The Major Genetic Defect Responsible for the Polymorphism of S-Mephenytoin Metabolism in Humans*

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The metabolism of the anticonvulsant drug mephenytoin exhibits a genetic polymorphism in humans, with the poor metabolizer trait being inherited in an autosomal recessive fashion. There are large interracial differences in the frequency of the poor metabolizer phenotype, with Oriental populations having a 5-fold greater frequency compared to Caucasians. Impaired metabolism of mephenytoin and a number of other currently used drugs results from a defect in a cytochrome P450 enzyme recently identified as CYP2C19. Attempts over the past decade to define the molecular genetic basis of the polymorphism have, however, been unsuccessful. We now report that the principal defect in poor metabolizers is a single base pair (G → A) mutation in exon 5 of CYP2C19, which creates an aberrant splice site. This change alters the reading frame of the mRNA starting with amino acid 215 and produces a premature stop codon 20 amino acids downstream, which results in a truncated, non-functional protein. We further demonstrate that 7/10 Caucasian and 10/17 Japanese poor metabolizers are homozygous for this defect, indicating that this is the major defect responsible for the poor metabolizer phenotype. Finally, the familial inheritance of the deficient allele was found to be concordant with that of the phenotypic trait.

Several genetic polymorphisms of drug metabolism have been documented in humans (1). One of the best characterized is that associated with the 4'-hydroxylation of the S-enantiomer of the anticonvulsant mephenytoin (2–4). Individuals can be characterized as either extensive (EM) or poor (PM) metabolizers. The latter phenotype is inherited in an autosomal recessive fashion (5, 6) with the EM phenotype comprising both the homozygous dominant and heterozygote genotypes. There are marked interracial differences in the frequency of this polymorphism. For example, the PM phenotype occurs in 2–5% of Caucasian populations but at higher frequencies (18–29%) in Oriental populations (2, 7). This polymorphism affects the metabolism of a number of other commonly used drugs, for example omeprazole (8), proganoil (9), certain barbiturates (10, 11), and citalopram (12). As a result, large interphenotypic differences occur in the disposition of these drugs, which may affect their efficacy and toxicity. The oxidation of propranolol (13), certain tricyclic antidepressants (14–16), and possibly diazepam (17) is also affected, albeit to a lesser extent. Recent studies have shown that CYP2C19 is the enzyme responsible for the 4'-hydroxylation of S-mephenytoin in human liver and that the levels of CYP2C19 protein correlate with microsomal S-mephenytoin 4'-hydroxylase activities in human livers (18, 19). However, the molecular basis of the PM phenotype is not known. The purpose of the present study was to determine the molecular genetic mechanism of the defect that is responsible for the polymorphism of S-mephenytoin metabolism in humans.

MATERIALS AND METHODS

Analysis of Human Liver Microsomes—Liver microsomes were prepared by differential centrifugation from 13 human liver samples selected from organ donors that had been previously characterized in vitro (20) as varying markedly in their S-mephenytoin 4'-hydroxylase activity. For immunoblot analysis of CYP2C19, liver microsomal proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and developed with a polyclonal antibody to CYP2C9 that also recognizes CYP2C19 using the ECL chemiluminescence kit (Amersham Corp.) as previously described (19). Results were confirmed with a specific peptide antibody to CYP2C19. Liver microsomal R- and S-mephenytoin 4'-hydroxylase activities were measured by high performance liquid chromatography analysis (21).

Amplification of CYP2C19 mRNA—Total liver RNA was isolated from liver samples using a single-step method (22) with Tri-reagent (Molecular Research Center Inc.) and reverse-transcribed as previously described (19). In initial experiments, the polymerase chain reaction (PCR) was used to amplify overlapping CYP2C19 cDNA fragments encompassing the full-length cDNA from three selected human liver samples, which had low microsomal S-mephenytoin 4'-hydroxylase activity; a high ratio of hydroxylation of the R/S enantiomers of mephenytoin (20), and the virtual absence of CYP2C19 by immunoblotting as described above. The cDNA was amplified in 1 x PCR buffer (67 mM Tris-HCl, pH 8.8, 17 mM (NH₄)₂SO₄, 10 mM β-mercaptoethanol, 7 μM EDTA, 0.2 mg/ml bovine serum albumin) containing 50 μM dATP, dCTP, and dTTP, and 0.25 μM PCR primers, 2.5 units of AmpliTaq DNA polymerase (Perkin-Elmer), and 1.0 mM MgCl₂. The amplification was performed using a Perkin-Elmer thermocycler for 30 cycles consisting of denaturation at 94 °C for 1 min, annealing at the appropriate temperature for 30 s, and extension at 72 °C for 1 min. An initial denaturation step at 94 °C for 3 min and a final extension step at 72 °C for 10 min were also performed. The PCR fragments were then subcloned into the Smal site of pBluescript II SK+ (Stratagene). Plasmids were purified with Qiagen kits and sequenced with an automated sequencer, using the cycle sequencing reaction employing fluorescence-tagged dye terminators (PRISM, Applied Biosystems).

RNA from all 13 human liver donors was subsequently reverse-transcribed and amplified using the forward primer G-ATGGAATGAAA-

The abbreviations used are: EM, extensive metabolizer; PM, poor metabolizer; PCR, polymerase chain reaction; bp, base pair(s).
CATCAGGATG-3' and the reverse primer 5'-GTAGTACGAGCTG-CAGTGAAT-3' and the strategy shown in Fig. 1 to detect aberrant splicing of exon 5. PCR conditions were similar to those described above. PCR products were analyzed on 3% agarose gels and stained with ethidium bromide. Selected PCR products were sequenced directly.

Phenotyping Procedures—The in vivo phenotype of most of the Swiss subjects was based on their "hydroxylation index" values (2), where a value above 5.6 identifies a PM. The phenotype of American subjects, Japanese subjects, and one Swiss subject was based on the urinary S/R ratio as described previously (3), with a poor metabolizer being defined as having a ratio >0.9. One American subject was of a rare intermediate phenotype characterized by the extent of 4'-hydroxylation being greater than in PMs, but with the rate of the metabolite's formation being slower than in EMs (23).

Genotyping Procedure—DNA was isolated (24) from human blood of selected Caucasian and Japanese subjects who had been previously phenotyped as described above. These populations contained an intended overrepresentation of PMs. PCR conditions were similar to those described previously, except that reactions used 200 ng of genomic DNA, 3 mm MgCl₂, and an initial denaturation at 94 °C for 5 min. The forward primer was 5'-AATTACACCCAGAGCGTGGC-3' and the reverse primer 5'-TATGCACTTCCATATAAGGAAG-3'. PCR products were restricted with Smal in the PCR buffer, without purification. Digested PCR products were analyzed on 4% agarose gels stained with ethidium bromide. PCR products of genomic DNA from three individuals who were homozygous-extensive, heterozygous-extensive, and homozygous-poor metabolizers (based on their Smal restriction digests and their in vivo phenotypes) were sequenced using an automated sequenator (Applied Biosystems). PCR products were purified using Microcon columns and sequenced using the same forward primer used in the PCR reaction.

RESULTS AND DISCUSSION

We initially amplified and sequenced overlapping CYP2C19 cDNA fragments from liver samples of selected human organ donors, which had low microsomal S-mephenytoin 4'-hydroxylase activity, a high ratio of hydroxylation of the R/S enantiomers of mephenytoin (20), and the virtual absence of CYP2C19 by immunoblotting. One aberrant CYP2C19 cDNA fragment was identified with a 40-bp deletion at the beginning of exon 5 (from bp 643 to bp 682), which included the deletion of a Smal restriction site. This alteration results in the deletion of amino acids 215-227 and shifts the reading frame beginning at amino acid 215, producing a premature stop codon 20 amino acids downstream. The resultant truncated 234-amino acid protein would lack the heme-binding region and, therefore, would be catalytically inactive.

RNA was then reverse-transcribed from a total of 13 liver donors, which were selected based on a wide range of S-mephenytoin hydroxylase activities, and the virtual absence of CYP2C19 by immunoblotting. One aberrant CYP2C19 cDNA fragment was identified with a 40-bp deletion at the beginning of exon 5 (from bp 643 to bp 682), which included the deletion of a Smal restriction site. This alteration results in the deletion of amino acids 215-227 and shifts the reading frame beginning at amino acid 215, producing a premature stop codon 20 amino acids downstream. The resultant truncated 234-amino acid protein would lack the heme-binding region and, therefore, would be catalytically inactive.

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RNA was then reverse-transcribed from a total of 13 liver donors, which were selected based on a wide range of S-mephenytoin hydroxylase activities as determined in vitro, and the PCR strategy shown in Fig. 1A was used to amplify the region encompassing the 40-bp deletion. Only the aberrantly spliced mRNA (244-bp fragment) was found in sample 35, the characteristic of which was most consistent with the PM phenotype (low liver microsomal S-mephenytoin 4'-hydroxylase activity and a ratio of >1.0 for metabolism of the R/S enantiomers) (Fig. 1B). In contrast, liver samples with the highest S-mephenytoin 4'-hydroxylase activities and R/S ratios of <0.1 contained only the normally spliced mRNA, while several liver samples with intermediate catalytic activities, R/S ratios of 0.1-0.8, and low CYP2C19 levels possessed both types of spliced mRNA. The relatively low liver microsomal S-mephenytoin 4'-hydroxylase activities in some of the heterozygotes may simply reflect the selection process, which included samples with highly variable S-mephenytoin 4'-hydroxylase activities.

The genomic sequence of CYP2C19 is not currently known; hence, primers for the intron 4/exon 5 junction were developed empirically. This involved the use of multiple primers for intron 4 based on the sequence of this region in CYP2C9 (25), which is a closely related gene that shows 95% similarity to CYP2C19 in the upstream region and several exons, and a specific reverse primer for exon 5 of CYP2C19. One primer pair amplified a DNA fragment with the correct predicted size in both EMs and PMs; however, only the fragment from EMs could be digested with SmaI. Sequencing of this fragment yielded sequence information from which a specific forward primer was generated and used in subsequent PCR reactions to genotype individuals.

DNA from 28 unrelated Swiss and American Caucasian subjects whose phenotype had been established in vivo was amplified using specific primers and restricted with SmaI using the strategy outlined in Fig. 2A. Fig. 2C shows that 11 of 17 EMS were homozygous for the normal CYP2C19 gene and 6 EMs were heterozygous. Among the 10 PMs, 7 were homozygous for

Fig. 1. The splicing patterns of CYP2C19 mRNA in 13 selected human liver donor samples exhibiting a wide range of S-mephenytoin 4'-hydroxylase activities and CYP2C19 protein levels. A, diagram of strategy used to amplify CYP2C19 transcripts from human liver samples. This PCR strategy yielded a 284-bp band for the normal cDNA (wt/wt), a 244-bp band for the aberrant cDNA (m/m), and both bands with CDNA from heterozygous (wt/m) individuals. The hatched area indicates the 40-bp deletion in exon 5 of the aberrant cDNA. B, relationship between genotype as assessed by reverse transcription PCR of human liver mRNA, hepatic content of CYP2C19 protein estimated by immunoblotting, liver microsomal S-mephenytoin 4'-hydroxylase activities, and the ratio of metabolism of the R/S enantiomers of mephenytoin in vitro. Arrows indicate the migration of recombinant CYP2C9 and CYP2C19 protein standards on the immunoblot (19).

2. S. M. F. de Morais, J. Blaisdell, and J. A. Goldstein, unpublished data.
Fig. 2. Analysis of genomic DNA. A, diagram showing the strategy used to genotype genomic DNA from human blood. The strategy utilizes a PCR reaction followed by Smal digestion. The predicted sizes of digested DNA fragments are shown. B, diagram of genotypic tree of proband (arrow) and the gel of Smal-digested PCR products. The genotype agrees with the previously published and indicated phenotype of Family C (5). C, analysis of genomic DNA from selected Caucasian subjects from Switzerland and the United States. The phenotype (EM, IM (intermediate phenotype), or PM) is indicated by the brackets above the gel. D, analysis of genomic DNA from selected Japanese subjects. M represents the molecular weight markers.

the aberrant gene, 1 individual was heterozygous, and 2 were homozygous for the wild-type gene. The individual with the rare intermediate phenotype was also heterozygous. Thus, the mutant CYP2C19 accounted for 15 of the 20 alleles (75%) tested in Caucasian PMs.

Oriental populations have a much greater frequency of the mephenytoin PM phenotype compared to Caucasians (2, 3, 7). Accordingly, we analyzed DNA from 29 unrelated Japanese subjects (Fig. 2D). Eight of the 12 EMS were homozygous and 4 were heterozygous for CYP2C19. CYP2C19 accounted for a similar percentage (74%) of the alleles (25 of 34) in Japanese PMs as found in Caucasian PMs. Ten of 17 PMs were homozygous for the mutant allele, and 5 were heterozygous. Thus, the major mutation responsible for the PM phenotype in Japanese is identical to that found in Caucasians. However, the defect was not present in all PMs regardless of whether they were Caucasian or Japanese. It is therefore likely that additional mutations exist, which result in the PM phenotype in both populations. In a similar manner, a point mutation at a splice site consensus sequence is the single most common mutation in CYP2D6, accounting for >75% of mutant alleles in PMs of debrisoquine (26, 27). However, several minor mutant alleles have also been identified that contribute to this phenotype.

We also genotyped a Japanese family that had been studied previously with respect to the inheritance of the PM trait (5) (Fig. 2B). There was complete concordance between the CYP2C19 genotype and the in vivo phenotype consistent with the previously reported Mendelian autosomal recessive mode of inheritance (5, 6).

DNA from individuals representative of the two CYP2C19
genotypes was amplified as described above and then directly sequenced (Fig. 3). The sequence information verified that only CYP2C19 was amplified in the genotyping test. Surprisingly, the sequence of intron 4 of the defective gene was identical to that of the normal gene. The only difference was a (11YNCAAGG) (Y, pyrimidine; N, any base) for the 3' splice site of the normal and cryptic splice junctions. Moreover, we have amplified the exon 4-exon 5 junction of the defective gene was identical to that of the normal gene. The only difference was a (11YNCAAGG) (Y, pyrimidine; N, any base) for the 3' splice site. There are also YYYYYYYYYYYYYNCAGG as the normal 3' splice site.

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