**ABSTRACT**

Olmesartan medoxomil (OM) is a prodrug-type angiotensin II receptor antagonist. OM is rapidly converted into its active metabolite olmesartan by multiple hydrolases in humans, and we recently identified carboxymethylenebutenolidase homolog (CMBL) as one of the OM bioactivating hydrolases. In the present study, we further investigated the interindividual variability of mRNA and protein expression of CMBL and OM-hydrolase activity using 40 individual human liver and 30 intestinal specimens. In the intestinal samples, OM-hydrolase activity strongly correlated with the CMBL protein expression, clearly indicating that CMBL is a major contributor to the prodrug bioactivation in human intestine. The protein and activity were highly distributed in the proximal region (duodenum and jejunum) and decreased to the distal region of the intestine. Although there was high interindividual variability (16-fold) in both the protein and activity in the intestinal segments from the duodenum to colon, the interindividual variability in the duodenum and jejunum was relatively small (3.0- and 2.4-fold, respectively). In the liver samples, the interindividual variability in the protein and activity was 4.1- and 6.8-fold, respectively. No sex differences in the protein and activity were shown in the human liver or intestine. A genetically engineered Y155C mutant of CMBL, which was caused by a single nucleotide polymorphism rs35489000, showed significantly lower OM-hydrolase activity than the wild-type protein although no minor allele was genotyped in the 40 individual liver specimens.

**Introduction**

Esterases, which are typically represented by carboxylesterases (CES), cholinesterases, and paraoxonases (PON), have been well investigated in particular as key enzymes responsible for the bioactivation of ester-based prodrugs (Satoh, 2005; Liederer and Borchardt, 2006; Fukami and Yokoi, 2012). The ester-prodrug strategy has been used frequently to overcome problematic drug-like properties such as chemical and enzymatic instability, low aqueous solubility, and low passive intestinal absorption, and consequently to improve the oral bioavailability of drugs (Beaumont et al., 2003; Ettmayer et al., 2004). Although these esterases are widely distributed in the blood, liver, intestine, and many other biological fluids and tissues (Testa and Mayer, 2003), in most cases, intestinal and hepatic esterases serve as the major enzymes in bioactivation of oral prodrugs during the first pass through the gut and liver after absorption.

Variability in the activity of drug metabolizing enzymes may determine the pharmacokinetics of the drug entities and also affect their therapeutic efficacy and safety when drug responses are closely related to drug exposure. In the case of prodrugs, insufficient or excessive conversion of the prodrugs into their active forms may directly lead to a lack of therapeutic response or unexpected adverse effects. In addition to physiological variables such as age, gender, and diseases, genetic polymorphism in the genes encoding the enzymes is one of the main factors contributing to the interindividual variability (Liederer and Borchardt, 2006). For example, Zhu et al. (2008) reported that two single-nucleotide polymorphisms (SNPs) in the CES1 gene altered pharmacokinetics and enhanced pharmacodynamic effects of CES1 substrate methylenidate. Furthermore, SNPs with amino acid substitutions of R34W and V142M in the CES2 gene have been reported to cause a lack of enzyme activity and to reduce conversion of CPT-11 (camptothecin-11, irinotecan) to its active metabolite (Kubo et al., 2005).

Olmesartan medoxomil (OM) is a prodrug-type angiotensin receptor blocker that is prescribed worldwide as monotherapy and in combination with a thiazide diuretic and/or a calcium channel blocker (Chrysant, 2008; Scott and McCormack, 2008; Rump and Sellin, 2010; Deeks, 2011). As shown in Fig. 1, prodrug OM was rapidly absorbed and converted to its active metabolite olmesartan. After oral administration of radiolabeled OM in healthy volunteers, no components other than the active metabolite olmesartan were detected.
in plasma, feces, or, with the exception of trace amount of polar material, in urine (Laes et al., 2001), which suggests that the prodrug is completely converted to its pharmacologically active form in the gastrointestinal mucosa, portal blood, and liver before it reaches the systemic circulation. Olmesartan is not further metabolized and is excreted both into urine and feces (Laes et al., 2001; Schwocho and Masonson, 2001). OM has a low potential for pharmacokinetic drug-drug interactions via cytochrome P450 (P450) enzymes or interindividual variability due to variation of P450 activities, as OM does not undergo metabolism via P450s (Scott and McCormack, 2008).

We recently identified human carboxymethylenebutenolidase homolog (CMBL, EC 3.1.1.45) as a bioactivating enzyme for OM in the liver and intestine (Ishizuka et al., 2010), demonstrating its function as hydrolase in humans for the first time. CMBL is a novel hydrolase, and its biological functions and enzyme characteristics mostly remain uninvestigated in contrast to the well-characterized prodrug bioactivating esterases like CES and PON. Therefore, in the present study, we quantitatively characterized CMBL’s mRNA and protein expressions and the interindividual variation of its enzymatic activity with a large number of individual human tissue samples, 40 liver and 30 intestinal specimens. Furthermore, to clarify the CMBL’s contribution to OM bioactivation in human liver and intestine, we performed correlation analyses between the protein level and OM-hydrolase activity using those individual sample sets. Also, we investigated the effects of two nonsynonymous SNPs of human CMBL on OM-hydrolase activity using site-directed mutants.

Materials and Methods

Materials. Olmesartan medoxomil (OM), olmesartan, and RNH-6272 (2-butyl-4-(1-hydroxy-1-methylethyl)-1-[2′-(1H-tetrazole-5-yl)-4-biphenylyl]-1H-imidazole-5-carboxylic acid; a structural analog of olmesartan) used as the internal standard for olmesartan determination, were synthesized in Daiichi Sankyo (Tokyo, Japan). Pooled human liver and intestinal cytosolic fractions were purchased from XenoTech LLC (Lenexa, KS).

Individual Human Liver and Intestine. Forty individual human liver (14 female and 26 male; median age 63 years, range 16–95 years) and 30 intestinal (10 female and 20 male; median age 68 years, range 44–84 years) specimens were collected and used to isolate DNA and RNA and to prepare cytosolic fractions at Daichii Sankyo Europe GmbH (Munich, Germany). The human liver and intestinal tissue sections were obtained from Caucasian patients undergoing partial hepatectomy surgery and gastrointestinal surgery for cancer, respectively, the normal portions of the tissue sections were used in the study. Informed consent was obtained from all patients, and the study was approved by the local ethics committees of the University of Regensburg (Germany) and Ludwig-Maximilian University (Munich, Germany). Each individual liver and intestinal specimen was made anonymous and assigned an individual code by the charitable state-controlled Human Tissue and Cell Research Foundation (Regensburg, Germany) (Thasler et al., 2003). The data of the liver and intestinal sample donors are shown in Supplemental Tables 1 and 2, respectively.

Preparation of Hepatic and Intestinal Cytosols and Microsomes. Liver and intestinal cytosolic fractions were prepared as described previously elsewhere (Paine et al., 1997) with some modifications. Briefly, frozen liver tissue or intestinal mucosa was thawed by directly adding a 4-fold volume (v/v) of homogenizing buffer (50 mM Tris/HCl, 154 mM potassium chloride, 250 mM sucrose, and 1 mM EDTA; pH 7.4) in the case of intestinal tissue, the homogenizing buffer was enriched with protease inhibitor cocktail (Complete EDTA-Free; Roche Applied Science, Indianapolis, IN). After thawing, the tissue was homogenized using an Ultra Turrax homogenizer (IKA Labortechnik, Staufen, Germany) with a motor-driven pestle (18 K, 18 mm diameter) at 13,500 rpm for 10 seconds, 3 times. The homogenate was centrifuged at 677g for 10 minutes, subsequently at 6940g for 10 minutes and finally at 10,844g for 10 minutes. The obtained supernatant was filtered through gauze and centrifuged at 111,000g for 60 minutes. The supernatant (cytosol) was collected and stored at −80°C until use. The protein concentration was measured using the Lowry method (Lowry et al., 1951) with bovine serum albumin as the reference standard.

Expression Analysis of Human CMBL Transcript. The total cellular RNA was isolated from tissue samples submersed in RNALater solution (Qiagen, Hilden, Germany) using an RNeasy Mini kit (Qiagen) in accordance with the manufacturer’s instructions. After determination of the quantity and quality of isolated RNA using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), cDNA was prepared from the total cellular RNA using an Omniscript RT Kit (Qiagen) with oligo-dT primers, according to the manufacturer’s instructions. Quantitative real-time reverse transcriptase-polymerase chain reaction (quantitative real-time RT-PCR) was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems; Foster City, CA) with the reaction mixture containing the cDNA, TaqMan Universal PCR Master Mix, and TaqMan Gene Expression Assays (both from Applied Biosystems). A TaqMan gene expression assay for human CMBL (Hs00540853_m1) and Pre-Developed TaqMan Assay Reagent human glyceraldehyde-3-phosphate dehydrogenase as the internal reference for normalization of mRNA expression levels were purchased from Applied Biosystems.

Semiqualitative Western Blot Analysis of Human CMBL. The following protein samples were subjected to Western blot analysis of human CMBL: 500 ng of 40 human individual liver and 30 intestinal cytosolic fractions; 500 and 250 ng of human pooled liver and intestinal cytosolic fractions, respectively; and 250 ng of human CMBL-overexpressed mammalian Freestyle 293-F cell lysate as a positive reference. The protein samples were separated by SDS-PAGE using 12.5% or 12% sodium dodecyl sulfate-polyacrylamide gel (Ready Gels J 12.5% or Mini-PROTEAN TGX precast gel 12%; Bio-Rad Laboratories, Hercules, CA) and were transferred electrophoretically onto a polyvinylidene difluoride membrane (Immun-Blot PVDF membrane, 0.2 μm; Bio-Rad Laboratories). The native CMBL proteins expressed in the human tissue preparations were detected with affinity-purified rabbit polyclonal IgG against a human CMBL peptide (1:5000 dilution; Immuno-Biologic Laboratories, Takasaki, Japan) as a primary antibody and an
anti-rabbit IgG conjugated to horseradish peroxidase (1:10,000 dilution; Amersham Biosciences/GE Healthcare, Little Chalfont, UK) as a secondary antibody. These immunoblots were visualized by enhanced chemiluminescence with an ECL Advance Western Blotting Detection Kit (Amersham Biosciences). The immunoreactive signals were detected by a luminoimage analyzer (LAS-4000UV mini; Fujifilm, Tokyo, Japan), and the signal intensities were semiquantified by Science Laboratory 2005 Multi Gauge software (ver. 3.0; Fujifilm). Each band intensity of individual liver and intestinal samples was normalized to those of pooled liver and intestinal cytosols, respectively.

Hydrolyase Activity Measurement. The OM-hydrolyase activity in human liver or intestinal cytosolic fractions (0.05 mg protein/ml) was measured in 100 mM HEPES buffer (pH 7.4, incubation volume of 0.25 ml) with OM as a substrate. After a 5-minute preincubation at 37°C, the reaction was initiated by adding the substrate (final substrate concentration: 10 μM of OM except as otherwise noted; final solvent concentration: 2.5% acetonitrile). After a 5-minute incubation at 37°C, the reaction was terminated by adding a 4-fold volume of ice-cold 87.5% acetonitrile containing RH-6272 as an internal standard for the determination of omesartan concentration and 0.25% formic acid for preventing the nonenzymatic degradation of OM. The mixture, with the volume of approximately 200 μl, was filtered using a Captiva 96-well filter plate, mixed with 200 μl of 50% methanol containing 1% formic acid, and was analyzed by liquid chromatography with tandem mass spectrometry consisting of a Prominence LC-20A system (Shimadzu Corp., Kyoto, Japan) and an API3200 (Applied Biosystems/MDS SCIEX).

Olmesartan was separated with a reverse-phase C18 column (Atlantis T3, S-5 μm, 2.1 mm ID × 150 mm; Waters Corp., Milford, MA) and a mobile phase of 64% methanol containing 0.2% formic acid at a flow rate of 0.2 ml/min, and was determined by monitoring the ion transition of m/z 447 to m/z 445 with multiple reaction monitoring in the positive electrospary ionization mode. The lower limit of quantitation was set at 20 nM.

The enzymatic activity was expressed as a metabolite formation rate (nmol/min/mg protein) based on the production of omesartan for the reaction by each enzyme source, from which that found in the buffer control was subtracted as nonenzymatic hydrolysis.

Expression of Human CMBL in E. coli and Mutant Generation. The plasmid vector containing the open reading frame of the full-length human CMBL (Ishizuka et al., 2010) was used as the template for generating mutants. A QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to create two single mutants in human CMBL (Y155C or A150T) in this study. The forward and reverse primers used for the mutagenesis were shown as follows: 5’-GGTGTCGTCGTGACACCCCTGTCCTGAATTCTGAGTATTTCATCATCAAATG-3’ and 5’-GACAAGTCACACAGCAGAAGACAGCTCCCTCCTGTCCT-3’ for the mutant Y155C, and 5’-CTCAGAATATTCCAGACCGGGGTTGTCCTGCTATGGC-3’ and 5’-GGACACCCCTCTCCTGGAATTCTGAGTATTTCATCATCAAATG-3’ for the mutant A150T.

To confirm the desired mutation and verify the absence of unintended mutations, the constructs were sequenced. Each plasmid for the native CMBL and the mutants was transformed into Escherichia coli BL21(DE3) competent cells (Novagen, Madison, WI), which were then grown in MagicMedia E. coli expression medium (Invitrogen, Carlsbad, CA) containing ampicillin (100 μg/ml). The wild-type protein and two mutants were expressed as N-terminal 6×His-tagged proteins and were purified using a column packed with His-select Nickel affinity gel (Sigma-Aldrich, St. Louis, MO). From the eluted recombinant proteins, the tag was cleaved by biotinylated thrombin (Novagen) treatment. The affinity-purified proteins with or without the thrombin treatment were analyzed by SDS-PAGE with GelCode Blue (Pierce, Rockford, IL) staining. The resulting protein solutions were stored at 4°C until use.

Kinetic Analysis. The enzyme kinetics for OM hydrolysis by the recombinant CMBL proteins were evaluated at substrate concentrations [S] ranging from 7.81 to 1000 μM. The buffer control was incubated in parallel as nonenzymatic hydrolysis. The enzymatic activity was measured as stated earlier. Kinetic parameters, namely, the Michaelis constant (K_m) and maximum velocity (V_max), were estimated from the data of the substrate concentrations [S] and initial velocity (v) using WinNonlin Professional (version 5.2.1; Pharsight, Sunnyvale, CA) by a nonlinear least-square regression analysis fitted to the Michaelis-Menten equation: v = V_max × [S]/(K_m + [S]).

Statistical Methods. Unpaired, two-tailed Student’s t test was performed with Microsoft Excel 2010 (Microsoft, Redmond, WA) for statistical analyses.

Ethics Approval. This study was performed under a protocol approved by the Institutional Human Ethics Committee of Daiichi Sankyo. All experimental procedures for human tissue collection and sample preparation at Daiichi Sankyo Europe GmbH were performed according to the guidelines of the Human Tissue and Cell Research Foundation (Thasler et al., 2003).

Results

Correlation Analysis of CMBL mRNA, Protein, and Activity in the Intestine. The intestinal sample set contained all intestinal segments from duodenum to sigmoid colon. An approximately 11-fold difference in the CMBL mRNA levels was observed among all the tested 20 individual samples, as shown in Fig. 2. Although the CMBL protein and OM-hydrolyase activity were detected in all the tested 30 individual samples, both showed greater variability, ~16-fold, than the mRNA levels. Correlations between the CMBL mRNA and protein expressions (n = 20), and between the protein expression and OM-hydrolyase activity (n = 30) in human intestine are shown in Fig. 2. A weak positive correlation was observed between the mRNA and protein expression levels (r = 0.669). Remarkably, the OM-hydrolyase activity strongly correlated with the CMBL protein expression, resulting in a correlation coefficient of 0.958.

Distribution of OM-Hydrolase Activity in Intestinal Segments. We analyzed the distribution of the CMBL protein expression and OM-hydrolyase activity in different intestinal segments, namely, the duodenum (n = 11), jejunum (n = 2), ileum (n = 3), ascending colon (n = 5), transverse colon (n = 3), descending colon (n = 2), and sigmoid colon (n = 4). The OM-hydrolyase activity was highly distributed in the proximal region of the intestine and was less distributed in the distal region, as was the CMBL protein level (Fig. 3). The mean value of the activity in each intestinal region was duodenum > jejunum > ileum > colon (ascending colon to sigmoid colon). The OM-hydrolyase activity among 13 individual duodenal and jejunal specimens showing abundant activity was relatively consistent and came within the range of 0.814–1.97 nmol/min/mg protein; the lowest value of a transverse colon specimen was 0.126 nmol/min/mg protein. Also, the activity in the 13 individual duodenum and jejunal specimens was well correlated with the CMBL protein expression level at a correlation coefficient of 0.968 (unpublished data).

Correlation Analysis of CMBL mRNA, Protein, and Activity in the Liver. CMBL mRNA was detected in all the liver samples except one out of 34 individuals. The interindividual variation in the mRNA levels in the liver samples was much greater (136-fold difference) than that in the intestinal samples (11-fold difference). In contrast to the great variation in the mRNA expression levels, the interindividual variation in the protein levels was only 4.1-fold. The correlation analysis (n = 34) in Fig. 4A shows that the CMBL protein levels followed a saturation curve against the mRNA levels. All 40 individual samples showed substantial OM-hydrolyase activity; a 6.8-fold difference in the activity was observed. The OM-hydrolyase activity showed a weak positive correlation with the CMBL protein expression (Fig. 4B; n = 40; r = 0.619). The low OM-hydrolyase activity in two individual samples (compared with the others) was attributed to their low mRNA and protein expression levels of CMBL.

Sex Differences in OM-Hydrolase Activity in Human Liver and Intestine. We also analyzed the sex differences in the CMBL protein expression and OM-hydrolyase activity in human liver (26 males and 14 females) and intestine (8 males and 5 females). For analysis of intestinal samples, we employed the data from the duodenum and jejunum, in which the OM-hydrolyase activity was highly distributed.
In both the liver and intestine, no statistically significant sex differences in the protein level and activity were observed (Fig. 5). The P values in the analysis of liver and intestinal samples were 0.24 and 0.21 in the protein level and 0.07 and 0.27 in the activity, respectively.

**Mutant Generation of CMBL and OM-Hydrolase Activity.**

The mutant proteins of human CMBL, Y155C, and A150T, were successfully created by site-directed mutagenesis to evaluate the effects of two nonsynonymous CMBL SNPs rs35489000 and rs34487157 (Supplemental Table 3), respectively, which were previously reported in the National Center for Biotechnology Information (NCBI) dbSNP (build 130; http://www.ncbi.nlm.nih.gov/SNP), on OM-hydrolase activities. All the recombinant proteins were purified using their affinity tag, and the tag was cleaved from the proteins by thrombin treatment. SDS-PAGE analysis indicated the high levels of purity and complete tag cleavage; gel filtration analysis demonstrated that both mutants behaved in the same manner as the wild-type protein (unpublished data).

In the kinetic analysis, OM was hydrolyzed and converted to pharmacologically active olmesartan by the mutant proteins as well as the wild-type protein in a simple Michaelis-Menten kinetics manner (Fig. 6). As summarized in Table 1, the mutant Y155C showed slightly higher $K_m$ and lower $V_{\text{max}}$, and A150T showed slightly higher $V_{\text{max}}$. The intrinsic metabolic clearance calculated as the $V_{\text{max}}/K_m$ for the hydrolysis by the mutant Y155C was approximately half of that by the wild-type (13.1 and 29.5 ml/min/mg protein, respectively), whereas the mutant A150T showed an almost consistent value (35.4 ml/min/mg protein) as the wild-type.

**Discussion**

We had previously identified CMBL as one of the OM bioactivating hydrolases (Ishizuka et al., 2010), and in the present study further investigated the interindividual variability of mRNA and protein expression of CMBL and OM-hydrolase activity using 40 individual human liver and 30 intestinal specimens.

The clear positive correlation between OM-hydrolase activity and CMBL protein expression among 30 individual human intestinal cytosolic fractions demonstrated that CMBL is a major contributor to the OM bioactivation in the in vitro preparations. In our previous work, we demonstrated that the recombinant CMBL produced in mammalian cells exhibited OM-hydrolase activity; the $K_m$ value agreed well with those in human liver and intestinal cytosols, and that the chemical inhibition pattern in the recombinant CMBL was consistent with those in human liver and intestinal cytosols, where strong inhibition by $p$-chloromercuribenzoate, a free thiol modifier, and partial inhibition by bis-$p$-nitrophenylphosphate, a carboxylesterase inhibitor, were observed (Ishizuka et al., 2010), suggesting a substantial contribution of the enzyme to the OM hydrolysis in these in vitro preparations. However, the contribution of CMBL had yet to be demonstrated because specific chemical inhibitors or neutralizing antibodies against this novel enzyme are not available.
In our present study, the correlation analysis using a large number of human individual specimens revealed that CMBL must be the key enzyme responsible for OM bioactivation in human intestinal cytosol by showing a significant correlation between CMBL protein and the target activity, in addition to the previously published data on enzyme kinetics and chemical inhibition. The intestine is considered to be the first site of exposure of orally administered prodrug OM to be converted to its active moiety olmesartan by metabolic bioactivation. According to the computer-simulated intestinal first-pass availability ($F_g$) of the prodrug OM (Ishizuka et al., 2012), the majority of the prodrug is converted to active olmesartan in the intestinal epithelial cells. In fact, active olmesartan is reported to be the only species in human blood circulation (Laeis et al., 2001). Taken together, where the intestinal involvement of CMBL in the intestinal bioactivation of OM and the extensive conversion to olmesartan that may occur in the intestine, the evidence is convincing that the intestinal CMBL is the key enzyme in bioactivation of prodrug OM.

The distribution of OM-hydrolase activity, corresponding with CMBL protein expression, was not uniform along the length of the intestine. The activity was significantly higher in the proximal region (duodenum > jejunum > ileum) than in the distal region (colon), with some extent of variation within each region. Likewise, the distribution of most P450 enzymes representing the most important class in phase-I drug metabolism is not uniform along the length of the small intestine and is generally higher in the proximal regions of the small intestine (Zhang et al., 1999; Thelen and Dressman, 2009). As for other known hydrolases, there is a relative paucity of knowledge of enzyme distribution in human enterocytes despite the potential for intestinal hydrolases to play major roles in oral prodrug bioactivation in the drug absorption process. However, Imai and Ohura (2010) recently reported that intestinal mRNA expression of carboxylesterases belonging to the CES2 gene family (hCE2) and their activities are nearly constant along the jejunum and ileum, and Schwer et al. (1997) reported higher mRNA expression of hCE2 in the jejunum than the ileum. In our study, the CMBL protein and OM-hydrolase activity varied considerably, both with approximately 16-fold interindividual differences in the sample set from duodenum to colon. However, by focusing on the data of the 11 duodenum and 2 jejunum specimens that exhibited abundant CMBL distribution, the activity and protein content did not greatly vary, showing only a 3.0- and 2.4-fold difference.

Our computer simulation with the previously reported method (Ishizuka et al., 2012) estimated the $F_g$ value of the prodrug OM to be only 20%, even with the lowest activity (0.814 ng/min/mg protein) among 13 cytosolic fractions of the duodenum and jejunum in the present in vitro study. Notably, a clear positive correlation was observed between OM-hydrolase activity and the CMBL protein expression level among the 13 samples as well as in all 30 samples from the duodenum to colon (unpublished data). In addition, our in situ closed gastrointestinal loop experiment in rats presented high regional absorption rates of radiolabeled OM from the duodenal and jejunal loops compared with those of ileum and stomach (unpublished data), suggesting efficient bioactivation of OM owing to the agreement of the primary sites of OM absorption and bioactivation in the intestine.

From the analysis using a sample set of 40 individual human liver specimens, the variability seen in CMBL mRNA expression levels was much higher than those observed for the protein level and activity.
(136-, 4.1-, and 6.8-fold difference, respectively). Looking at carboxylesterases, Hosokawa et al. (1995) reported a more than 8-fold interindividual difference in hCES1 protein levels among 12 human liver microsomal preparations, and Xu et al. (2002) reported a 3-fold difference for hCES2 among 13 human liver microsomal preparations. In addition, age-dependent mRNA expression (adult > child > fetus) in the liver has been reported for both hCES1 and hCES2 (Yang et al., 2009), so further research is needed for CMBL to demonstrate its developmental expression. In the correlation analysis among the liver samples, OM-hydrolase activity exhibited a poor correlation with CMBL protein content in contrast with the analysis for the intestine. One possible reason for the poor correlation in liver cytosols is the significant contribution of other enzymes, including PON1, another OM-hydrolase (Ishizuka et al., 2012), which is localized in the liver microsomal fraction (Leviev et al., 1997; Marsillach et al., 2007) as well as in plasma. The possible contribution of the CMBL protein to OM hydrolysis might be obscured by contamination with PON1 from the liver microsomal fraction to cytosolic fraction during their separation process.

Moreover, we investigated the effects of two nonsynonymous SNPs rs35489000 and rs34487157 (NCBI dbSNP build 130; Supplemental Table 3), which result in the two single amino acid substitutions of Y155C and A150T, respectively. According to our previous work (Ishizuka et al., 2010), the prediction of a three-dimensional structure of CMBL protein showed that 155Tyr was located near the active center of the protein and could interact with its ligands. In accordance with that prediction, the mutant Y155C by the site-directed mutagenesis led to a decrease of the OM-hydrolase activity while the mutant A150T did not. In our present study, two outliers out of 40 individual livers demonstrated considerably low mRNA and protein expression levels of CMBL and consequently low OM-hydrolase activities. To elucidate whether the low activities arose from some genetic variations of CMBL, we genotyped those two nonsynonymous SNPs (rs35489000 and rs34487157) and six other SNPs chosen from NCBI dbSNP build 130 (rs10067744, rs1287736, rs1287735, rs3995688, rs687670, and rs10072686) (Supplemental Table 3) using DNA extracts from the liver specimens of 40 individual Caucasians. No mutations of the two nonsynonymous SNPs were detected among these, which is consistent with the minor allele frequencies in the Caucasian population reported in dbSNP (Supplemental Table 3). In addition, we found no relationship between the CMBL mRNA/protein/activity and the genetic variations of the other six SNPs, although variants were detected for all the SNPs with well-matched minor allele frequencies previously reported to dbSNP (Supplemental Table 3). Given the high metabolic clearance of intestinal CMBL that has been estimated using human intestinal cytosol (Ishizuka et al., 2010), it is unlikely that the mutation leads to a substantial in vivo decrease of the first-pass conversion rate of the prodrug OM into its active metabolite in human intestine. Also, plasma and hepatic esterase PON1 (Ishizuka et al., 2012) as well as hepatic CMBL are considered to play a supplemental role to complete the in vivo prodrug bioactivation. These multiple bioactivating enzymes in multiple sites allow us to disregard the genetic polymorphism in each bioactivating enzyme that may cause varying production of the pharmacologically active metabolite.

In the present study, no statistically significant sex-related differences were observed in the liver or intestinal CMBL protein levels or the OM-hydrolase activities. In the previous population pharmacokinetic analysis of olmesartan after oral administration of its prodrug OM in healthy volunteers and hypertensive patients (Yoshihara et al., 2005), female sex was reported to be one of the effective covariates of a lower apparent oral clearance. However, the observed change was slight and thus not considerable (13.5% lower in women than men) to adjust the dosage.

In conclusion, the correlation analysis using a number of individual human specimens clearly showed that CMBL is the key enzyme for OM bioactivation in the intestine, where the prodrug OM is practically and predominantly converted into its active metabolite olmesartan after oral administration. The interindividual variations in the OM-hydrolase activity and CMBL protein in the duodenum and jejunum, where the activity and protein were highly distributed, were relatively low, with only 3.0-fold and 2.4-fold differences, respectively. In the liver, the variations in the activity and protein were 4.1-fold and 6.8-fold differences, respectively.

Acknowledgments

The authors thank Ursula Hoepner and Christine Baumhauer for their technical assistance of the preparation of the human subcellular fractions and DNA extraction, and the preparation of mRNA, respectively; Osamu Ubukata for expert instruction for the site-directed mutagenesis experiments; and Dr. Yumi Nishiya and Satoru Yasuda for helpful support and advice on the human sample collection and in vitro metabolic experiments.
Authorship Contributions

Participated in research design: Ishizuka, Yoshigae, Kurihara.
Conducted experiments: Ishizuka, Rozehnal, Fischer, Kato.
Performed data analysis: Ishizuka, Rozehnal, Fischer, Kato, Endo.
Wrote or contributed to the writing of the manuscript: Ishizuka, Rozehnal, Yoshigae, Kurihara, Izumi.

References


Address correspondence to: Tomoko Ishizuka, Drug Metabolism and Pharmacokinetics Research Laboratories, Daiichi Sankyo Co., Ltd., 1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140-8710, Japan. E-mail: ishizuka.tomoko.xx@daiichisankyo.co.jp