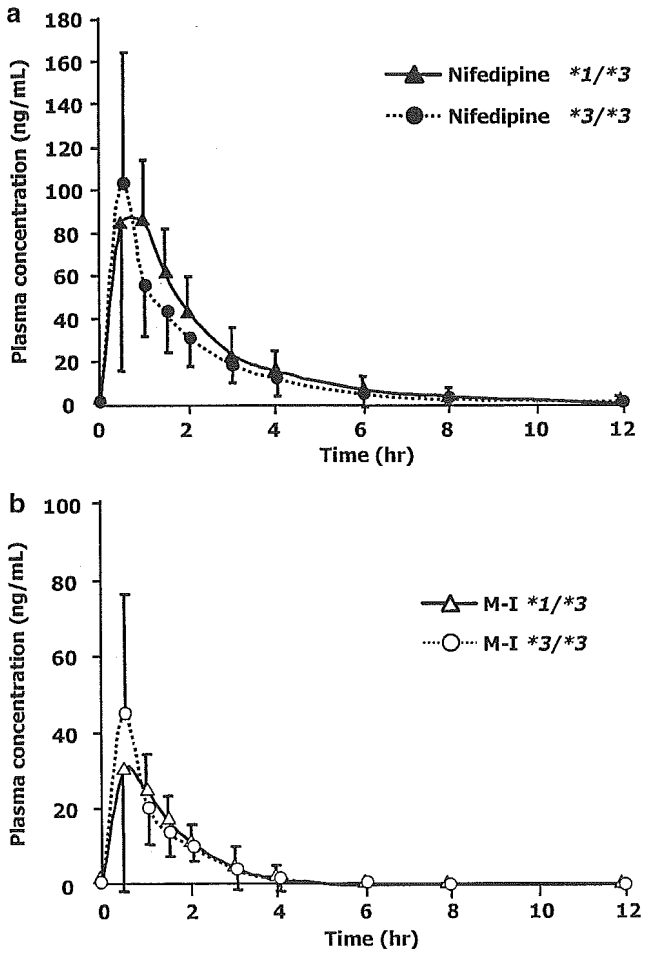

Figure 1 Plasma concentration–time curves of nifedipine (a) and M-I (b) in the *CYP3A5*1/*3* and *CYP3A5*3/*3* subjects. Values represent the means with SD ($n=8$).

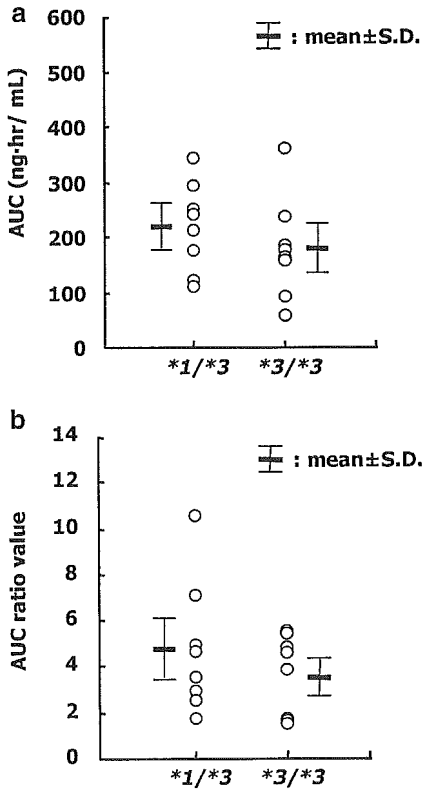

Figure 2 Individual (dot) and mean (line) values with SD of the $=AUC_{0-12h}$ of nifedipine (a) and the ratio of the nifedipine $=AUC_{0-12h}$ to the M-I $=AUC_{0-12h}$ (b) in the two genotypes.

Table 3 Pharmacokinetic parameters of nifedipine after oral administration to subject with *CYP3A5*1/*3* and *CYP3A5*3/*3* genotypes (mean ± SD)

Genotype	C_{max} *(ng/ml)*	$t_{1/2}$ *(h)*	AUC_{0-12} *(ng h/ml)*	*CL/F (ml/min)*
*CYP3A5*1/*3* ($n=8$)	116 ± 43.3	1.77 ± 0.66	219 ± 80.9	877 ± 375
*CYP3A5*3/*3* ($n=8$)	111 ± 53.8	1.81 ± 1.09	178 ± 92.8	1246 ± 837

liver samples. That is, they indicated that Kuehl *et al*[3] might have overestimated the level of CYP3A5 protein, possibly due to problems with the method of quantification. On the other hand, Williams *et al*[19] reported that the Km value of nifedipine was much lower for CYP3A4 than CYP3A5 under the detailed conditions with cytochrome *b*5. Cytochrome *b*5 was suggested to be an essential component in CYP3A4-catalyzed nifedipine oxidation in human liver microsomes.[22] Although these data seem to support the present findings *in vivo*, the same was not consistent in the case of midazolam. Based on all these observations, we speculated that the amount of CYP3A5 protein in the liver is much lower than CYP3A4, although we should consider the limitations of our study design. This speculation might be demonstrated by using CYP3A5-specific substrates, although no drugs metabolized specifically by CYP3A5 have been reported yet. With respect to mRNA expression, it was reported that CYP3A genes exhibit a degree of tissue-specific expression and CYP3A5 is predominantly expressed in the adrenal grand, prostate and kidney.[23] Therefore, CYP3A5 polymorphism might have a physiological and pharmacological effect, which is related to the extrahepatic tissues. This possibility remains to be verified in further studies.

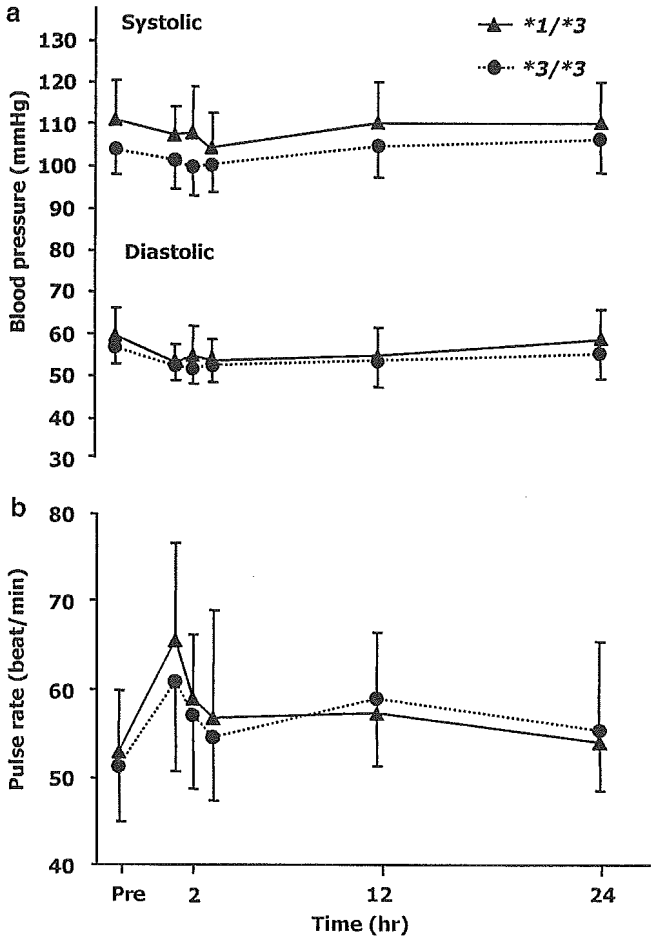

Figure 3 Blood pressure (a) and pulse rate (b) after nifedipine administration. Values represent the means with SD ($n=8$).

Interindividual differences in nifedipine pharmacokinetics remain to be elucidated genetically. Here, we propose that polymorphic regulation of CYP3A4 gene transcription, including the polymorphisms of the promoter activities and transcriptional factors, may have to be taken into account to explain the variation of nifedipine pharmacokinetics. However, a polymorphism, which plays a significant role in the activity of CYP3A4, has not yet been identified in the 5′-regulatory region of the CYP3A4 gene.[24] Therefore, we have focused on human PXR (hPXR) as a factor effecting CYP3A expression and identified splicing variants of hPXR as a possible factor in interindividual variation caused in CYP3A activity.[25] Interestingly, we have found that mRNA expression of wild-type hPXR is well correlated with mRNA expression of CYP3A4 in liver sample (unpublished data), which is consistent with the recent report.[21]

In summary, we revealed that nifedipine disposition *in vivo* is not affected by the *CYP3A5*3* allele. Owing to a discrepancy between the *in vitro* and *in vivo* contribution of CYP3A5, the functional relevance of CYP3A5 in humans should be re-evaluated by clinical studies for each drug.

MATERIALS AND METHODS

In vitro Screening for the Contributions of CYP3A4 and CYP3A5

Nifedipine and phenytoin were purchased from Wako Pure Chemicals Co. (Osaka, Japan) and oxidized nifedipine (ULTRAFINE) was obtained from Funakoshi (Tokyo, Japan). Microsomes from baculovirus-infected insect cells expressing human CYP3A4 and CYP3A5 with NADPH cytochrome *P*450 reductase (GENTEST) were obtained from Daiichi Pure Chemicals Co. (Tokyo, Japan). NADPH was purchased from Oriental Yeast Co. (Tokyo, Japan). Other reagents and

Table 4 Systolic and diastolic blood pressures change rate (%) after administration of nifedipine

	1 h after administration of nifedipine		*2 h after administration of nifedipine*	
	Systolic	*Diastolic*	*Systolic*	*Diastolic*
*CYP3A5*1/*3* ($n=8$)	3.0±7.2	9.3±10.4	2.7±5.7	6.9±11.3
*CYP3A5*3/*3* ($n=8$)	2.3±2.8	6.9±5.2	3.8±3.5	8.5±3.7

Values were the mean±SD of blood pressure changes (%). Each blood pressure change (%) was calculated by the formula ((measured–baseline)/baseline × 100).

organic solvents were obtained from Nakarai Tesq Chemical Industries (Kyoto, Japan).

All incubation, extraction and other handling of samples were carried out in amber vials. A mixture (0.50 ml) containing 10 pmol of *P*450 protein from baculovirus-expressed human CYP3A4 and CYP3A5 and 1 mM NADPH in 0.1 M potassium phosphate buffer (pH 7.4) was incubated with nifedipine (final concentration: 1, 2, 5, 10 and 40 μM) at 37°C after 2 min of preincubation without NADPH. The incubation was continued with gentle shaking at 37°C for 30 min and the reaction was stopped with ethyl acetate (3 ml). A measure of 50 μl of methanol and 12.5 μl of 20 mM phenytoin (internal standard) were added to each sample. The organic layers were transferred to other vials after centrifugation at 1500*g* for 10 min and evaporated to dryness. The residues were dissolved in 200 μl of the mobile phase. A measure of 50 μl were analyzed by high-performance liquid chromatography (HPLC) with a reverse-phase column (Mightysil RP-18 GP250, $4.6 \times 250\,mm^2$, Kanto Chemical Industries Ltd, Kyoto, Japan). The column temperature was set at 40°C. The mobile phase was composed of 40% acetonitrile (pH 3.0 with perchloric acid) and was delivered at a constant flow rate of 1.2 ml/min. Nifedipine, M-I and phenytoin were detected by a UV detector (Nanospace, SHISEIDO, Tokyo, Japan) with a wavelength at 254 nm. In determining kinetic parameters, nifedipine concentration ranged from all points.

Subjects

Institutional Review Board approval of the study protocol was obtained. In all, 16 healthy male Japanese volunteers participated in this study (Table 2). The subjects gave written informed consent to participate.

A total of 33 healthy volunteers was screened by the genotyping test to find the eight *CYP3A5*1/*3* subjects and eight *CYP3A5*3/*3* subjects. The genotyping test of CYP3A5 was conducted according to the previous study.[6,26] All subjects were healthy as assessed by medical history, physical examination, hematologic tests, blood chemistry and urinalysis, and the results of a positive test for hepatitis B and C, human immunodeficiency virus and syphilis.

Eight *CYP3A5*1/*3* and eight *CYP3A5*3/*3* subjects who showed normal results on routine laboratory tests described above and the negative results of virus tests were selected.

Genotyping Test

Genomic DNA was isolated from peripheral leukocytes using QIAGEN blood kit. The genotypes of each individual at the *CYP3A5*3* and *CYP3A5*6* alleles were determined using PCR-restriction fragment length polymorphism analysis according to the previous report.[6] For the analysis of the *CYP3A5*3* allele, the forward (CYP3A5 6956Fm; 5′-CTT TAA AGA GCT CTT TTG TCT CTC A-3′) and reverse (CYP3A5 7155R; 5′-CCA GGA AGC CAG ACT TTG AT-3′) primers were used (GenBank accession no. AC005020). For the analysis of the *CYP3A5*6* allele, the forward (CYP3A5 14505F; 5′-GTG GGT TTC TTG CTG CAT GT-3′) and reverse (CYP3A5 14741R; 5′-GCC CAC ATA CTT ATT GAG AG-3′) primers were also created based on the published sequence. These PCR reactions were carried out in 25 μl of solution consisting of 2.5 μl of 10 × PCR buffer, 0.2 mM of each dNTP, 0.4 μM of each primer, 90 ng of genomic DNA as a template and 1 U of AmpliTaq Gold (Perkin-Elmer, Branchburg, NJ, USA). After initial denaturation at 95°C for 10 min, the amplification for the *CYP3A5*3* or **6* alleles was performed using 37 cycles of 94°C for 30 s, 56°C (**3*) or 58°C (**6*) for 30 s and 72°C for 30 s, followed by 72°C for 5 min for final extension. After PCR amplification, 5 μl of each PCR product was digested for a minimum of 2 h at 37°C with 5 U of *Dde*I before electrophoresis using a 3% agarose gel.

*CYP3A5*5* and *CYP3A5*7* alleles were also determined according to the method of van Schaik *et al*[26] with some modifications.

Study Design

A single oral dose of 10 mg of nifedipine (Adalat® Bayer, Germany) with 200 ml of water was administered to the subjects at 01000 after overnight fasting. Blood samples (7 ml each) were collected before administration and at 0.5, 1, 1.5, 2, 3, 4, 6, 8 and 12 h after administration. During this study, adverse effects were assessed on an ongoing basis as subjects offered information. Blood pressure and pulse rate were measured before administration and at 1, 2, 3, 12 and 24 h after administration. A 12-lead electrocardiogram was recorded before administration and at 2.5 and 24 h after administration. Hematologic tests, blood chemistry and urinalysis were performed before administration and at 24 h after administration.

Grapefruit juice, St John's Wort, alcohol, caffeine-containing beverages, tobacco and exercises were not allowed during the study.

Assays

Blood samples were collected in heparinized tubes and adequately protected from light and centrifuged to obtain serum. The samples were stored frozen at –20°C until analyzed. Plasma concentrations of nifedipine and M-I were measured by HPLC as described previously with minor modifications.[27–29] Briefly, nifedipine and M-I were extracted with dichloromethane/pentane (3 : 7, v/v) and separation of the compounds was achieved using an Inertsil ODS-3 column (3 mM particles, $4.0 \times 100\,mm^2$, GL Sciences Inc., Tokyo, Japan). The compounds were detected at a wavelength of 230 nm using a UV detector (SPD-10Avp, SHIMADZU, Tokyo, Japan).

Pharmacokinetic Parameters

The peak plasma concentration (C_{max}) and the time to reach the C_{max} (T_{max}) were obtained as measured values. The apparent first-order elimination rate constant (K) of nifedipine was determined by linear regression analysis of the slope of the terminal phase using the last three or four points on the log plasma drug concentration–time curve. The terminal elimination half-life ($t_{1/2}$) was calculated from the relation $t_{1/2} = 0.693/K$. The area under the plasma concentration–time curve from 0 to 12 h after administra-

tion ($AUC_{0-12\,h}$) was calculated by the linear trapezoidal method. The area under the drug concentration–time curve from time to infinity (AUC_{∞}) was determined by the linear trapezoidal method with extrapolation to infinity. Clearance (CL/F) was calculated as dose/(AUC_{∞}). The plasma nifedipine/M-I ratio was calculated from the $AUC_{0-12\,h}$ value of nifedipine and M-I.

Data Analysis

The pharmacokinetic parameters and the plasma nifedipine/M-I ratios were first analyzed by one-way ANOVA. If the overall F ratio was significant, further comparison of the means was performed with the Student's *t*-test. Statistical analysis of the pharmacodynamic variables of systolic and diastolic blood pressure, and pulse rate made under resting conditions, were performed using ANOVA with repeated measurements. Time was used as a repeated variable. A probability level of $P<0.05$ was considered to be statistically significant.

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DUALITY OF INTEREST

None declared

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Association of plasma PAF acetylhydrolase gene polymorphism with IMT of carotid arteries in Japanese type 2 diabetic patients

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Abstract

The aim of this study was to investigate association of a missense mutation in plasma PAF acetylhydrolase (G994T) with intima media thickness (IMT) of the carotid arteries. One hundred and forty Japanese type 2 diabetic patients aged from 40 to 79 years without severe nephropathy were enrolled in this study. The genotype of the patients was determined by allele specific PCR. IMT of the carotid arteries of the subjects was recorded by B-mode ultrasound imaging. The patients were divided into two groups by genotyping, one carrying two wild alleles (wild group), and another carrying one or two mutant alleles (mutant group). Each group was further divided into two subgroups according to age; one subgroup consisted of 40s or 50s, and another consisted of 60s or 70s. The prevalence of the G994T mutation in the subjects was 28.6% (24.3% heterozygote, and 4.3% homozygote). IMT of the elderly patients of the mutant group was significantly greater (0.98 ± 0.22 mm, $n = 26$) than of the elderly patients of the wild group (0.87 ± 0.20 mm, $n = 50$, $P = 0.0292$). There was no significant difference in clinical characteristics between the two subgroups. The results of this study indicate that the missense mutation in plasma PAF acetylhydrolase is associated with development of atherosclerosis in the elderly.

Keywords: Plasma PAF acetylhydrolase; G994T polymorphism; Intima media thickness; Type2 diabetes; Elderly patient

1. Introduction

Platelet-activating factor (PAF) is a phospholipid with wide variety of activities and is implicated as a pathologic mediator. PAF is inactivated by plasma PAF acetylhydrolase, which removes the sn-2 acetyl group. Human plasma PAF acet-

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ylhydrolase is associated with low and high density lipoproteins (LDL and HDL). Stafforini et al. reported that phospholipids with PAF-like activity were formed during the oxidation of LDL and these phospholipids stimulated smooth muscle cells and neutrophils [1]. The activity of the phospholipids disappeared by treatment with PAF antagonist or plasma PAF acetylhydrolase [1]. Thus, the phospholipids generated during oxidative modification of LDL could participate in atherogenesis and the activity could be abolished by plasma PAF acetylhydrolase.

Deficiency of plasma PAF acetylhydrolase activity in Japanese population was described first by Miwa et al. [2]. Stafforini et al. identified a missense mutation in the gene of plasma PAF acetylhydrolase (G994T, Val279Phe) as the cause of deficiency of enzyme activity [3]. They showed that this mutation as a heterozygotous trait is 27% in the Japanese population [3]. It has been reported that the mutation is a risk factor for myocardial infarction in men, stroke, atherosclerotic occlusive disease and abdominal aortic aneurysm in Japanese population [4–7]. Atherosclerosis is responsible for the etiology of these diseases. Gene transfer of plasma PAF-acetylhydrolase inhibited injury-induced neointima formation and spontaneous atherosclerosis in apolipoprotein E-deficient, atherosclerosis-susceptible mice [8].

Therefore, we investigated the association of the mutation of plasma PAF acetylhydrolase with early stage of atherosclerosis using a non-invasive method. We used ultrasound B-mode imaging of carotid arteries for evaluation intima media thickness (IMT) correlated with the progression of systemic atherosclerosis [9]. We have chosen Japanese patients with type 2 diabetes as subjects, in whom atherosclerosis progresses rapidly.

2. Subjects and methods

2.1. Study subjects

The study subjects consisted of consecutive 140 Japanese patients with type 2 diabetes aged from 40 to 79 years who had attended the NTT West Osaka Hospital. These subjects were free from major cardiovascular events and severe nephropathy. The study was approved by the institutional review committee. Informed consent for participation was obtained from each subject. Since homo mutant group was very small, the subjects were divided into two groups by genotyping of plasma PAF acetylhydrolase. One group consisted of patients carrying no mutant allele (wild group) and another consisted of patients carrying one or two mutant alleles (mutant group). Each group was further divided to two subgroups according to age. One subgroup consisted of 40s or 50s (middle aged) and another consisted of 60s or 70s (elderly). Kawamori et al. reported that IMT values increased rapidly in the type 2 diabetic patients who were over 60 years [10], thus we divided the groups to the subgroups by the age. The characteristics of the subjects are presented in Table 1.

2.2. Genotyping of plasma PAF acetylhydrolase

Genomic DNA was extracted and purified from peripheral blood with QIAamp Blood Kit (QIAGEN, Germany). The genotype of plasma PAF acetylhydrolase was determined by an allele-specific PCR as previously described with two sets of the sense A and antisense primer B or C as follows [3]. The fragment using a set of sense A and antisense primer D was produced for restriction enzyme assay [3].

Sense primer A, 5′-CTATAAATTTATATCATGCTT-3′;
Antisense primer B, 5′-TCACTAAGAGTCTGAATAAC-3′;
Antisense primer C, 5′-TCACTAAGAGTCTGAATAAA-3′;
Antisense primer D, 5′-TTTACTATTCTCTTGCTTTAC-3′.

Reactions were performed in a total volume of 50 μl containing 90 ng genomic DNA, 50 pmol of each primer, 0.2 mM each of dATP, dGTP, dCTP, and dTTT, 0.5U Ampli *Taq* Gold DNA polymerase (Perkin–Elmer, New Jersey, USA), 50 mM KCl, 1.5 mM MgCl2, and 10 mM Tris–HCl (pH 8.3). The thermocycling procedure consisted of initial denaturation at 94 °C for 5 min; five cycles

Table 1
Clinical characteristics in plasma PAF-AH genotype groups

Characteristics	Genotype [a] and P value					
	Wild (middle aged[b])	Mutant (middle aged[b])	P value[d]	Wild (elderly[c])	Mutant (elderly[c])	P value[e]
N	50	14		50	26	
Sex (M/F)	30/20	8/6	0.847[f]	34/16	14/12	0.225[f]
Age (years)	52.4±5.0	52.6±3.8	0.866[g]	67.3±4.9	67.6±4.1	0.780[g]
Diabetes duartion (years)	8.5±7.8	5.5±4.4	0.160[g]	12.3±9.9	13.5±8.1	0.600[g]
Body mass index (kg/m^2)	23.6±3.2	24.6±3.2	0.318[g]	23.5±3.3	24.5±3.7	0.240[g]
Hemoglobin A_{1C} (%)	8.6±2.7	8.5±2.7	0.874[g]	7.7±2.1	7.7±1.4	0.940[g]
Fasting glucose (mg/dl)	198.8±85.2	212.7±80.7	0.587[g]	176.2±87.1	201.1±113.5	0.290[g]
Total cholesterol (mg/dl)	205.4±38.1	207.6±28.1	0.847[g]	196.6±30.2	203.0±42.4	0.455[g]
HDL cholesterol (mg/dl)	54.9±15.6	55.2±15.7	0.947[g]	54.3±15.6	55.5±13.9	0.739[g]
Triglyceride (mg/dl)	145.2±96.7	252.57±379.75	0.072[g]	146.94±90.97	146.96±92.51	0.999[g]
Creatine (mg/dl)	0.898±0.878	0.693±0.138	0.390[g]	0.906±0.589	0.821±0.217	0.496[g]
Systolic blood pressure (mmHg)	137.8±18.2	135.4±22.1	0.670[g]	143.6±20.2	144.7±22.2	0.820[g]
Diastolic blood pressure (mmHg)	81.9±10.1	85.5±14.6	0.298[g]	79.2±10.5	79.1±12.7	0.975[g]

Data are mean±S.D. $P < 0.05$ was regarded significant.
[a] Genotype, Wild: patients carrying no mutant allele, Mutant: patients carrying one or two mutant alleles.
[b] middle aged: 40–59 years old.
[c] elderly: 60–79 years old.
[d] P value compared individuals (middle aged) carrying wild genotype with individuals (middle aged) carrying mutant genotype.
[e] P value compared individuals (elderly) carrying wild genotype with individuals (elderly) carrying mutant genotype.
[f] χ^2-test.
[g] Unpaired t-test.

of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min; 30 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 7 min. PCR products were analyzed by 8% acrylamide gel electrophoresis and visualized by ethidium bromide staining. The expected sizes of the PCR products were 108 base pairs (bp) with antisense primer B or C and 160 bp with antisense primer D. Because the G→T transversion at nucleotide 994 produces a new restriction site for *Mae* II, genotypes were designated normal, heterozygous and homo mutant.

2.3. Measurements of plasma PAF acetylhydrolase activity

Plasma PAF acetylhydrolase activities were measured in a total 67 subjects that were randomly chosen from each genotype (wild $n = 48$, heterozygote $n = 15$, homo mutant $n = 4$) by the spectrophotometric assay method [11].

2.4. Laboratory measurements

Fasting blood samples were drawn from the subjects, and serum total and HDL cholesterol, triglyceride, creatinine, plasma glucose, and HbA_{1C} levels were determined by the clinical research center in NTT West Osaka Hospital, following standard laboratory protocols.

2.5. B-mode ultrasonography

We used B-mode ultrasound imaging with 7.5-MHz transducers giving an axial resolution of 0.1 mm (apparatus, Toshiba SSA-370A; probe, PLM-703AT). All the examinations were performed by a single trained sonographer. The IMT measurements were conducted according to a previous report [12]. IMT was measured in the far wall on a

longitudinal scan of common carotid arteries at a point of about 10 mm proximal from the beginning of the dilation of the bulb and we chose the point that was and its vicinity was smooth, and we avoid focal thickening areas. We measured IMT values of left and right carotid arteries for a patient and the averaged IMT value was used for statistical analysis as the representative value for each individual.

2.6. Statistical analysis

Data are expressed in terms of mean ± S.D., and statistically significant differences in IMT values were evaluated by Fisher's Protected Least Significant Difference and other clinical data were evaluated by unpaired t-test. Data of plasma PAF acetylhydrolase activity were compared among the genotypes by one-way ANOVA (Bonferroni/Dunn). We performed these analyses using STAT VIEW 5.0 software for Macintosh (SAS Institute Inc., NC USA) and a $P < 0.05$ was regarded significant.

3. Results

3.1. Statistical analysis of clinical characteristics of study subjects

In clinical characteristics, there were no significant differences between the wild and mutant genotype subgroups in the middle aged and elderly patients (Table 1).

3.2. Genotyping of plasma PAF acetylhydrolase

The genotypes determined by the allele-specific PCR agreed with the results obtained by digestion with the restriction enzyme *Mae* II. The sequenced PCR products were accordant to the reported data (data not shown). Results of the genotypes were listed in Table 2. The prevalence of the G994T mutation was 28.6% (24.3% heterozygote, and 4.3% homozygote); these values being almost similar to the data reported in previous report [3].

3.3. Measurements of plasma PAF acetylhydrolase activity

The results indicated that the values of plasma PAF acetylhydrolase activity in the wild genotype were 496.9 ± 120.3 ($n = 48$), in the heterozygote were 242.5 ± 47.9 ($n = 15$) and in the homo mutant were 34.5 ± 3.5 ($n = 4$) nmol/min per ml (Table 3). The differences between three genotypes reached statistic significance. The relationship between genotypes and plasma enzyme activity is consistent with the data reported in previous studies [3–5,7]. In the wild genotype, PAF-AH activities have a tendency to be increased according to age. In the middle aged patients ($n = 21$), PAF-AH activity was 474.6 ± 119.2 and in the elderly patients ($n = 27$), it was 514.2 ± 120.5 nmol/min per ml. But there were no statistical significance ($P = 0.26$).

3.4. IMT measurements

IMT values increased with age both in wild and mutant group, but the subjects of the mutant group showed greater IMT values than those of the same generation of the wild group. Especially IMT value of the elderly mutant subgroup was significantly greater than that of the elderly wild subgroup ($P = 0.0292$, Table 2).

4. Discussion

The aim of this study was to investigate the association between the missense mutation of plasma PAF acetylhydrolase (G994T) and IMT values of carotid arteries that correlate with the progression of systemic atherosclerosis. The results reveal that the mutation is associated with development of atherosclerosis of carotid arteries in the elderly.

Recently, Yamada et al. reported that plasma PAF acetylhydrolase activity in healthy persons with the wild genotype increased with aging in a large population of Japanese, but the activity was less increased in the hetero genotype, while in homo mutant genotype the activity diminished [13]. They supposed that in the wild genotype, increasing activity of plasma PAF acetylhydrolase

Table 2
Plasma PAF acetylhydrolase genotypes and IMT values of both genotypes in type 2 diabetic patients

	Wild		Mutant	
Age (years)	*N*	IMT (mm)	*N* (heterozygote/homozygote)	IMT (mm)
40–59	50	0.79±0.20	14 (12/2)	0.83±0.16
60–79	50	0.87±0.20[a]	26 (22/4)	0.98±0.22[b, c]
Total	100 [71.4%]		40 (34/6) [28.6% (24.3%/4.3%)]	

Data are mean±S.D. [a]$P = 0.0437$ versus 40s or 50s in the wild group; [b]$P = 0.0320$ vs. 40s or 50s in the mutant group; [c]$P = 0.0292$ vs. 60s or 70s in the wild group. P values were calculated by the Fisher's Protected Least Significant Difference.

Table 3
Plasma PAF acetylhydrolase activities in the three genotypes*

PAF-AH genotype	
Wild	496.9±120.3 ($n = 48$)
Heterozygote	242.5±47.9 ($n = 15$)[a]
Homo mutant	34.5±3.5 ($n = 4$)[a, b]

*, Enzyme activity is expressed nmol/min per ml. [a]$P < 0.0001$ vs. wild group. [b]$P < 0.01$ vs. heterotygote group. P values were calculated by one-way ANOVA (Bonferroni/Dunn).

with aging may protect vascular wall from atherosclerosis and thus, atherosclerosis would progress to a larger extent with aging in patients having the mutant allele. Our study indicates that plasma PAF acetylhydrolase activity increased according to age but not statistically significant because of a smaller number of the patient.

We have selected diabetic patients who were free from major cardiovascular events, because we expected to investigate the association between the mutation and progression of atherosclerosis in the early stage. We excluded patients with severe nephropathy, because atherosclerosis is highly accelerated by renal insufficiency. The present data suggested that type 2 diabetic patients with the mutant allele should be prevented from risk factors for atherosclerosis. The data in the present study together with those in previous studies indicate that PAF-like activity is associated with atherosclerosis, and suggest that treatment with a PAF antagonist is a potential therapeutic strategy for prevention and intervention in atherosclerosis. Further studies in larger numbers of patients are necessary to manifest the relationship between the three genotypes, i.e. wild, heterozygote and mutant homozygote.

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PHARMACOKINETICS AND DISPOSITION

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No major difference in inhibitory susceptibility between CYP2C9.1 and CYP2C9.3

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Abstract *Objective*: CYP2C9 is a polymorphic enzyme, and *CYP2C9*3* is associated with decreased metabolic activity. In addition to the impaired metabolism, we investigated whether the *CYP2C9*3* exhibited altered inhibitory susceptibility compared with *CYP2C9*1*.
Method: In the present study, CYP2C9.1 and CYP2C9.3 were expressed in yeast. Using typical CYP2C9 substrates (diclofenac, tolbutamide and *S*-warfarin) and a potent CYP2C9 inhibitor (nicardipine), the K_i values for nicardipine on the three metabolisms in *CYP2C9*1* and *CYP2C9*3* were determined.
Result: The ratios of K_i(*CYP2C9*3*) /K_i(*CYP2C9*1*) on tolbutamide, diclofenac and *S*-warfarin metabolisms were 1.2, 3.1 and 0.8, respectively.
Conclusion: In conclusion, there are no significant differences in the inhibitory susceptibility between the two CYP2C9 enzymes.

Keywords CYP2C9 · Polymorphism · Inhibition

Introduction

Cytochrome P_{450} (CYP) enzymes have an important functional role for the oxidative conversion of numerous drugs to hydrophilic metabolites. In particular, CYP2C9 has been increasingly recognized to play a major role in the metabolism of several important drugs, such as losartan, phenytoin, tolbutamide, torsemide, *S*-warfarin and many non-steroidal anti-inflammatory drugs (NSAIDs), including ibuprofen, diclofenac and naproxen [1]. With regard to functional CYP2C9 polymorphisms, two variants have been described. CYP2C9.2 has Arg^{144} to Cys^{144} substitution, whereas CYP2C9.3 (the Leu^{359} variant) has Ile^{359} to Leu^{359} substitution [1]. There is an interethnic difference in the allele frequency of *CYP2C9*2*, which has not been detected in Asians [2]. Also, it is unknown whether the impact of *CYP2C9*2* on metabolism would be significant. However, the allele frequency of *CYP2C9*3* overlaps among the different ethnic groups [2]. Moreover, CYP2C9.3 is attributed to interindividual variability in drug response in vivo, and the magnitude of the effect on pharmacokinetics varies depending on CYP2C9 substrate [3].

Since there are some CYP2C9 substrates with the narrow therapeutic indices, we should pay particular attention to the impaired metabolism resulting from CYP2C9.3. Also, harmful side effects caused by co-administration of the CYP2C9 inhibitors could possibly occur. With the view of predicting and preventing drug–drug interactions, it is clinically significant to assess the inhibitory property of CYP2C9.3 compared with CYP2C9.1. In the present study, we compared K_i values between CYP2C9.1 and CYP2C9.3 using typical CYP2C9 substrates.

Materials and methods

Tolbutamide, sodium diclofenac and nicardipine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *S*-warfarin, 7-hydroxywarfarin, hydroxytolbutamide and 4-hydroxydiclofenac were purchased from Daiichi Pure Chemicals Co. (Tokyo, Japan). Reduced nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from the Oriental Yeast Co. (Tokyo, Japan). Microsomes from yeast cells expressing CYP2C9.1 and CYP2C9.3 were prepared as described previously [4].

The incubation mixture (200 µl) containing 10 pmol P_{450} protein and 1 mM NADPH in 0.05 M potassium phosphate buffer (pH 7.4) was incubated with substrate at 37°C after 3 min of preincubation without NADPH (in case of inhibition study, inhibitor was added). The incubation for the assay of tolbutamide, diclofenac and *S*-warfarin metabolic activity continued with gentle shaking at 37°C for 10, 15 and 30 min, respectively. The reaction

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was stopped with acetonitrile (200 µl). Denatured protein was precipitated by centrifugation and the supernatant (100 µl) was analyzed using high-performance liquid chromatography (HPLC).

In the present study, a Shimadzu LC-10A HPLC system equipped with a LC-10AT pump, a SPD-10A UV detector and a C-R6A integrator (Shimadzu, Kyoto, Japan) was used. The column temperature was set at 30°C. For the determination of hydroxytolbutamide, the chromatography was conducted on a Purospher RP-18 column (4.6×250 mm; Kanto Chemical Co., Tokyo, Japan). The mobile phase was composed of H_2O/acetonitrile (69:31, vol/vol) containing 0.086% perchloric acid and was delivered at a flow rate of 1 ml/min. The eluate was detected at a wavelength 230 nm. For the determination of 4-hydroxydiclofenac, the chromatography was conducted on a Capcell Pak C18 UG120 column (4.6×250 mm; Shiseido, Tokyo, Japan). The mobile phase was composed of 50 mM potassium phosphate buffer (pH 7.4)/acetonitrile (70:30, vol/vol) and was delivered at a flow rate of 0.8 ml/min. The eluate was detected at a wavelength 282 nm. For the determination of 7-hydroxywarfarin, the chromatography was conducted on a J's sphere ODS H-80 column (4.6×150 mm; YMC, Kyoto, Japan). The mobile phase was composed of H_2O/acetonitrile (62:38, vol/vol) containing 0.5% phosphoric acid and was delivered at a flow rate of 1.3 ml/min. The eluate was detected at a wavelength 313 nm.

Results

The kinetic parameters of CYP2C9.1 and CYP2C9.3 for the metabolism of tolbutamide, diclofenac and *S*-warfarin are summarized in Table 1. For all three metabolisms, the CYP2C9.3 showed higher K_m values than CYP2C9.1, and the difference in K_m values between the CYP2C9.1 and CYP2C9.3 varied from 1.5-fold to 2-fold. However, the difference in V_{max} values between the two varied among the CYP2C9 substrates. There were differences in the V_{max}/K_m values between CYP2C9.1 and the CYP2C9.3 for hydroxytolbutamide (33-fold) and 7-hydroxywarfarin (3.2-fold), not but for 4-hydroxydiclofenac.

Summary of K_i values for nicardipine via CYP2C9.1 and the CYP2C9.3-mediated metabolism of tolbutamide, diclofenac and *S*-warfarin are shown in Table 2. For the determination of apparent K_i values, Dixon plots were constructed. The ratios of K_i values in CYP2C9.3 compared with those in CYP2C9.1 for nicardipine varied from 0.8 to 3.1.

Discussion

Consistent with a previous report [5], all three substrates exhibited higher K_m values in the CYP2C9.3 than those in CYP2C9.1 expressed in yeast (Table 1). Surprisingly, the CYP2C9.3 had a higher V_{max} value than CYP2C9.1 for diclofenac 4-hydroxylation. This matter might relate to the specificity of diclofenac, which is fairly flexible within the binding pocket of CYP2C9 due to the high degree of torsional freedom [6]. Recent reports using yeast microsomes showed that the V_{max} value for 4-hydroxydiclofenac in CYP2C9.3 was similar [5] or higher [7] than that in CYP2C9.1, as well as our observation.

Subsequently, we studied whether CYP2C9.3 exhibited altered inhibitory susceptibility compared with CYP2C9.1 using these substrates. We used nicardipine, a 1,4-dihydropyridine calcium antagonist, as a CYP2C9 inhibitor since it was reported that nicardipine strongly inhibited for CYP2C9 [8]. Moreover, nicardipine is likely to be co-administrated with these CYP2C9 substrates in clinical practice. As a result, the K_i values in CYP2C9.3 on tolbutamide and *S*-warfarin metabolisms were close to those in CYP2C9.1, whereas the K_i values in the two CYP2C9 enzymes on diclofenac metabolism appeared to be different (Table 2). This difference may be caused by the specificity of diclofenac. We also studied inhibition of sulfaphenazole, a typical CYP2C9 inhibitor, for *S*-warfarin 7-hydroxylation in CYP2C9.1 and CYP2C9.3. The K_i values in the two enzymes, CYP2C9.1 and CYP2C9.3, were 1.3 µM and 1.2 µM, respectively. These results suggest that there are no significant differences in the inhibitory susceptibility between CYP2C9.1 and CYP2C9.3.

In conclusion, our findings indicate that CYP2C9.3 exhibits a lower metabolic capability and a similar inhibitory susceptibility compared with CYP2C9.1. Furthermore, the outcome may allow for the prediction of the magnitude of metabolic inhibition in carrier for CYP2C9.3. In addition to further in vitro study, in vivo study is necessary to verify this matter since in vitro data does not always reflect in vivo condition.

Table 1 Kinetic parameters for the formation of hydroxytolbutamide, 4-hydroxydiclofenac and 7-hydroxywarfarin by CYP2C9.1 and CYP2C9.3 expressed in yeast. Values were the mean from two independent experiments

Substrate	K_m (µM)	V_{max} (nmol/min/nmol P_{450})	V_{max}/K_m (µl/min/nmol P_{450})	Substrate concentration range (µM)
Tolbutamide				
2C9.1	286	73.2	25.6×10^{-2}	25–1000
2C9.3	431	3.4	0.8×10^{-2}	25–750
Diclofenac				
2C9.1	25.9	57.9	2.2	1–100
2C9.3	46.9	138.1	3.0	1–100
S-warfarin				
2C9.1	28.0	0.22	7.9	10–100
2C9.3	55.1	0.13	2.4	10–100

Table 2 Inhibitory effect of nicardipine on the metabolism of three CYP2C9 substrates by CYP2C9.1 and CYP2C9.3 expressed in yeast. Values were the mean from two independent experiments. For the assay of tolbutamide, diclofenac and *S*-warfarin metabolic activity, nicardipine ranged 0.1–5, 0.1–1 and 0.1–1 μM, respectively

Substrate	K_i (2C9.1) (μM)	K_i (2C9.3) (μM)	K_i (2C9.3)/K_i (2C9.1)	Substrate concentration range (μM)
Tolbutamide	0.38	0.44	1.2	100–500 (2C9.1), 250–750 (2C9.3)
Diclofenac	0.19	0.58	3.1	10–50 (2C9.1, 2C9.3)
S-wafarin	0.14	0.11	0.8	10–50 (2C9.1), 25–100 (2C9.3)

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MTHFR Gene Polymorphism as a Risk Factor for Diabetic Retinopathy in Type 2 Diabetic Patients Without Serum Creatinine Elevation

Diabetic retinopathy (DR), a serious microangiopathic complication of diabetes, is the leading cause of catastrophic loss of vision in Japan. Methylenetetrahydrofolate reductase (MTHFR) is an enzyme involved in remethylation of homocysteine to methionine. A point mutation (C677T) in the MTHFR gene leads to impaired activity and is the most common genetic determinant of moderate hy-