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ABSTRACT:

Cytochrome P450 2C8 is one of the primary enzymes responsible for the metabolism of a wide range of drugs such as paclitaxel, cerivastatin, and amiodarone. We have sequenced the CYP2C8 gene from 201 Japanese subjects and found five novel nonsynonymous single nucleotide polymorphisms (SNPs): 511G>A (G171S), 556C>T (R186X; X represents the translational stop codon), 556C>G (R186G), 740A>G (K247R), and 1149G>T (K383N), with the allele frequency of 0.0025. The CYP2C8 variants were heterozygously expressed in COS-1 cells and functionally characterized in terms of expression level, paclitaxel 6α-hydroxylase activity, and intracellular localization. The prematurely terminated R186X variant was undetectable by Western blotting and inactive toward paclitaxel 6α-hydroxylation. The G171S, K247R, and K383N variants exhibited properties similar to those of the wild-type CYP2C8. Paclitaxel 6α-hydroxylase activity of the R186G transfectant was only 10 to 20% that of wild-type CYP2C8. Furthermore, the R186G variant displayed a lower level of protein expression in comparison to the wild type, which was restored by the addition of a proteasome inhibitor (MG-132; Z-Leu-Leu-Leu-aldehyde). The reduced CO-difference spectral analysis using recombinant proteins from an insect cell/baculovirus system revealed that the R186G variant has a minor peak at 420 nm in addition to the characteristic Soret peak at 450 nm, suggesting the existence of improperly folded protein. These results indicate that the novel CYP2C8 SNPs, 556C>T (R186X) and 556C>G (R186G), could influence the metabolism of CYP2C8 substrates such as paclitaxel and cerivastatin.
jima et al. (2003), no coding-region SNPs in CYP2C8 have been found in the Japanese population except for the CYP2C8*5 allele (475delA) (Soyama et al., 2002), which causes a frame-shift leading to premature termination at residue 177. Recently, this allelic variant has been identified in a patient suffering from rhabdomyolysis after administration of cerivastatin (Ishikawa et al., 2004). Accordingly, we have searched for novel coding-region SNPs in the CYP2C8 gene from the Japanese population.

In the present study, we report five novel nonsynonymous SNPs in the CYP2C8 gene from the Japanese population. The wild-type and variant CYP2C8s were heterologously expressed in COS-1 cells and Sf9 cells and functionally characterized for their enzyme activity (paclitaxel 6α-hydroxylation), intracellular localization, proteasomal degradation, and reduced CO-difference spectra.

Materials and Methods

Chemicals. Paclitaxel and 6α-hydroxypaclitaxel were obtained from BD Gentest (Woburn, MA). 7,13-Diacetyl baccatin III was purchased from Alexis Biochemicals (San Diego, CA). Emulgen 911 was a generous gift from KAO (Tokyo, Japan).

Preparation of Human Genomic DNA Samples and Sequencing of CYP2C8 Gene. All of the 201 subjects in this study were Japanese cancer or epileptic patients. The ethical review boards of the National Cancer Center, the National Center of Neurology and Psychiatry, and the National Institute of Health Sciences approved this study. Written informed consent was obtained from all participating subjects. DNA extraction from blood leukocytes, PCR amplification of CYP2C8 exons, and DNA sequencing of each fragment were carried out as described previously (Soyama et al., 2001).

Construction of Plasmids. Construction of the wild-type CYP2C8 expression plasmid (pC3R.1/CYP2C8/WT) was described previously (Soyama et al., 2001). Mutations (511G→A, 556C→G, 740A→G, and 1149G→T) were introduced into the pC3R.1/CYP2C8/WT using a QuikChange multi site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the following 5'-phosphorylated primers: 5′-TCCACCTTTACATCGATGCTGCTCCTCC-TGCA-3′ for 511G→A, 5′-GCTGGTGTTTTCGACAAGAAGTTTGAT-TATAAAGATC-3′ for 556C→G, 5′-GCTCGGTTTGTCCGACAAGAAGTTTGAT-TATAAAGATC-3′ for 556C→G, 5′-GCTCGGTTTGTCCGACAAGAAGTTTGAT-TATAAAGATC-3′ for 556C→G, 5′-GCTCGGTTTGTCCGACAAGAAGTTTGAT-TATAAAGATC-3′ for 556C→G, 5′-GCTCGGTTTGTCCGACAAGAAGTTTGAT-TATAAAGATC-3′ for 556C→G, 5′-GCTCGGTTTGTCCGACAAGAAGTTTGAT-TATAAAGATC-3′ for 556C→G, 5′-GCTCGGTTTGTCCGACAAGAAGTTTGAT-TATAAAGATC-3′ for 556C→G, 5′-GCTCGGTTTGTCCGACAAGAAGTTTGAT-TATAAAGATC-3′ for 556C→G. The bases exchanged are boldface and underlined. To ensure that no errors had been introduced during the amplification process, all the plasmid constructs were verified by DNA sequencing of both strands.

For the construction of C-terminal ECFP-tagged CYP2C8 expression plasmids, CYP2C8 cDNAs were reamplified by PCR using each cDNA in pC3R.1 as a template with the following primers: 5′-GCCATTGGAACCTTTTGT-GTGGC-3′ and 5′-GACAGGGATGAAAGCATGTCG-3′. The underlined sequence was introduced for directional TOPO cloning. The latter reverse primer lacks the stop codon in the native CYP2C8 cDNA. The resulting CYP2C8 cDNAs (designated CYP2C8Δ100) were first cloned into pENTR/D-TOPO vectors (Invitrogen, Carlsbad, CA), and then subcloned into pDONR207 vectors (Invitrogen). Conversion of the pECPF-N1 vector (BD Biosciences Clontech, Palo Alto, CA) to a Gateway-compatible destination vector was carried out using the Gateway Vector Conversion System (Invitrogen) according to the manufacturer’s instructions. Specifically, the pECPF-N1 vector was digested with Xhol and BamHI, blunted with T4 DNA polymerase, and then ligated with Gateway reading-frame cassette B fragment. The resulting pECPF vector was designated pECPF-DEST. Each CYP2C8Δ100 cDNA in pDONR207 was subcloned into pECPF-DEST vector by the Gateway LR reaction.

Heterologous Expression of CYP2C8s in COS-1 Cells. Transfection of COS-1 cells with pC3R.1/CYP2C8 vectors were essentially the same as described previously (Jinno et al., 2003a). Mock transfections were carried out using pC3R.1 vector without an insert. Forty-eight hours after transfection, microsomes from COS-1 cells were prepared by sequential centrifugation according to the standard procedure described by Ekins et al. (1999). Microsomes were then suspended in 100 mM potassium phosphate buffer (pH 7.4) containing 10% glycerol and stored at −80°C until use.

In the experiments on the proteasomal degradation of CYP2C8, COS-1 cells were treated with 10 μM MG-132 (Sigma-Aldrich, St. Louis, MO) after 24 h post-transfection. The cells were incubated for another 8 h and harvested in ice-cold buffered sucrose. The whole-cell lysates were prepared by sonication and subjected to Western blot analysis.

Determination of CYP2C8 mRNA by Real-Time RT-PCR. Total cellular RNA was isolated from the COS-1 cells using the RNeasy Mini kit (QIAGEN, Tokyo, Japan) in combination with the RNase-free DNAse treatment to minimize plasmid DNA contamination of samples. First-strand cDNA was prepared from 1 μg of total cellular RNA using a High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) with random primers. Real-time PCR assays were performed on an ABI7000 using TaqMan gene expression assays for CYP2C8 (Hs00426387_m1; Applied Biosystems) according to the manufacturer’s recommendations. The relative abundance of mRNA levels was determined from calibration curves generated from a serial dilution of the pooled cDNA from COS-1 cells expressing wild-type CYP2C8. Samples without reverse transcriptase were routinely included in the RT-PCR reactions to measure the possible interference of the contaminated DNA, which was usually less than 1% of the mRNA-derived amplification. Transcripts of β-actin were quantified as internal controls using TaqMan β-actin Control Reagent (Applied Biosystems), and CYP2C8 mRNA levels were normalized on the basis of their β-actin content.

Intracellular Localization of ECFP-Tagged CYP2C8 in COS-1 Cells. COS-1 cells, plated in a six-well culture plate at a density of 4 × 10⁴ cells/cm², were transfected with 4 μg of pECPF-N1/CYP2C8s or pEYFP-EF (BD Biosciences Clontech), an endoplasmic reticulum localization vector, using LipofectAMINE 2000 reagent. Twenty-four hours after transfection, fluorescence microscopic observation of the ECFP-tagged CYP2C8s and EYFP protein was performed using an Eclips TE2000 inverted microscope (Nikon, Tokyo, Japan) equipped with Cyan GFP V2 and Yellow GFP BP filters (Chroma Technology Corp., Brattleboro, VT). Images were captured using a cooled charge-coupled device camera (ORCA-ER; Hamamatsu Photonics, Shizuoka, Japan).

Heterologous Expression of CYP2C8 in Spodoptera frugiperda (Sf9) Cells. The intact wild-type and R186G variant CYP2C8 cDNAs were cloned into pENTR/D-TOPO vectors using a strategy similar to that for CYP2C8Δ100 cDNAs except that a different reverse primer was used for the amplification: 5′-TCAGACGGGTAGATGGCGAGC-3′. Baculovirus construction and protein expression in Sf9 cells were performed according to the BaculoDirect Baculovirus Expression System protocol from Invitrogen. Briefly, Sf9 cells were directly transfected with the recombinant baculovirus DNA, which was generated by the LR reaction between the entry clone (pENTR/CYP2C8s) and the BaculoDirect linear DNA. The cells were selected with ganciclovir for 5 days, and the resulting viral stock was amplified twice by infecting the Sf9 cells. Finally, a high-titer viral stock (>10⁶ pfu/ml) was obtained.

S9 cells were infected with BaculoDirect/CYP2C8 with a multiplicity of infection of 2. After 24 h postinfection, δ-aminolevulinic acid (0.1 mM) and ferric citrate (0.1 mM) were added to the S9 cells to compensate for a deficiency in heme biosynthesis (Schwarz et al., 2001). The cells were harvested 72 h postinfection. Cells were lysed in 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 0.4% Emulgen 911 (KAO) by incubation for 30 min on ice with occasional agitation. After centrifugation, the resulting whole-cell lysate was diluted to a total protein concentration of 10 mg/ml. The reduced CO-difference spectra were recorded as described by Gonzalez et al. (1991) using an UV-visible spectrophotometer (Shimadzu UV-2400PC; Shimadzu, Kyoto, Japan).

Western Blotting. Microsomal fractions (30 μg/lane), or whole-cell lysates (10 μg/lane) from COS-1 cells or S9 cells (0.5 μg/lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electro-blotted onto a polyvinylidene difluoride membrane (ATTO, Tokyo, Japan). The membrane was incubated with rabbit anti-human CYP2C8 antibody (diluted at 1:2000; Research Diagnostics, Flanders, NJ) as the primary antibody and then with horseradish peroxidase-conjugated donkey anti-rabbit Ig (1:2000; Amersham Biosciences Inc., Piscataway, NJ) as the secondary anti-
body. Immuno-reactive proteins were visualized with chemifluorescence (ECL-Plus reagents; Amersham Biosciences), and the band densities were quantified using the Typhoon 9400 Variable Mode Imager and ImageQuant analysis software. A commercially available human CYP2C8 protein expressed in insect cells (Supersomes; BD Gentest, Woburn, MA) was used as a calibration standard. To confirm that the samples were evenly loaded, the blot derived from the COS-1 cells was subsequently stripped and reprobed with a polyclonal anti-calnexin antibody (diluted at 1:100,000; StressGen Biotechnologies, San Diego, CA) as described previously (Jinno et al., 2003a).

Enzyme Assay. The paclitaxel 6o-hydroxylation activities of wild-type and variant CYP2C8s were assayed as described previously (Soyama et al., 2001) at eight different substrate concentrations between 0.5 and 20 μM. The limit of quantitation for 6o-hydroxypaclitaxel was 30 pmol/ml, which corresponded to the enzyme activity of 1.3 pmol/min/mg protein. Kinetic parameters were calculated with Prism 4.0 (GraphPad Software Inc., San Diego, CA) using nonlinear regression of the Michaelis-Menten equation.

Data Analysis. Statistical comparisons were performed by one-way analysis of variance with Dunnett’s post hoc test using Prism 4.0. Differences were considered to be statistically significant when the p value was <0.01.

Results

Novel Genetic Variations of Human CYP2C8. From the sequence analysis of 201 Japanese subjects, five novel nonsynonymous SNPs were identified in the coding region of the CYP2C8 gene. These results were confirmed by repeating the PCR on genomic DNA and sequencing the newly amplified products. The novel SNPs include 511G>A (exon 4, resulting in the amino acid substitution of G171S), 556C>T (exon 4, R186X; X represents the translational stop codon), 556C>G (exon 4, R186G), 740A>G (exon 5, K247R), and 1149G>T (exon 7, K383N): these alleles have been designated CYP2C8*6, CYP2C8*7, CYP2C8*8, CYP2C8*9, and CYP2C8*10, respectively, by the Human CYP Allele Nomenclature Committee (http://www.imm.ki.se/CYPalleles/).

Each SNP was found in a distinct subject as a heterozygote with an allele frequency of 0.0025. CYP2C8*5 allele (475delA) was not found in the present study. Figure 1 depicts the representative electropherograms of the SNPs.

Expression of the Wild-Type and Variant CYP2C8s in COS-1 Cells. To functionally characterize the nonsynonymous CYP2C8 variants, the wild-type and variant CYP2C8 proteins were transiently expressed in COS-1 cells. As shown in Fig. 2, A and B, the protein expression levels of the R186G variant was only ~40% that of the wild-type (p < 0.01 by one-way analysis of variance and Dunnett’s test), whereas those of G171S, K247R, and K383N were slightly reduced (not statistically significant). These differences in the levels of recombinant protein within the cell were reproducible in three independent transfection experiments. R186X variant protein was undetectable even as proteolytic fragment bands under this experimental condition (data not shown).

CYP2C8 mRNA expression levels in the transfected COS-1 cells were measured by real-time RT-PCR. As shown in Fig. 2C, no significant differences were detected in the level of mRNA among the four CYP2C8 variants, suggesting that the observed disparity in the level of CYP2C8 protein was not due to variation in the transcription/translation efficiency of the plasmid constructs.

Paclitaxel 6o-Hydroxylase Activities of the Wild-Type and Variant CYP2C8s Expressed in COS-1 Cells. The CYP2C8 variants were functionally characterized for the hydroxylase activity toward a representative CYP2C8 substrate, paclitaxel. In Fig. 3, paclitaxel 6o-hydroxylase activities of the CYP2C8s were expressed on the basis of microsomal protein (Fig. 3A) or CYP2C8 content (Fig. 3B). Except for R186X and R186G, enzyme activities of the variants were almost comparable to that of the wild type, regardless of the low (0.5 μM) and high (20 μM) substrate concentrations. In contrast, the R186G variant showed a markedly lower turnover rate; no 6o-hydroxylated metabolite was detected at the low substrate concentration, and the metabolite generated at the high concentration was approximately 20% of that of wild-type on the basis of pmol/min/pmol CYP2C8 (p < 0.01). For the R186X variant, no enzyme activity was observed even at the high substrate concentration of 20 μM, indicating that the residual activity was less than 2% of the wild-type.

We attempted to determine the kinetic parameters for the paclitaxel 6o-hydroxylase reaction of the CYP2C8 variants. The representative nonlinear regression curves of the Michaelis-Menten kinetics are shown in Fig. 4. Kinetic parameters for the R186G variant could not be accurately established using high performance liquid chromatography analysis due to the low turnover rates. As summarized in Table 1, the apparent K_m and V_max values of wild-type were 7.08 ± 0.30 μM, 87.4 ± 8.6 pmol/min/mg protein, and 32.4 ± 3.4 pmol/min/pmol CYP2C8, respectively. The K_m value was almost comparable to those previously reported for the yeast-expressed CYP2C8 (10 μM) and human liver microsomes (12–15 μM) (Cretei et al., 2002; Melet et al., 2004; Tamiguchi et al., 2005).

Intracellular Localization of CYP2C8 Proteins in COS-1 Cells. In eukaryotes, P450 enzymes are localized either on the outer side of the endoplasmic reticulum or on the inner membrane of mitochondria. To test whether the lower than expected level of R186G variant in the microsomal fraction could be the result of altered intracellular location, we tested the localization of CYP2C8 proteins using C-terminal ECFP-tagged CYP2C8s. pEYFP-ER was included as a control to demonstrate the representative localization in endoplasmic reticulum. As shown in Fig. 5, no apparent difference was recorded in the intracellular localization of each CYP2C8, which was similar to the localization of the microsome-oriented EYFP protein. These results indicate that intracellular localization does not account for the decreased R186G level.

Proteasomal Degradation of R186G Variant. To further clarify the molecular mechanisms underlying the decreased expression of R186G, involvement of the proteasomal degradation pathway was investigated by using a proteasome inhibitor, MG-132. COS-1 cells expressing either wild-type or R186G CYP2C8 were treated with MG-132 (10 μM) for 8 h. Upon treatment of the cells with MG-132, the decrease in the expression level of R186G variant was restored to a level almost comparable with that of wild-type CYP2C8 (Fig. 6). This finding suggests that the enhanced proteasomal degradation of the R186G variant could explain the observed decrease in the level of protein.

Spectral Analysis of Wild-Type CYP2C8 and R186G Variant. The reduced CO-difference spectral analysis was carried out using recombinant wild-type CYP2C8 and R186G variant proteins heterologously expressed in Sf9 cells. The relative expression level of the R186G variant protein was approximately 70% of the wild-type, as shown in Fig. 7. The reduced CO-difference spectrum of wild-type CYP2C8 comprised a single Soret peak at 450 nm. In contrast, an equivalent spectrum of the R186G variant had a minor peak at 420 nm in addition to a major peak at 450 nm. It is likely that the 420-nm peak corresponds to an inactive form of the variant. These results, together with those on the proteasomal degradation, suggest that a proportion of the R186G variant might be improperly folded or unstable.

Discussion

To date, several SNPs have been reported in the human CYP2C8 gene. Dai et al. (2001) identified two CYP2C8 alleles designated as CYP2C8*2 (805A>T, I269F) and CYP2C8*3 (416G>A and 1196A>G, R139K and K399R). CYP2C8*2 was only found in African-Americans (allele fre-
frequency, 0.18) and CYP2C8*3 primarily in Caucasians (allele frequency, 0.13), whereas neither allele was found in Asians. Bahadur et al. (2002) reported the CYP2C8*4 allele (792C>G, I264M) at an allele frequency of 0.075 and a rare SNP, 1169T>C (L390S). In addition, we discovered a CYP2C8*5 allele (475delA, resulting in an early stop codon at residue 177) in a Japanese subject and a 1210C>G substitution (P404A) in an established cell line derived from a Japanese individual (Soyama et al., 2001, 2002). Among these allelic variants, CYP2C8.3 (R139K/K399R) exhibits reduced paclitaxel 6α-hydroxylase activity (Dai et al., 2001; Bahadur et al., 2002), whereas CYP2C8.2 has a 2-fold higher $K_m$ and a 2-fold lower intrinsic clearance, or $V_{max}/K_m$ value, compared with CYP2C8.1 (Dai et al., 2001). A similar decrease in the intrinsic clearance was observed for P404A, which was mainly caused by the reduced protein expression level (Soyama et al., 2001). Recently, Nakajima et al. (2003) have screened more than 200 Japanese individuals for the known SNPs in the coding region of CYP2C8. From this screen they found only a heterozygous CYP2C8*5 allele at a frequency of 0.0025, indicating that the coding-region SNPs in CYP2C8 are relatively rare in Japanese. The present study supports these findings, except that we identified five novel nonsynonymous substitutions: 511G>A (G171S), 556C>T (R186X), 556C>G (R186G), 740A>G (K247R) and 1149G>T (K383N). These SNPs were found in distinct subjects as heterozygotes. The allele frequency of each SNP was 0.0025.

To characterize the functional alteration(s) of the novel CYP2C8 variants, their paclitaxel 6α-hydroxylase activities were first determined using the recombinant proteins heterologously expressed in COS-1 cells. The enzyme activity of the R186X and R186G variants were undetectable (R186X, data not shown) or almost one tenth of the wild-type (R186G, Fig. 2). The null enzyme activity of the R186X variant was fully expected given that it lacks approximately 60% of the C-terminal region, including the heme-binding site. However, R186G exhibited a markedly reduced paclitaxel 6α-hydroxylase ac-
activity despite an intact heme-binding site and substrate recognition sites (SRSs) (Gotoh, 1992). Furthermore, the lower than expected level of R186G protein was reproducibly observed without any significant reduction in the level of corresponding mRNA (Fig. 2). No difference in the intracellular distribution of the R186G variant could be detected (Fig. 5). These results suggest that the R186G variant is less stable and/or more rapidly degraded than the wild-type protein in the heterologous expression system. Recent findings on the degradation of the ER-resident proteins including some P450 isoforms revealed a significant contribution from the ubiquitin-proteasome pathway (Correia, 2003; Bandiera et al., 2005). We examined the effect of a proteasome inhibitor, MG-132, on the expression level of R186G variant in COS-1 cells. MG-132 treatment restored the expression level of R186G to a level comparable to that of wild-type (Fig. 6). This clearly indicates that the decreased level of R186G protein compared with wild-type is the result of enhanced proteasome-mediated degradation.

Reduced enzyme activity and extensive degradation of the R186G variant might be indicative of a structural defect of CYP2C8 apoprotein. Although the transient expression system in COS-1 cells has been successfully applied to the functional analysis of P450 variants including CYP2B6, CYP2E1, and CYP1B1 (Hanioka et al., 2003; Jinno et al., 2003b; Bandiera et al., 2005), the primary drawback of this heterologous system is insufficient levels of recombinant proteins...
The amino acid residue R186 is well conserved among the majority of the CYP2 subfamily enzymes including CYP2C9 and CYP2C19. The X-ray crystal structure of CYP2C8 shows that R186 is located in the loop between helices E and F (Schoch et al., 2004; Protein Data Bank, 1PQ2). To date, several functionally significant regions and residues in the P450s have been identified based on homology modeling and site-directed mutagenesis studies. For instance, the proline-rich motif (PGPXPXPXGN motif) in the N-terminal region of P450s plays a significant role in the proper assembly of the native enzyme (Kemper, 2004). In addition, Kerdpin et al. (2004) have reported that SRS1 and SRS5 of CYP2C8 are important for paclitaxel hydroxylase activities of wild-type and variant CYP2C8

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In summary, we identified five novel nonsynonymous SNPs from screening 201 Japanese subjects: 511G>A (G171S), 556C>T (R186X), 556G>G (R186G), 740A>G (K247R), and 1149G>T (K383N). Functional analyses of these CYP2C8 variants revealed that R186X has no detectable enzyme activity. The R186G variant showed a much reduced enzymatic activity in comparison to the wild-type enzyme. Although the allele frequencies of both these variants were rather low, it would be of clinical significance to evaluate the influence of these CYP2C8 variant alleles on the pharmacokinetics/pharmacodynamics of drugs such as paclitaxel and cerivastatin.

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References


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