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Nicotine Metabolic Capability Following Cigarette Smoking in Japanese Smokers with CYP2A6*4/*9 Genotype

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ABSTRACT

Objectives : To investigate whether smoking induced-nicotine metabolism is reduced in Japanese intermediate metabolizers (IMs) possessing the cytochrome P450 (CYP) 2A6*4/*9 genotype, we compared urinary excretion of nicotine and its metabolites (cotinine and *trans*-3′-hydroxycotinine [3OH-cotinine] catalyzed by CYP2A6) between IMs and extensive metabolizers (EMs). We also compared the saliva levels of nicotine and cotinine with blood levels after cigarette smoking and analyzed the kinetic disposition.

Study design : The subjects were 10 male smokers : 3 IMs (CYP2A6*4/*9) and 7 EMs (1 with CYP2A6*1/*4, 2 with *1/*9, and 4 with *1/*1). After abstaining from smoking for 3 days, the subjects smoked 3 cigarettes, and then serial blood and saliva samples were collected at given time intervals and timed urine specimens were collected. Percent urinary excretion of nicotine and its metabolites and the metabolic ratio or index as an indication of *in vivo* CYP2A6 activity were compared between IMs and EMs.

Results : The 24-hour urinary excretion of nicotine as a percentage of total nicotine intake was significantly higher in IMs (37.0±6.9) than in EMs (16.3±2.4%, $p<0.05$). Conversely, urinary excretion of cotinine + 3OH-cotinine as a percentage of total nicotine intake was slightly but not significantly lower in IMs (63.0±6.9) than in EMs (83.8±2.4%, $p=0.30$). Metabolic clearance (239.8±98.4 vs. 959.9±264.4 ml/min, $p<0.05$) and metabolic index (10.4±21.3 vs. 39.3±11.7 mL/min, $p<0.05$) were also lower in IMs than in EMs. Nicotine and cotinine levels in saliva were much higher than in serum (nicotine : 21 to 37 times higher during the first 3 hours ; cotinine : 1.9 to 3.4 times higher up to 24 hours).

Conclusion : These results suggest that nicotine metabolism via CYP2A6 is moderately suppressed in Japanese subjects with the CYP2A6*4/*9 genotype compared with that in EMs. Nicotine and cotinine appear to be secreted to a great extent in saliva, and the salivary level may be an excellent substitute for the serum level. (Jikeikai Med J 2007 ; 54 : 11-9)

Key words : nicotine, cotinine, cigarette smoking, CYP2A6, saliva

Introduction

Nicotine, a major constituent of tobacco, rapidly enters the blood after being absorbed through the lung during tobacco smoking to an extent similar to that after intravenous administration. Nicotine is metabolized primarily by C-oxidation to cotinine, and to a lesser extent by *N*-oxidation to nicotine *N*-1′-oxide, *N*-demethylation, and N-glucuronidation (conjugated). Cotinine is further hydroxylated to *trans*-3′-hydroxycotinine and 5′-hydroxycotine, *N*-oxidation to cotinine *N*-1-oxide, and N-glucronida-

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tion (conjugated). *Trans*-3′-hydrocotinine is further metabolized by *O*-glucuronidation (conjugated)[1,2]. Typically, 70% to 80% of nicotine is converted to cotinine, which is then converted to trans-3′-hydroxycotinine[3]. Cytochrome P450 (CYP) 2A6, a super family of CYPs, is a responsible enzyme for these catabolic pathways.

Large intersubject variability in the kinetic disposition of nicotine and its metabolites after tobacco smoking has been documented. Several factors have been proposed to explain this large variability, and the most likely factor is the individual difference in metabolic capability[4,5]. For the responsible enzyme CYP2A6, several mutated alleles have been reported[1,5–8]. Among the major mutations which decrease the activity of CYP2A6 in Japanese, CYP2A6*4 (deletion type)[4,9,10] and CYP2A6*9 (T-48G)[11,12] have allele frequencies of 15.8% and 22.3%, respectively. Another major factor affecting intersubject variability is the difference in nicotine intake after tobacco smoking. The depth of tobacco smoke inhalation, the volume of each puff, and the force of drawing would affect the intake of nicotine after smoking. Even by unifying smoking status, significant intrasubject variability in nicotine intake would exist.

Considering the above two factors, the present study analyzed the CYP2A6*4 and CYP2A6*9 mutations in Japanese smokers as a first step, using Invader methods combined with copy number assay[13]. In the second step, we attempted to unify nicotine bioavailability after smoking by assuming total nicotine intake as the sum of 24-hour urinary excretions of nicotine, cotinine (unconjugated : free+conjugated), and *trans*-3′-hydroxycotinine (free+conjugated)[1,3]. We then calculated on a molecular basis the percentage of urinary excretion of nicotine and that of nicotine metabolites relative to the total urinary excretion of nicotine and its metabolites and compared the results in intermediate metabolizers (IMs) and extensive metabolizers (EMs). In addition, we also evaluated the nicotine metabolic clearance or index obtained from serum and salivary data, respectively, in the two groups to assess individual catabolic capability of CYP2A6 following cigarette smoking.

Another aim of present study was to evaluate the degree of passage of nicotine and cotinine into secretions, such as saliva, after tobacco smoking. Smokers often experience a persistent bitter taste for hours. Many studies have demonstrated that nicotine levels are much higher in saliva than in blood[14–17]. Rose et al.[16] have demonstrated that the salivary nicotine level may be used as an alternative to the blood nicotine level during nicotine skin patch administration. Tenneggi et al.[17] have also suggested that salivary measurements are a useful marker of blood levels. These findings suggest that saliva collection is advantageous, especially when which blood collection is impractical. Moreover, if blood levels cannot be measured because of lower sensitivity, measurement of salivary levels is more practical because they are probably higher than blood levels. In the present study, we measured and compared serial serum and saliva concentrations of nicotine and cotinine simultaneously over time following tobacco smoking.

MATERIALS AND METHODS

Subjects

Ten healthy men (28 to 48 years of age ; mean± SD, 41±7 years ; 52 to 82 kg in weight ; mean weight, 72±10 kg) who were regular cigarette smokers and had mild to heavy smoking habits (2 to 35 cigarettes per day) were studied. The subjects refrained from consumption of coffee and caffeine-containing foods and beverages and grapefruit juice for 5 days before and during the study period. Cigarette smoking was prohibited for 3 days before and during the study period. None of the subjects were taking any medication. At 9 : 00 AM on the study day the subjects smoked three cigarettes (Long Peace, 1.9 mg nicotine, 21 mg tar, Japan Tobacco Inc., Tokyo, Japan) within 30 minutes. To measure nicotine and nicotine metabolite levels in biological fluids, blood sample were collected from the antecubital vein before and 1, 2, 3, 6, 9, 12, and 24 hours after smoking. Simultaneously, 2 ml of saliva was collected without stimulation after the subjects had rinsed and gargled at least twice. To accurately determine salivary nicotine levels, repeated rinsing and gargling were required after smoking and before each collection to avoid

contamination directly by cigarette smoke or by residual saliva from previous sampling. Urine samples were collected before smoking (spotted urine) and for the periods 0 to 6, 6 to 12. and 12 to 24 hours after smoking. Samples of serum, saliva, and urine were stored at −20°C until analyzed.

The study protocol was approved by our university's ethics committee, and written informed consent was obtained from all subjects.

Genetic analysis of CYP2A6

*CYP2A6*9 (T-48G) Genotyping Assay*: We analyzed a single nucleotide polymorphism (SNP) *CYP2A6*9* T-48G with the PCR-Invader assay (Third Wave Technologies, Madison, WI, USA)[13]. Genomic DNA was extracted from EDTA-treated venous blood using the procedure of Kunkel et al.[18]. Polymerase chain reaction (PCR) of genomic DNA was performed using a modification of the method of Pitarque et al.[11]. The region containing the polymorphic site *CYP2A6*9* T-48G was amplified with PCR using a pair of forward and reverse oligonucleotide primers *9/S1 and ex1R (Table 1), in a 25-μL mixture of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% gelatin containing 200 μM of dNTP, 0.5 μM of each primer, and 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA). After an initial denaturation step of 10 minutes at 95°C, 35 cycles of amplification (0.5 minute at 94°C for denaturation; 0.5 minute at 65°C for annealing; 1 minute at 72°C for extension) were carried out with a thermal cycler (GeneAmp 9700, Applied Biosystems), followed by a final extension period of 7 minutes at 72°C.

The signal probes and the Invader oligonucleotides for detection of the *CYP2A6*9* T-48G polymorphism was designed using the Invader Creator software program (Third Wave Technologies) (Table 2). Invader reactions were performed using 384-well plates with reagents containing Cleavase XI and both FAM dye and Redmond Red (RED) dye (Epoch Biosciences, Redmond, WA, USA) FRET cassettes. In brief, 3 μL of a 1/20 dilution of the *CYP2A6*-specific PCR products containing the region of T-48G or the no target control (NTC: 10 ng/μL tRNA) was added to the appropriate wells, followed by addition of 1.2 μL of signal probes/Invader Oligo Mix, 1.4 μL of FRET Mix and 0.4 μL of Cleavase/$MgCl_2$ solution for amplified DNA (Third Wave Technologies) and an overlay of 6 μL of molecular biology-grade mineral oil (Sigma-Aldrich, St. Louis, MO, USA). The plates were then spun for 10 seconds at 1,000 rpm (120 g), incubated at 63°C for 10 minutes in a thermal cycler (PTC-100, MJ Research, Waltham, MA, USA), and then directly read with a fluorescence plate reader (CytoFlour 4000, Applied Biosystems) with excitation at 485 nm/20 nm (wavelength/bandwidth) and emission at 530 nm/25 nm for FAM; excitation at 560 nm/20 nm and emission at 620 nm/40 nm for RED.

*CYP2A6*4 (deletion) Invader copy number assay*: We used the quantitative capability of the Invader system to determine gene copy number by comparing the target gene signal (*CYP2A6*) with that of a reference gene such as *α-actin*. The *CYP2A6* probe set was designed to react specifically with only the *CYP2A6* sequence (Table 1). The relative ratios

Table 1. PCR and Invader Signal Probes and Invader Oligonucleotide Sequences

Oligonucleotide Locus	Reaction type	Oligonucleotide type	Sequence
*9/s1	PCR	PCR sense	CAGGATTCATGGTGGGGCATGT
ex1R	PCR	PCR antisense	CTTCATGAGGGAGTTGTACATC
*CYP2A6*9(T-48G)*	Invader	invader oligo	TGACGGCTGGGGTGGTTTGCCTTTA
		Signal probe 1	cgcgccgaggATACTGCCTGAAAAAGAGG
		Signal probe 2	acggacgcggagCTACTGCCTGAAAAAGAGG
CYP2A6*4 *copy number assay*	Invader	2A6 Invader oligo	GTATCTAGGGGTCTCAGAGCAGGAAATGATAGTCCGAATAG
		α-actin Invader oligo	AAGAGTAGCCACGCTCGGTGAGGATCTTCATT
		2A6 signal probe	acggacgcggagGCAAAATGGGGTGG
		α-actin signal probe	cgcgccgaggCAGGTAGTCGGTGAGATC

Table 2. Subject Characteristics of Smoking Status and Putative Genetic Polymorphism of CYP2A6

Subject	Age (years)	Weight (kg)	Smoking rate (cigarettes/day)	Total[a)] (μg/24 hr)	Copy number[b)]	Effect (T-48G)	Putative genotype[c)]
A	46	80	2	295.7	1	G	*4/*9
B	35	75	10	1,563.2	1	G	*4/*9
C	37	71	30	3,504.3	1	G	*4/*9
D	39	79	35	7,595.7	1	T	*1/*4
E	37	82	4	979.5	2	T/G	*1/*9
F	46	82	15	1,932.9	2	T/G	*1/*9
G	48	63	15	2,495.2	2	T	*1/*1
H	43	63	20	3,359.7	2	T	*1/*1
I	47	73	25	3,567.9	2	T	*1/*1
J	27	52	15	2,981.8	2	T	*1/*1

a) Total: Total of 24-hour urinary excretion (μg/24 hours) of nicotine, cotinine, and 3 hydroxycotinine, measured as nicotine bioavailability: b) Data derived from Invader copy number assay; c) The genotype in the present study was putative, derived from *4 (copy number) and *9 (T-48G point mutation); therefore, other mutations cannot be ruled out

of the *CYP2A6* and reference gene signals from each assay allowed us to identify and quantify the deletion alleles of *CYP2A6*.

Genomic DNA was extracted using the method described above. Invader reactions were performed using 384-well plates with reagents containing Cleavase XI enzyme for genomic DNA, and both FAM and RED FRET cassettes. In brief, 3 μL of predenatured DNA samples (20 ng/μL) or NTC (10 ng/μL tRNA) was added to the appropriate wells, followed by the addition of 1.2 μL of signal probes/Invader oligo Mix, 1.4 μL of FRET Mix and 0.4 μL of Cleavase/$MgCl_2$ solution for Genomic DNA and an overlay of 6 μL of molecular biology-grade mineral oil. Following reagent dispensing, the plates were spun for 10 seconds at 1,000 rpm (120 g), incubated at 63°C for 3 hours in a PTC-100 thermal cycler, and then directly read with a CytoFlour 4000 fluorescence plate reader using the settings described above[13]. For the genotype determination, Fold-over-zero (FOZ) values were used to confirm the validity of each assay of the samples[13]. The values were calculated with a program provided by Third Wave Technologies. For the copy number assay, the NET FAM and RED FOZ values (for CYP2A6 and α-actin) were calculated, and the ratio of the CYP2A6 to α-actin NET FOZ was calculated to identify CYP2A6 copy number[13].

Analysis of nicotine, cotinine, and their metabolites in biological fluids

Levels of nicotine, cotinine, and their metabolites in serum, saliva, and urine were determined with high-performance liquid chromatography (HPLC). The assay procedures were slightly modified from the methods reported by Zuccaro et al.[19] and by Oddoze et al.[20]. For the extraction procedure, a 200-μL aliquot of urine, 600 μL of sodium hydrochloride, and 200 μL of 10 mg/mL 2-phenylimidazole methanol solution (internal standard), for a total volume of 1 mL, were transferred to Extrelut-1 glass columns (Merck Sharp & Dohme, Whitehouse Station, NJ, USA) that were prepacked and filled with 700 mg of diatomaceous earth and preconditioned with 6 mL of dichloromethane 1 day before assay. After 10 minutes, the components were eluted under gravity with 5 mL of dichloromethane-isopropyl alcohol (9 : 1, v/v). The extracted fluid was mixed with 100 μL of methanol HCl (25 mM) and then evaporated to dryness under nitrogen and redissolved in 200 μL of water. For glucuronide-conjugated compound, 250 μL urine was mixed with 20 μL of 400 U β-glucuronidase. After overnight reaction, 200 μL of the reaction mixture was measured in a manner similar to that used for the conjugated compound. For HPLC assay (L-6000, Hitachi Medical Corporation, Tokyo), an Inertsil C8 column (5-μm particle size spherical silica, 25 cm × 4.6 mm I.D., GL Sciences, Tokyo) was used with a mobile

phase of water-acetonitrile (80 : 9, v/v) containing 5 mL of triethylamine, 670 mg/L sodium heptanesulfonate, and 0.034 M each of K_2HPO_4 and citric acid (pH＝4.4), at a flow-rate of 1.6 mL/min.

The saliva sample was measured in a similar manner as for urine. For extraction of the serum samples, 1.5 mL of serum sample, 1.4 mL of sodium hydrochloride (0.5 M) and 100 μL of internal standard solution, for a total of volume of 3 mL, were transferred to a Extrelũt-1 glass column prewashed with 12 mL of dichloromethane. After 15 minutes, 10 mL of the extracted fluid was added to 300 μL of methanol HCl (25 mM), evaporated to dryness under nitrogen, and redissolved in 100 μL of water for injection into the HPLC system. The absolute recovery rates of the extraction ranged from 85% to 95% for nicotine, 95% to 110% for cotinine, and 80% to 90% for trans-3′-hydroxycotinine. The within-day coefficient of variation ranged between 1.9% and 4.4% for the lower levels and between 0.6% and 1.6% for the higher levels.

Pharmacokinetic and statistical analyses

Nicotine and cotinine concentrations in serum and saliva were fitted to a pharmacokinetic model by the non-linear least squares methods using the microcomputer program Window-Nonline (Version 4.0, SCI Software, Pharsight Corp., Lexington, KY, USA)[21]. The half-life was determined with 0.693/Kel. The peak plasma concentration (Cmax) and the time to reach Cmax (Tmax) were obtained from real data points. The area under the concentration time curve (AUC_{0-t}) after cigarette smoking was calculated with log-linear trapezoidal approximation from time 0 to the time of the last observed measurement (Cplast) and extrapolated to infinity by adding Cplast/Kel to obtain $AUC_{0-\infty}$. As a marker for *in vivo* CYP2A6 activity, 24-hour urinary excretion of nicotine metabolites (cotinine＋*trans*-3′-hydroxycotinine, free and conjugated) as a percentage of the total urinary excretion of nicotine and metabolites (estimated nicotine intake) excreted was calculated. Furthermore, as another marker for *in vivo* CYP2A6 activity, metabolic clearance was calculated from the 24-hour urinary excretion of cotinine (free and conjugated) and *trans*-3′-hydroxycotinine (free and conjugated) divided by serum nicotine AUC[22]. We also calculated the metabolic index as a surrogate for metabolic clearance using the salivary nicotine AUC instead of serum nicotine AUC. We attempted to use salivary nicotine AUC instead of the serum nicotine AUC because serum nicotine levels were extremely low, near the detection limit, and might not be accurate. Moreover, the serum samples from 1 subject (G) could not be measured because of opaque serum, probably caused by increased triglycerides.

Data are shown as means±standard errors of the mean (S.E.M.) if not otherwise indicated. The Mann-Whitney U test was performed for the statistical comparison between the IM and EM groups. A p value of < 0.05 was considered to indicate statistical significance.

Results

Nicotine disposition in CYP2A6 genotype

Ten healthy male subjects were enrolled in present study; all were smokers, but their smoking habit ranged from 2 to 35 cigarettes per day (Table 2). After the smoking of 3 cigarettes, total nicotine bioavailability estimated from 24-hour urinary excretion of nicotine and its metabolites (nornicotine and norcotinine levels were not detected in any urine sample) was also variable and ranged from 295.7 to 3,567.9 μg/day. The genetic analysis of CYP2A6 revealed CYP2A6*4/*9 (IM) in 3 subjects, *1/*4 in 1 subject (supposed EM), *1/*9 in 2 subjects (supposed EM), and *1/*1 in 4 subjects (supposed EM) (Table 3). Table 3 shows urinary excretions of nicotine, cotinine, and *trans*-3′-hydroxycotinine (free＋conjugated) during the 24 hours after smoking. Of the total nicotine and metabolites excreted in urine over 24 hours, nicotine showed significantly higher percentages in IMs (37.0±6.9) than in EMs (16.3±2.4%, $p<0.05$). Conversely, the percentage of cotinine ＋ 3-hydroxycotinine was lower in IMs (63.0±6.9%) than in EMs (83.8±2.4%, $p=0.30$). Both the mean metabolic clearance (239.8±98.4 vs. 959.9±246.4 mL/min, $p<0.05$) and metabolic index (10.4±21.3 vs. 39.3±11.7 mL/min, $p<0.05$) were significantly lower in IMs than

Table 3. Urinary excretion data of nicotine and nicotine metabolites, and metabolic clearance of nicotine

Group	Subject	Nicotine[a] (%)	Cotinine(%)	3-Hydroxycotinine (%)	% of cotinine + % 3-hydroxycotinine (%)	Metabolic clearance[b] mL/min	Metabolic index[c] (mL/min)
IM	A	25.5	31.7	42.8	74.5	95.6	5.9
	B	49.5	32.2	18.3	50.5	196.0	13.3
	C	36.1	53.6	10.2	63.9	427.9	11.9
	Mean±S.E.M.	37.0±6.9*	39.2±7.2	23.8±9.8*	63.0±6.9	239.8±98.4†	10.4±2.3*
EM	D	9.6	37.3	53.1	90.4	2,145.7	103.4
	E	9.4	31.6	59.0	90.6	720.0	14.7
	F	18.8	27.9	53.4	81.2	571.0	35.9
	G	17.8	31.3	50.9	82.2	—	25.0
	H	11.8	35.5	52.7	88.2	851.5	17.6
	I	26.3	38.7	35.0	73.7	951.4	26.9
	J	20.0	56.2	23.9	80.0	519.5	51.9
	Mean±S.E.M.	16.2±2.4	36.9±3.5	46.9±4.7	83.8±2.4	959.9±246.4	39.3±11.7

3-hydroxycotinine: trans-3′-hydroxycotinine. a) The percentage data was calculated on the molecular basis of nicotine; b) metabolic clearance was obtained from urinary excretion of cotinine + 3 hydroxycotinine divided by the serum nicotine AUC on a molecular basis. c) The metabolic index was calculated in the same manner as metabolic clearance, but the salivary nicotine AUC was used instead of the serum AUC. *$p<0.05$ compared with the EM group ($n=7$); †: $p=0.020$ compared with the EM group ($n=6$)

in EMs.

Nicotine and cotinine levels in serum and saliva following smoking

Nicotine and cotinine levels in saliva were much higher than those in serum (nicotine: 21 to 37 times higher during the first 3 hours; cotinine: 1.9 to 3.4 times higher up to 24 hours; Figure 1). The $AUC_{0-\infty}$ for nicotine was approximately 19 times greater in saliva than in serum, whereas the $AUC_{0-\infty}$ for cotinine was approximately 2.7 times greater in saliva than in serum. The Cmax for nicotine was 30 times higher, and the Cmax for cotinine was approximately 3 times higher in saliva than in serum (Table 4). The mean half-life for nicotine was 1.20±0.23 hours in serum and 2.02±0.80 hours in saliva, and the half-life for

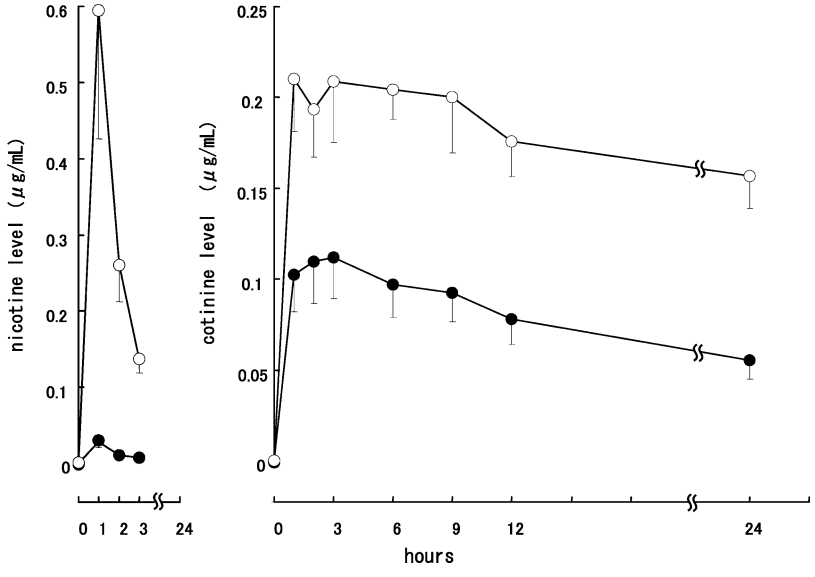

Fig. 1. Serum (●) and saliva (○) nicotine (left) and cotinine (right) concentration-time curves following cigarette smoking in 10 healthy Japanese men. Data are means±S.E.M.

Table 4. Pharmacokinetic Variables of Nicotine and Cotinine in Blood and Saliva

Variable	Unit	Blood		Saliva	
		Nicotine	Cotinine	Nicotine	Cotinine
$AUC_{0-\infty}$	(μg・hr/mL)	0.08±0.01	1.59±0.06	1.49±0.28	4.28±0.36
Cmax	(μg/mL)	0.02±0.01	0.11±0.02	0.60±0.17	0.28±0.03
Tmax	(hour)	1.16±0.16	2.67±0.17	1.00±0.00	5.89±2.42
half-life	(hour)	1.20±0.23	20.75±2.11	2.02±0.80	23.11±2.09
mean resident time	(hour)	1.96±0.31	30.67±3.02	3.31±1.01	33.85±3.44

Data are expressed as means±S.E.M. The above variables were obtained from observing the actual data or from Win-Nonlin pharmacokinetic analysis or both.

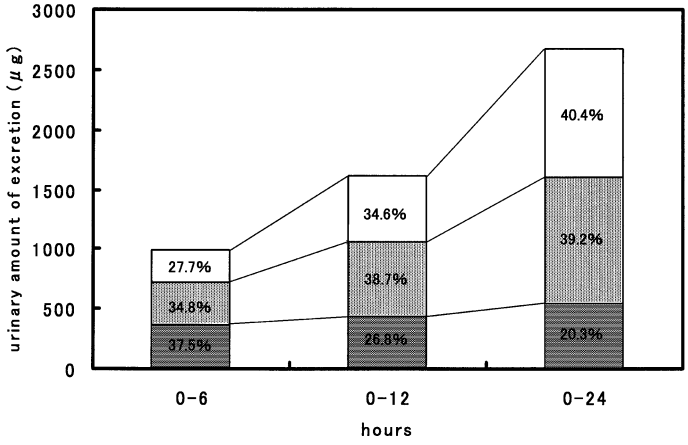

Fig. 2. Urinary excretion of nicotine (dark box), cotinine (gray box), and *trans*-3′-hydroxycotinine (white box) with the time following cigarette smoking in 10 healthy Japanese men. Values in the boxes indicate the percentage of total excretion in each time interval calculated on a molecular basis.

cotinine was 20.75±2.11 hours in serum and 23.11±2.09 hours in saliva (Table 4).

Urinary excretions of nicotine and its metabolites : time relationship

Figure 2 shows the urinary excretion of nicotine, cotinine, and *trans*-3′-hydroxycotinine over time following cigarette smoking in 10 healthy Japanese men. The urinary excretion of *trans*-3′-hydroxycotinine increased with time to 40.4% of total excretion, whereas nicotine urinary excretion decreased gradually from 37.5% to 20.3% of total excretion.

Discussion

Of the 10 Japanese male smokers enrolled in the study, 3 had the CYP2A6*4/*9 genotype. According to a recent study by Yoshida et al. of 92 Japanese subjects[12], the *in vivo* CYP2A6 enzyme activity expressed by the blood cotinine/nicotine ratio 2 hours after chewing 2 mg of nicotine gum is lower in subjects with this genotype than in subjects with other genotypes (1.9±0.9 for *4/*9 vs. 4.9±4.4 for *1/*1; 3.3±1.7 for *1/*4; 3.1±0.8 for *1/*9; 3.1±0.8 for *9/*9). The frequency of this genotype is 8.7% in the Japanese population. Furthermore, Yoshida et al. have also reported that the expression levels of CYP2A6 mRNA and coumarin 7-hydroxylase activity in liver speciments from subjects with this genotype decreased to approximately 37% and 20%, respectively, of those in specimens from subjects with the wild-type genotype (*1/*1). Indeed, no activity is detected in the complete deletion genotype (*4/*4). Therefore, while subjects with the complete deletion genotype *4/*4 are classified as poor metabolizers, subjects with the *4/*9 genotype appears to be

Ims[4,5,9,10,12]. On the basis of this classification, the 10 subjects of the present study were divided into 3 IMs and 7 EMs, and none had the deletion genotype of poor metabolizers.

We assessed the *in vivo* CYP2A6 enzyme activity with the percentage urinary excretion and metabolic clearance or index in the subjects after smoking. The urinary excretion of the unchanged form of nicotine in IM subjects was 2 times greater than that in EM subjects. Conversely, IM subjects excreted lower levels of nicotine metabolites, which are catabolized by CYP2A6, in urine than did EMs. In a recent report using methods similar to ours[22], the metabolic clearance of nicotine tended to be lower in IMs than in EMs, and the metabolic index (using salivary data) was significantly lower than that in EMs. The results of metabolic clearance or index were also in agreement with urinary excretion data in the two groups. The serum data from 1 EM subject were not available for the calculation of metabolic clearance because of sample turbidity, probably due to triglycerides. Therefore, we used the salivary data to obtain the metabolic index. Additionally, when saliva collection is an advantage because blood collection is impractical or because blood levels cannot be measured owing to lower sensitivity, the metabolic index using salivary data may be an effective tool to indicate individual *in vivo* CYP2A6 activity. Many investigators have demonstrated that serum nicotine-time decay curves after administration of nicotine through skin patch or gum differ among several genotypes[1,5,6]. However, in a practical setting for cigarette smoking, the bioavailability of nicotine varies greatly with the depth of inhalation, the volume of each puff, and the force of drawing, all of which depend of the degree of nicotine dependence or the smoking behavior. Therefore, the total bioavailability of nicotine in our subjects varied widely from 295.7 to 3,567.9 μg of nicotine (Table 3). However, the present study used the percent urinary excretion data, and the metabolic clearance or index indicates the individual metabolic capability via CYP2A6.

According to many previous studies[14-17], nicotine and cotinine levels in saliva are approximately 25 times and 2.3 times higher, respectively than those in serum during the first 12 hours after smoking. The variation in the saliva/serum concentration ratio in different studies may be due to differences in saliva flow rates resulting from different collection methods[16,17,23]. A previous study has shown that the cotinine concentration in saliva depends on the saliva flow rate and is lower in stimulated saliva than in nonstimulated saliva[24]. In the present study, we collected saliva samples without stimulation. The present pharmacokinetic disposition data showed longer a half-life for cotinine than in previous studies[14,15,25,26]. The reason for this difference is unclear ; however, 3 days' abstention from smoking may not be long enough to determine the half-life of cotinine precisely. A long half-life previously reported[14,15,25,26] suggests that the present sample collection schedule of up to 24 hours also may not be sufficient to determine the cotinine half-life precisely. Because a recent report has suggested that cotinine is eliminated from more slowly from the saliva than from the blood or urine[27], our data concerning saliva half-life may be in better agreement with those of previous studies. Nevertheless, the above issues should be evaluated in future investigations.

In conclusion, the present results suggest that nicotine metabolism via CYP2A6 is moderately suppressed in Japanese subjects with the genotype of CYP2A6*4/*9 compared with that in EMs. Nicotine and cotinine appear to be secreted in large quantities in saliva, and the salivary level may be an excellent substitute for the serum level.

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References

1. Tricker AR. Nicotine metabolism, human drug metabolism polymorphisms, and smoking behaviour. Toxicology 2003 ; 183 : 151–73.
2. Tutka P, Mosiewicz J, Wielosz M. Pharmacokinetics and metabolism of nicotine. Pharmacological Reports 2005, 57 : 143–53.
3. Benowitz NL, Jacob III P, Fong I, Gupta S. Nicotine metabolic profile in man : comparison of cigarette smoking and transdermal nicotine. J Pharmacol Exp Ther 1994 ; 268 : 296–303.
4. Cholerton S, Arpanahi A, McCracken N, Boustead C, Taber H, Johnstone E, et al. Poor metabolisers of nicotine and CYP2D6 polymorphism. Lancet 1994 ; 343 : 62–3.
5. Nakajima M, Kuroiwa Y, Yokoi T. Interindividual differences in nicotine metabolism and genetic polymorphisms of human CYP2A6. Drug Metab Rev 2002 ; 34 : 865–77.
6. Yoshida R, Nakajima M, Watanabe Y, Kwon J–T, Yokoi T. Genetic polymorphisms in human CYP2A6 gene causing impaired nicotine metabolism. Br J Clin Pharmacol 2002 ; 54 : 511–17.
7. Tyndale RF, Sellers EM. Variable CYP2A6–mediated nicotine metabolism alters smoking behavior and risk. Drug Metab Dispos 2001 ; 29 : 548–52.
8. Fujieda M, Yamazaki H, Saito T, Kiyotani K, Gyamfi MA, Sakurai M, et al. Evaluation of CYP2A6 genetic polymorphisms as determinants of smoking behavior and tabacco–related lung cancer risk in male Japanese smokers. Carcinogenesis 2004, 25 : 2451–8.
9. Nakajima M, Yamagishi S, Yamamoto H, Yamamoto T, Kuroiwa Y, Yokoi T. Deficient cotinine formation from nicotine is attributed to the whole deletion of the CYP2A6 gene in humans. Clin Pharmacol Ther 2000 ; 67 : 57–69.
10. Zhang X, Ameno K, Ameno S, Kinoshita H, Kubota T, Kumihashi M, et al. Effects of whole deletion of CYP2A6 on nicotine metabolism in humans. Drug Chem Toxicol 2002 ; 25(2) : 203–13.
11. Pitarque M, Richter O, Oke B, Berkkan H, Oscarson M, Ingelman–Sandberg M. Identification of a single nucleotide polymorphism in the TATA box of the CYP2A6 gene : impairment of its promoter activity. Biochem Biophys Res Commun 2001 ; 284 : 455–60.
12. Yoshida R, Nakajima M, Nishimura K, Tokudome S, Kwon JT, Yokoi T. Effects of polymorphism in promoter region of human CYP2A6 gene (CYP2A6*9) on expression level of messenger ribonucleic acid and enzymatic activity in vivo and in vitro. Clin Pharmacol Ther 2003 ; 7 : 69–76.
13. Neville M, Selzer R, Aizenstein B, Maguire M, Hogan K, Walton R, et al. Characterization of cytochrome P450 2D6 alleles using the Invader™ system. Bio Techniques 2002 ; 32 : S34–43.
14. Jarvis MJ, Russell MAH, Benowitz NL, Feyerabend C. Elimination of cotinine from body fluids : implications for noninvasive measurement of tobacco smoke exposure. Am J Public Health 1988 ; 78 : 696–8.
15. Curvall M, Elwin C–E, Kazemi–Vala E, Warholm C, Enzell CR. The pharmacokinetics of cotinine in plasma and saliva from non–smoking healthy volunteers. Eur J Clin Pharmacol 1990 ; 38 : 281–7.
16. Rose JE, Levin ED, Benowitz N. Saliva nicotine as an index of plasma levels in nicotine skin patch users. Ther Drug Monit 1993 ; 15(5) : 431–5.
17. Teneggi V, Squassante L, Iavarone L, Milleri S, Bye A, Gomeni R. Correlation and predictive performances of saliva and plasma nicotine concentration on tobacco withdrawal–induced craving. Br J Clin Pharmacol 2002 ; 54 : 407–14.
18. Kunkel LM, Smith KD, Boyer SH, Borgaonkar DS, Wachtel SS, Miller OJ, et al. Analysis of human Y–chromosome–specific reiterated DNA in chromosome variants. Proc Natl Acad Sci (USA) 1977 ; 74 : 1245–9.
19. Zuccaro P, Altieri I, Rosa M, Passa AR, Pichini S, Pacifici R. Solid–phase extraction of nicotine and its metabolites for high–performance liquid chromatographic determination in urine. J Chromatogr 1995 ; B668 : 187–8.
20. Oddoze C, Pauli AM, Pastor J. Rapid and sensitive high –performance liquid chromatographic determination of nicotine and cotinine in nonsmoker human and rat urines. J Chromatogr 1998 ; B708 : 95–101.
21. Gibaldi M, Perrier D, editors. *Pharmacokinetics*, 2nd edn. New York : Marcel Dekker : 1982.
22. Ushiama H, Echizen H, Nachi S, Ohnishi A. Dose–dependent inhibition of CYP3A activity by clarithromycin during *Helicobacter pyroli* eradication therapy assessed by changes in plasma lansoprazole levels and partial cortisol clearance to 6β–hydroxycortisol. Clin Pharmacol Ther 2002 ; 72 : 33–43.
23. Wall MA, Johnson J, Jacob P, Benowitz NL. Cotinine in the serum, saliva and urine of nonsmokers, passive smokers, and active smokers. Am J Public Health 1988, 78 : 699–701.
24. Scheider NG, Jacob III P, Nilsson F, Leischow SJ, Benowitz NL, Olmstead RE. Saliva cotinine levels as a function of collection methods. Addiction 1997 ; 92(3) : 347–51.
25. Benowitz NL, Jacob III P. Nicotine and cotinine elimination pharmacokinetics in smokers and nonsmokers. Clin Pharmacol Ther 1993 ; 53 : 316–23.
26. Zevin S, Jacob III P, Geppetti P, Benowitz NL. Clinical pharmacology of oral cotinine. Drug Alcohol Depend 2000 ; 60 : 13–8.
27. Sepkovic DW, Haley NJ. Biomedical application of cotinine quantitation in smoking related research. Am J Public Health 1985 ; 75 : 663–5.