POLYMORPHISMS IN HUMAN ORGANIC ANION TRANSPORTING POLYPEPTIDE 1A2 (OATP1A2): IMPLICATIONS FOR ALTERED DRUG DISPOSITION AND CNS DRUG ENTRY

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SUMMARY

Organic anion transporting polypeptide 1A2 (OATP1A2), is a drug uptake transporter known for broad substrate specificity, including many drugs in clinical use. Therefore, genetic variation in \textit{SLCO1A2} may have important implications to the disposition and tissue penetration of substrate drugs. In the present study, we demonstrate OATP1A2 protein expression in human brain capillary and renal distal nephron using immunohistochemistry. We also determined the extent of single nucleotide polymorphisms (SNPs) in \textit{SLCO1A2} upon analyses of ethnically defined genomic DNA samples (n=95 each for African-, Chinese-, European-, and Hispanic-Americans). We identified six nonsynonymous polymorphisms within the coding region of \textit{SLCO1A2} [T38C (Ile13Thr), A516C (Glu172Asp), G559A (Ala187Thr), A382T (Asn128Tyr), A404T (Asn135Ile) and C2003G (Thr668Ser)] whose allelic frequencies appeared to be ethnicity-dependent. \textit{In vitro} functional assessment revealed that the A516C and A404T variants had markedly reduced capacity for mediating the cellular uptake of OATP1A2 substrates, estrone 3-sulfate and two delta-opioid receptor agonists, deltorphin II and [D-penicillamine$^{2,5}$]-enkephalin. On the other hand, the G559A and C2003G variants appeared to have substrate-dependent changes in transport activity. Cell surface biotinylation and immunofluorescence confocal microscopy suggested that altered plasma membrane expression of the transporter may contribute to reduced transport activity associated with the A516C, A404T and C2003G variants. The A404T (Asn135Ile) variant also showed a shift in the apparent molecular size, indicative of alterations in glycosylation status. Taken together, these data suggest that \textit{SLCO1A2} polymorphisms may be an important, yet unrecognized contributor to inter-individual variability in drug disposition and central nervous system entry of substrate drugs.
FUNCTIONAL OATP1A2 POLYMORPHISMS AND DRUG TRANSPORT

INTRODUCTION

During the past decade there has been an increasing recognition of the critical interplay between drug transporters and drug metabolizing enzymes as determinants of drug disposition and response. Indeed, the extent of targeted tissue entry for many drugs may be facilitated by drug transporters, which are often expressed in a tissue-specific manner with broad substrate specificities. Among the uptake transporters, members of the organic anion transporting polypeptides (human: OATPs, rodents: Oatps) have been shown to be expressed in organs such as the central nervous system (CNS), liver, and intestine and mediate the cellular uptake of a large number of structurally divergent compounds (1). Within this family, OATP1A2 (SLCO1A2, also known as human OATP-A or OATP1) was the first human OATP to be cloned and characterized (2). OATP1A2 mRNA has been detected in various tissues including the brain, liver and kidney (2,3). Substrates of OATP1A2 include endogenous compounds such as bile acids, steroid hormones and their conjugates, thyroid hormones as well as drugs including fexofenadine, ouabain, peptides (e.g. deltorphin II, [D-penicillamine\textsuperscript{2,5}]-enkephalin, DPDPE) and the toxin, microcystin (2,4-7). In humans, OATP1A2 has been reported to be the only OATP transporter detected in the brain capillary endothelium at present, suggesting that OATP1A2 may play a critical role in the CNS penetration of many drugs and hormones across the blood brain barrier (BBB) (7,8). Given the extent of its substrate specificity and expression in organs of importance to drug disposition and response, genetic variations in SLCO1A2 may have significant pharmacologic and toxicologic consequences.

In the present study, we were able to confirm the expression of OATP1A2 at the level of the brain capillaries which make up the BBB. Moreover, we describe the identification and
functional characterization of SNPs in SLCO1A2 from a population of European-, Chinese-, Hispanic- and African-Americans. Genotypic frequencies of six nonsynonymous polymorphisms within the coding region of SLCO1A2 were dependent on ethnicity and some of the genetic variants were associated with markedly reduced uptake transport activity for estrone 3-sulfate and two delta-opioid receptor agonists, [D-penicillamine$^{2,5}$]-enkephalin and deltorphin II. Our data indicate that SLCO1A2 polymorphisms may contribute to inter-individual variability in drug disposition and may be a heretofore unrecognized factor governing the CNS entry of some drugs.
EXPERIMENTAL PROCEDURES

**Materials**—Radiolabeled $[^3]H]$estrone 3-sulfate (53 Ci/mmol), [Tyrosyl-3,5-$[^3]H$]deltorphin II (2-D-Ala, 38.5 Ci/mmol), [Tyrosyl-2,6-3H(N)]enkephalin (D-Penicillamine$^{2,5}$, DPDPE, 34.0 Ci/mmol) and unlabeled estrone3-sulfate were purchased from PerkinElmer Life Sciences (Boston, MA). Unlabeled deltorphin II and DPDPE were obtained from Bachem (King of Prussia, PA). Recombinant vaccinia virus containing the T7 RNA polymerase gene (vtf-7) was a gift provided by Dr. Bernard Moss (National Institutes of Health, Bethesda, MD). The pEF6/V5-His-TOPO® expression vector and the monoclonal mouse anti-V5 antibody were purchased from Invitrogen (Carlsbad, CA). Genomic DNA isolated from peripheral blood lymphocytes of healthy European-, African-, Chinese-, and Hispanic-American volunteers was purchased from Coriell Cell Repositories (Camden, NJ). Enzymatic deglycosylation kit was obtained from Glyko (San Leandro, CA) and tunicamycin was purchased from Calbiochem (San Diego, CA). Immunohistochemistry reagents were obtained from Biogenex (San Ramon, CA). All other chemicals and reagents, unless stated otherwise, were obtained from Sigma (St. Louis, MO) and were of the highest grade available.

**Immunohistochemistry of normal human brain, kidney and liver sections**—The rabbit polyclonal antiserum against human OATP1A2 was custom-made using a synthesized C-terminal polypeptide of OATP1A2 (ECKDIYKSTVLDDELKTL, Invitrogen) as the epitope. Paraffin-embedded sections from normal human brain, kidney and liver tissues (5 µm) were obtained from Human Tissue Acquisition Shared Resources at Vanderbilt-Ingram Cancer Center. Histopathological analyses were performed on H&E-stained sections from the paraffin tissue specimens. Sections were deparaffinized using EZ-Dewax (Biogenex), followed by the antigen retrieval procedure using Retrieve-It® (pH 8, Biogenex) in the case of brain and liver.
tissue sections. Sections were incubated with the peroxidase blocking reagent (Biogenex) for 10 min at room temperature to quench endogenous peroxidase activity. After rinsing with PBS, sections were incubated with blocking buffer (10% powerblock, Biogenex) for 60 min and subsequently incubated with anti-OATP1A2 antiserum diluted in blocking buffer (1:100 ~ 1:500 dilutions) for 2 hours at room temperature. After three washes, sections were incubated with the avidin and biotin blocking reagents (Avidin-biotin blocking kit, Biogenex) in order to reduce non-specific background staining due to endogenous biotin. After three washes with PBS containing 0.4% Triton-X 100, sections were incubated with biotinylated anti-rabbit IgG (Biogenex) for 20 min and then with streptavidin-HRP conjugate (Biogenex) for 20 min. After washes, the immune reaction was visualized using 3-amino, 9-ethyl-carbazole (AEC, Biogenex) and nuclei were counter-stained with hematoxylin (Biogenex). The specificity of immunoreactive signals for OATP1A2 was verified by various negative controls, which were incubated with the rabbit preimmune serum, blocking buffer or polyclonal antiserum that had been neutralized by pre-incubation with the antigenic peptide at 37 °C for 2 hours.

Identification of SNPs in SLCO1A2 and determination of genotypic frequencies—Initially, total genomic DNA was isolated from blood samples obtained from healthy volunteer subjects (46 European- and 32 African-Americans) residing in middle-Tennessee who were judged to be healthy on the basis of medical history, physical examination and laboratory test indicative of normal cardiac, renal and liver function. The protocol was approved by the Vanderbilt University Institutional Review Board and informed consent was obtained. Further analyses of genetic variations in the human SLCO1A2 gene used ethnically defined genomic DNA samples from healthy volunteers (European-, African-, Chinese- and Hispanic-American, n=95 each). Initial
screening involved the amplification of the 14 exonic regions of *SLCO1A2*, followed by nonisotopic single-strand conformational polymorphism (SSCP) analysis. PCR was typically carried out at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s for 30 cycles using 25 ng of genomic DNA previously digested with the restriction endonuclease *XhoI* (New England Biolabs, Inc., Beverly, MA). Variations in the single-strand mobility patterns were clearly visualized by ethidium bromide staining. A search of available SNP databases, including PharmGKB (http://www.pharmgkb.org), IMS-JST Japanese SNP (http://snp.ims.u-tokyo.ac.jp), NCBI dbSNP (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=snp) and GeneCards (http://bioinfo.weizmann.ac.il/cards), was performed to identify any additional polymorphisms. The genotypic frequencies of the nonsynonomous SNPs identified by SSCP and available database analyses (T38C, A516C, G559A, A382T, A404T and C2003G) were determined by either direct sequencing (ABI 3700 DNA Analyzer, Applied Biosystems Inc., Foster City, CA) or temperature-dependent capillary electrophoresis (Reveal®, SpectruMedix, State College, PA) of PCR products.

**Generation of Wild-type and Variant SLCO1A2 Expression Plasmids**—Original *SLCO1A2* plasmid containing the full-length cDNA in the pSPORT vector (pSPORT®/SLCO1A2) was a gift from Dr. P. Meier (University Hospital Zurich, Zurich, Switzerland). The full open reading frame (ORF) of human *SLCO1A2* cDNA was amplified by PCR using pSPORT®/SLCO1A2 as the template and ligated into the pEF6/V5-His-TOPO® vector (Invitrogen). The PCR primer pairs (5'-ATGGGAGAACTGAGAAAAGA-3' and 5'-CAATTTAGTTTTCATTCATCATC-3') were designed to amplify *SLCO1A2* ORF without the stop codon in order to generate epitope-tagged proteins when ligated into the pEF6/V5-His-TOPO® vector. Both pSPORT®/SLCO1A2
and pEF6/V5-His-TOPO®/SLCO1A2 with the SLCO1A2 ORF inserted in the sense orientation downstream from the T7 promoter region were verified by direct sequencing and found to fully match the published reference sequence (GenBank™ accession number NM_021094). This clone was termed SLCO1A2*1 and the position of the first base (“A”) of translation start site was assigned as “+1” position. Site-directed mutagenesis using the QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA) was performed to create the identified nonsynonymous allelic variants: T38C (Ile13Thr, *2), A516C (Glu172Asp, *3), G559A (Ala187Thr, *4), A382T (Asn128Tyr, *5), A404T (Asn135Ile, *6) and C2003G (Thr668Ser, *7). Presence of each mutation was sequence-verified.

Expression of Variant SLCO1A2 Alleles—For transport studies, HeLa cells grown in 12-well plates (~0.8x10⁶ cells/well) were infected with vaccinia at a multiplicity of infection of 10 pfu/cell in serum-free Opti-MEM I medium (Invitrogen) and allowed to adsorb for 30 min at 37 °C. Cells in each well were then transfected with 1 µg of wild-type or variant SLCO1A2 cDNA packaged into either pEF6/V5-His-TOPO® or pSPORT® vector (Invitrogen), along with Lipofectin™ (Invitrogen) and incubated at 37 °C for 16 h. The parental plasmid lacking any insert was used as vector control. Transport was then evaluated using labeled drug substrates as outlined previously (9). In order to measure estrone 3-sulfate, deltorphin II and DPDPE transport kinetics, radiolabeled drug uptake during the linear phase (first 3 min) was assessed in the presence of varying concentrations of unlabeled respective compounds. Passive diffusion was determined by carrying out parallel experiments using the parental plasmid DNA lacking the transporter cDNA insert, and this value was then subtracted from the total uptake rate seen in the presence of the transporter cDNA. Michaelis-Menten-type nonlinear curve fitting was carried out to obtain estimates of the maximal uptake rate (Vmax) and the concentration at which half the
maximal uptake occurs (Km) (Prism™, GraphPad, San Diego, CA). All experiments were carried out in duplicate on at least 2-3 experimental days.

**OATP1A2 cell surface expression**—HeLa cells were grown on 6-well plates and transfected with wild-type or variant SLCO1A2 cDNA packaged into pEF6/V5-His-TOPO® using a similar protocol for transport experiments. Sixteen hours post-transfection, cells were washed with ice-cold PBS-Ca²⁺/Mg²⁺ (138 mM NaCl₂, 2.7 mM KCl, 1.5 mM KH₂PO₄, 9.6 mM Na₂HPO₄, 1 mM MgCl₂, 0.1 mM CaCl₂, pH 7.3) and then treated with a membrane-impermeable biotinylating agent (sulfo-N-hydroxysuccinimide-SS-biotin, 1.5 mg/ml, Pierce, Rockford, IL) at 4 °C for 1 h. Subsequently, the cells were washed three times with ice-cold PBS-Ca²⁺/Mg²⁺ containing 100 mM glycine and then incubated for 20 min at 4 °C with the same buffer to remove the remaining labeling agent. After washing with PBS-Ca²⁺/Mg²⁺, cells were disrupted with 700 µl of lysis buffer (10 mM Tris base, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, pH 7.4) containing protease inhibitors (Complete, Roche Applied Science, Indianapolis, IN) at 4 °C for 1 h with constant agitation. Following centrifugation, 140 µl of streptavidin-agarose beads (Pierce) was added to 600 µl of cell lysate and incubated for 1 h at room temperature. Beads were washed four times with ice-cold lysis buffer, and the biotinylated proteins were released from the beads by adding 4x Laemmli buffer and boiling for 10 min at 95 °C. Similar to the biotinylated (cell surface-expressed) fractions, total cell lysates were subjected to Western blotting analysis for detection of immunodetectable OATP1A2 with monoclonal anti-V5 antibody (1:5000 dilution, Invitrogen) and the intracellular, endoplasmic reticulum-resident protein calnexin with polyclonal anti-calnexin antibody (1:1000 dilution, StressGen, Victoria, British Columbia, Canada) as described previously (10).
**Immunofluorescence Confocal Microscopy**—HepG2 and HeLa cells were grown on sterile uncoated 35-mm glass-bottomed microwell dishes (MatTek, Ashland, MA) and transfected at 70-80% confluency with 2 µg of V5-tagged wild-type or variant *SLCO1A2* alleles (*SLCO1A2*~1 ~ *7) using LipofectAMINE 2000 (Invitrogen). After 48 h, cells were fixed for 10 min in ice-cold 70% methanol. Cells were then permeabilized for 10 min in PBS containing 0.3% Triton X-100 (PBT). After rinsing with PBS, cells were placed in blocking buffer (2% bovine serum albumin in PBS) for 1 h at room temperature. Cells were then incubated with monoclonal antibody against V5 epitope (diluted 1:500 in blocking buffer) for 2 h at room temperature. After three washes in PBS containing 0.05% Tween 20 (PBST), cells were incubated with secondary goat anti-mouse antibody labeled with the fluorescent dye, Texas Red (Molecular Probes, Eugene, OR) for 30 min at 37 °C. During the final washes in PBST, SYTOX® Green (Molecular Probes, Eugene, OR) was added for nucleic acid staining. Confocal microscopy was performed with a Zeiss Axiovert 100-M inverted microscope equipped with a LSM510 laser scanning unit. A Zeiss 63x 1.4 numerical aperture plan Apochromat oil immersion objective was used for all experiments. Confocal images were obtained using single excitation (595 nm) and emission (610-630 nm Texas Red) filter sets. Cells transfected with the parental plasmid lacking any insert were used as control. Cells transfected with V5-tagged *SLCO1A2*~1 plasmid without incubation with primary antibody were also used as an additional control. For slow frame scanning, confocal images were obtained by scanning either laterally (top view, x-y scans) or axially (side view, x-z scans) across the cell. Image analysis and processing were performed with Zeiss LSM and Adobe Photoshop software.
Deglycosylation of Total and Cell Surface-expressed OATP1A2—HeLa cells were grown on 6-well plates and transfected with V5-tagged SLC01A2*1 and *6 using a protocol similar to that for the transport experiments. Total and cell-surface expressed OATP1A2 variants (*1 and *6) were isolated as described previously and subjected to enzymatic deglycosylation according to the instructions provided by the manufacturer (Glyko, San Leandro, CA). Briefly, total or cell surface-expressed OATP1A2 protein samples were subjected to denaturation and enzymatic deglycosylation by N-glycanase, sialidase A, and O-glycanase. Samples were separated by 7.5% SDS-PAGE and analyzed by Western blotting for detection of immunoreactive proteins with monoclonal anti-V5 antibody (1:5000 dilution). In separate experiments, tunicamycin, an inhibitor of the N-linked glycosylation of proteins was added to a final concentration of 0.3 μg/ml during 16-hour transient transfection period. Total and cell surface-expressed OATP1A2 protein samples were isolated and analyzed by Western blotting as described previously.

Statistical Analysis—Determination of the statistical differences between various group parameters was determined using analysis of variance (using Newman-Keuls multiple comparison test). p-Values of <0.05 were considered to reflect statistical significance.
RESULTS

**OATP1A2 Immunohistochemistry**—Immunohistochemical staining of OATP1A2 was performed in order to define the tissue distribution of OATP1A2 in the human brain and kidney using rabbit polyclonal antiserum against human OATP1A2. Different dilutions of the antiserum (1:100 ~ 1:500) were used to optimize the detection of different expression levels in immunohistochemical analyses. Non-specific background staining was absent even when immunohistochemical staining was performed with the highest antiserum concentration utilized (1:100). In normal human brain cortex sections, immunohistochemistry detected OATP1A2 expression almost exclusively in endothelial capillary cells (Fig. 1A). Preincubation of the polyclonal antiserum against OATP1A2 with the antigenic peptide abolished positive staining in brain capillary (Fig. 1A, inset). OATP1A2 expression in human kidney was localized to the apical domain of distal nephrons (Fig. 1B). In human liver sections, OATP1A2 expression appeared to be localized to the cholangiocytes (Fig. 1C).

**Genetic Variations in Human SLCO1A2 Gene**—Initial SSCP analyses of all 14 exons of *SLCO1A2* (using genomic DNA samples from 46 European- and 32 African-Americans) and a search of available SNP databases identified 11 nonsynonymous SNPs. Based on the reported genotypic frequencies and putative location of amino acid changes, we determined the allelic frequencies of six nonsynonymous SNPs (T38C, A516C, G559A, A382T, A404T and C2003G) using ethnically-defined genomic DNA (European-, African-, Chinese- and Hispanic-American, n=95 each) (Table I). T38C and A516C polymorphisms were more common in European-Americans (11.1% and 5.3%, respectively) than Hispanic- and African-Americans while these SNPs were not observed with Chinese-Americans. G559A variation was observed only in Hispanic-Americans (0.5%) while A382T was found only in African-Americans (1.0%).
However, A404T polymorphism was not detected in genomic DNA samples used in the present study. G2003G variation was observed in African- and Hispanic-Americans with varying allelic frequencies of 3.7% and 1.0%, respectively. None of the six nonsynonymous SNPs tested were observed in Chinese-Americans.

**Transport Properties of SLCO1A2 Variant Alleles**—An array of expression plasmids comprising 7 SLCO1A2 allelic variants (*1 ~ *7) were transiently overexpressed using the recombinant vaccinia system (vtf-7). OATP1A2*1 was capable of transporting known substrates, such as estrone 3-sulfate (Fig. 2A), deltorphin II (Fig. 2B) and DPDPE (Fig. 2C). To determine the relative transport efficiencies of the OATP1A2 variants, we compared the total cellular uptake (0.5 µM) at 30 min. Transport activities associated with OATP1A2*3 (A516C) and *6 (A404T) variants were significantly lower than the reference allele (*1) for all three substrates tested. The OATP1A2*4 (A404T) variant exhibited markedly reduced transport activity toward estrone 3-sulfate, but not toward deltorphin II and DPDPE. In contrast, the OATP1A2*7 (C2003G) variant appeared to have the reduced cellular uptake of deltorphin II and DPDPE, but normal uptake activity for estrone 3-sulfate.

For a more comprehensive characterization, concentration-dependent uptake kinetics, utilizing uptake data during the linear phase (first 3 min), of estrone 3-sulfate and deltorphin II were examined for all seven allelic variants (Figs. 3 and 4). Consistent with initial screening results, some of the OATP1A2 variants (e.g. *3, A516C and *6 A404T) possessed significantly lower Vmax values for both estrone 3-sulfate and deltorphin II than the reference allele (OATP1A2*1). On the other hand, the OATP1A2*4 (A404T) variant showed significantly altered Vmax and Km values.
values only for estrone 3-sulfate, but not for deltorphin II. The other variants, OATP1A2*5 (A382T) and *7 (C2003G) also appeared to have altered Vmax values with deltorphin II. Due to low Vmax values, it was not feasible to obtain reliable Km values for those variants.

**Total and Cell Surface Expression of OATP1A2 Variants**—Immunoblot analyses of total OATP1A2 protein expression (Fig. 5A) indicated that the reduced transport activity of some OATP1A2 variants (*6, A404T and *7, C2003G) may be associated with alterations in total protein expression. In addition to the band corresponding to the expected molecular size of ~74-kDa (2), the blots showed multiple band patterns, suggestive of various glycosylation status (Fig. 5, indicated by the arrows, a, b and c) as well as possible dimer formation (Fig. 5, indicated by the arrow, d). Interestingly, the OATP1A2*6 (A404T) variant also exhibited a shift in the mobility of immunoreactive bands, indicative of altered glycosylation status (Fig. 5). We also examined whether differences in cell surface expression could account for the decreased transport capacity of variant OATP1A2 transporters. Cell surface biotinylation experiments were performed to specifically capture plasma membrane-associated OATP1A2 (Fig. 5B). Similar to that seen with total cell lysate, OATP1A2*6 (A404T) and *7 (C2003G) variants showed significantly lower cell surface-associated expression of immunoreactive proteins. For OATP1A2*3 and *5, lower intensity of bands of ~71 and 100 kDa were noted (Fig. 5B). The enrichment of cell surface proteins within the biotinylated fractions was evidenced by the lack of immunodetectable calnexin (an intracellular protein) in the samples (Fig. 5B). In addition, expression of OATP1A2*6 (A404T) variant was associated with showed altered mobility the immunoreactive protein, indicative of possible changes in glycosylation status.
Deglycosylation of Total and Cell Surface-expressed OATP1A2—When expressed in HeLa cells, the total and cell surface-expressed OATP1A2 protein appeared to possess multiple molecular masses including the predicted (unglycosylated) size (∼59 kDa), as well as larger molecular weight forms of ∼71, 100, and 150 kDa (Figs. 5A-B). We performed enzymatic deglycosylation experiments to determine whether this apparent difference reflects various glycosylation status of total and cell surface-expressed OATP1A2. In addition, we also compared the deglycosylation pattern of the OATP1A2*6 (A404T) variant (which showed altered mobility in Fig. 5A) with that of OATP1A2*1. When subjected to enzymatic N-deglycosylation, both total and cell surface-expressed fractions of OATP1A2*1 and *6 showed a significant shift in mobility, resulting in similar apparent molecular sizes (Fig. 6). These observations are consistent with the presence of additional N-glycosylation sites in OATP1A2 protein as predicted by the NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/) and ScanProsite algorithms (http://us.expasy.org/tools/scanprosite/); five putative N-glycosylation sites were identified, including Asn residues at the amino acid positions of 62, 124, 412 and 483 as well as the 135 position corresponding to the OATP1A2*6 variant. Chemical deglycosylation experiments were also carried out using tunicamycin, an inhibitor of Asn-linked glycosylation. Similar to enzymatic deglycosylation results, tunicamycin treatment shifted the molecular masses of the immunoreactive bands of both total and cell surface-expressed OATP1A2*1 and *6 protein to the similar extent as observed with enzymatically N-deglycosylated samples (Fig. 6). These results confirm that Asn135 is a key glycosylation site in OATP1A2.

Plasma Membrane Localization of OATP1A2 Variants Using Immunofluorescence Analysis—Cell surface biotinylation experiments and Western blot analysis of total cell lysates
in transfected HeLa cells suggested that the reduced transport activities of certain OATP1A2 variants may be due to altered trafficking of the protein to the cell surface. To further assess our findings, we performed immunofluorescence confocal microscopy experiments from the liver-driven HepG2 as well as HeLa cells following transient transfection with the V5-tagged \textit{SLCO1A2} variants (*1 ~ *7). Using primary antibody against V5 epitope and secondary antibody labeled with Texas Red, we observed that wild-type OATP1A2*1 protein was targeted to the plasma membrane of both HepG2 and HeLa cells when viewed axially (x-z scans) and laterally (x-y scans) (Fig. 7). Expression pattern of certain OATP1A2 variants including, OATP1A2*3 (A516C), *6 (A404T) and *7 (C2003G), suggested substantial alterations in cell surface trafficking in both HepG2 and HeLa cells (Fig. 7).
DISCUSSION

Drug absorption, elimination and targeted tissue penetration are now widely recognized to be significantly dependent on drug uptake and efflux transporters (11,12). While the extent of our knowledge in terms of efflux transporters such as MDR1 (p-glycoprotein) is quite significant, only recently has there been the appreciation of the presence and importance of drug uptake transporters to the overall drug disposition process. A family of solute carriers, broadly referred to as OATP appears to have particular relevance to drug disposition. Targeted expression of OATPs in organs such as the liver, intestine, kidney and brain seems to alter the extent of drug bioavailability and possibly CNS penetration. Recently, a new classification and nomenclature system (OATP/SLCO superfamily) has been implemented to permit an unequivocal and species-independent identification of OATP genes and gene products (1). Clearly, a better understanding of OATP transporters at the molecular level is likely to aid the development of drugs with more predictable bioavailability and efficacy profiles.

In this report, we describe the differential expression pattern of OATP1A2 in human brain and kidney and the identification and functional characterization of nonsynonymous SNPs in SLCO1A2. We first assessed the expression of OATP1A2 using multiple human organ tissues via immunohistochemistry. We utilized an antibody raised against the C-terminal of OATP1A2 which appeared to be specific when compared to antibodies generated against OATP1B1 (OATP-C) and OATP1B3 (OATP-8) (data not shown). In the liver, OATP1A2 expression was localized specifically to cholangiocytes, but not in hepatocytes. In the kidney, OATP1A2 was expressed specifically on the apical side of distal nephrons. Consistent with the reported literature (7), we detected OATP1A2 mainly in the brain capillary.
Genotypic analyses of subjects from various ethnic population revealed that the allelic frequencies of six identified SNPs in \textit{SLCO1A2} are dependent on ethnicity (Table I), although the extent of genetic variation appeared modest compared to other members of OATPs such as OATP1B1 and OATP2B1 (10,13). When the transport function of the wild-type and six \textit{SLCO1A2} allelic variants was assessed \textit{in vitro}, the OATP1A2*3 (A516C, Glu172Asp) and *6 (A404T, Asn135Ile) variants demonstrated markedly reduced transport activity toward all three substrates tested, i.e. estrone 3-sulfate, deltorphin II and DPDPE (Figs. 2 - 4). Other OATP1A2 variants showed altered transport activity in a substrate-specific manner (Figs. 3 and 4). For example, OATP1A2*4 (G559A, Ala187Thr) exhibited reduced uptake transport activity with estrone 3-sulfate, but not with deltorphin II and DPDPE. However, the opposite was found to be the case with OATP1A2*5 (A382T, Asn128Tyr) and *7 (C2003G, Thr668Ser)

Upon analysis of transport kinetic data, it was evident that mutations at codons 172 (OATP1A2*3), 187 (OATP1A2*4), 128 (OATP1A2*5), 135 (OATP1A2*6) and 668 (OATP1A2*7) have functional consequences. An OATP1A2 membrane topology map based on hydropathy analysis suggested that mutations associated with OATP1A2*3 (A516C) and *6 (A404T) are located in the transmembrane domain 4 and the second external loop, respectively (Fig. 8). Both mutations appear to be located in relatively conserved amino acid sequence regions in comparison to other related OATP members. These regions have been suggested to have a potentially important function in terms of substrate specificity based on sequence homology with a evolutionarily primitive Oatp cloned from skate liver (14). Reduced transport activities of these OATP1A2 variants (OATP1A2*6 and *7) may relate in part to reduced total
OATP1A2 protein expression. In addition, OATP1A2*6 variant exhibited significantly reduced cell surface-associated expression in HepG2 and HeLa cells when assessed using cell surface biotinylation or immunofluorescence confocal microscopy. Similarly, confocal microscopy revealed significant loss of cell surface associated expression for OATP1A2*3 and *7. It should be noted that there may be a degree of cell-type dependent differences in the extent of cell surface expression for variants such as OATP1A2*7. However, for the most part, there appeared to be good concordance in terms of SNP-induced differences in cell surface expression between the two cell lines. In addition, for OATP1A2*6 (A404T, Asn135Ile), loss of asparagine at this position appeared to result in altered glycosylation status, as indicated by a modest but readily noticeable shift in molecular size on Western blot analysis (Fig. 6). Indeed, the A404T (Asn135Ile) mutation, putatively located in the second external loop, is at a predicted consensus sites (Asn-X-(Ser/Thr)) for $N$-glycosylation (Fig. 8). The $N$-glycosylation of membrane-bound proteins has been demonstrated to play a number of important roles including modulation of biological activity, regulation of intracellular targeting, protein folding and maintenance of protein stability. A recent study demonstrated that $N$-glycosylation also plays an important role in OAT1 transport activity (15).

When tissue expression pattern and genetic variation in OATP1A2 are considered together, a number of novel insights regarding organ specific elimination or drug entry become apparent. First, presence of OATP1A2 in the distal tubules of the nephrons suggests a potentially important role for this transporter to reabsorption of drugs which are filtered or secreted at the level of the proximal tubule. Its expression at the level of the blood brain barrier (BBB) is of particular clinical relevance given the broad substrate specificity for this transporter, including
opioid peptide analogues such as deltorphin II and DPDPE (Fig. 2). The BBB, formed by the tight intracellular junctions of brain capillary endothelial cells express a number of transporter proteins on the apical and basolateral membranes. Expression of transporters has been shown to be critical to the regulated and some time limited entry of xenobiotic compounds into the brain (16). Among multiple transporters expressed at the BBB, OATP1A2 may be one of the major uptake transporters that therapeutic drugs may use to gain the access to the brain (7). The efficacy of analgesics and peptide-mimetic compounds may depend critically on OATP1A2-mediated transport across membrane barriers to their cellular target in the CNS. Accordingly, genetic variations associated with OATP1A2 may not only affect the disposition of endogenous and xenobiotic compounds in organs such as the kidney, but also alter the extent of drug delivery to key tissue compartments such as the brain, thereby contributing to inter-individual variability in drug responsiveness.

In summary, we report the identification and functional characterization of polymorphisms in human SLCO1A2. To our knowledge, this represents the first detailed examination of tissue-selective expression and characterization of polymorphisms in human OATP1A2 and creates the framework for further investigations of the consequences of SLCO1A2 polymorphisms in vivo. Considering its substrate specificity and expression in organs such as the brain and kidney, genetic variations in SLCO1A2 may be an important contributor to inter-individual variability in drug disposition and CNS entry of substrate drugs.
REFERENCES

FOOTNOTES:

ACKNOWLEDGEMENTS

This work was supported by United States Public Health Service Grants GM54724, GM31304, RR00095, and the NIH/NIGMS Pharmacogenetics Research Network and Database (U01GM61374, http://pharmgkb.org/) under grant U01 HL65962. Experiments/data analysis/presentation were performed in part through the use of the VUMC Cell Imaging Core Resource, (supported by NIH grants CA68485, DK20593 and DK58404).
TABLE I

Allele frequencies of nonsynonymous SNPs in OATP1A2 among various ethnic populations

(African-, European-, Chinese-, and Hispanic-Americans, n=95 each)

<table>
<thead>
<tr>
<th>OATP1A2 variants</th>
<th>Base pair change</th>
<th>Exon</th>
<th>Amino acid change</th>
<th>Allelic frequency (%)</th>
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<td></td>
<td></td>
<td></td>
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<td>African-</td>
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<tr>
<td>OATP1A2*2</td>
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<td>1</td>
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<tr>
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<tr>
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<tr>
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<tr>
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<td>Thr668Ser</td>
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</table>

**FIGURE LEGENDS**

Fig. 1. Localization of OATP1A2 in human brain (A), kidney (B) and liver (C) sections. Paraffin-embedded tissue sections (5 µm) were stained with rabbit polyclonal antiserum against OATP1A2 and immunoreactive signals (red) were detected by avidin-biotin-peroxidase complex. The arrows indicate positive immunohistochemical staining and the inset figure (A) represents the signal obtained in the consecutive brain tissue section using the OATP1A2 antiserum preincubated with the antigenic peptide.

Fig. 2. Uptake transport activity of the OATP1A2 variants following recombinant vaccinia-mediated expression. Left panels: Uptake transport of radiolabeled estrone 3-sulfate (A), deltorphin II (B) and DPDPE (C) using the initial concentrations of 0.5 µM over time by HeLa cells transfected with the wild-type SLCO1A2*1 (closed circles) and vector only control (open circles). Right panels: Uptake transport of radiolabeled estrone 3-sulfate (A), deltorphin II (B) and DPDPE (C) using the initial concentrations of 0.5 µM at 30 min by HeLa cells transfected with the SLCO1A2 variants (*2 ~ *7) was assessed relative to the wild-type SLCO1A2*1. (Values are expressed as percent of cellular uptake by OATP1A2*1 (mean ± SE, n=4-6). ** p<0.01, and *** p<0.001, ANOVA followed by Newman-Keuls test for multiple comparisons.

Fig. 3. Transport kinetics of the OATP1A2 variants for estrone 3-sulfate using varying concentrations of 1 ~ 50 µM. Data are expressed as mean ± SE (n=4). Kinetic parameters were obtained using amount of estrone 3-sulfate uptake during the initial 3 minutes and non-linear curve fitting.
Fig. 4. Transport kinetics of the OATP1A2 variants for deltorphin II using varying concentrations of 5 ~ 100 µM. Data are expressed as mean ± SE (n=4). Kinetic parameters were obtained using amount of deltorphin II uptake during the initial 3 minutes non-linear curve fitting.

Fig. 5. Total and cell surface expression of OATP1A2 variants. Total cell lysate proteins (panel A, 0.5 µg total protein) containing both biotinylated and non-biotinylated proteins and cell surface-expressed proteins (panel B, 50% of biotinylated proteins captured on streptavidin beads) were subjected to SDS-PAGE, transferred onto nitrocellulose membranes and probed with anti-V5 antibody (top panels) and anti-calnexin antibody (bottom panels). Immunoblots for total and cell surface proteins were exposed to X-ray film for identical periods of time. In addition to the band corresponding to the expected (unglycosylated) molecular size of ~59-kDa of OATP1A2 (indicated by the arrow, a) and 74-kDa (glycosylated, indicated by the arrow, b), additional band patterns (indicated by the arrows, c and d), suggest various glycosylation status as well as possible dimer formation. The OATP1A2*6 (A404T) variant exhibited a shift in the mobility of immunoreactive bands.

Fig. 6. Enzymatic and chemical deglycosylation of total and cell surface-expressed OATP1A2 *1 and *6 variants. Enzymatic N-deglycosylation was carried out on the total and cell surface-expressed OATP1A2*1 and *6 and Western blot analyses were subsequently performed to probe immunoreactive bands and shifts in molecular sizes. Chemical deglycosylation experiments were carried out by adding tunicamycin (0.3 µg/ml) during the post-transfection period after recombinant vaccinia-mediated transfection. VC, vector control lacking any insert.
Fig. 7. Immunofluorescence confocal microscopy of OATP1A2 variants in HepG2 (A and B) and HeLa cells (C). Texas Red (red fluorescence) was used to detect V5-tagged OATP1A2 variants. OATP1A2*1 was targeted to the cell surface in transiently transfected HepG2 and HeLa cells as demonstrated by lateral (x-y) and axial (x-z) confocal imaging across the cell. Certain OATP1A2 variants (*3, *6 and *7) appeared to have substantial alterations in plasma membrane trafficking in both HepG2 and HeLa cells.

Fig. 8. Predicted membrane topology of OATP1A2 based on hydrophobicity analysis. Locations of the six nonsynonymous polymorphisms in OATP1A2 are indicated with arrows.
FIG. 1

Brain

Kidney

Liver

200x 400x
FIG. 2

A

**Estrone Sulfate**

B

**Deltorphin II**

C

**DPDPE**
FIG. 3

**OATP1A2*1**

Estrone Sulfate (µM) Uptake, pmol mg protein⁻¹ min⁻¹

Kₘ = 16.1 ± 11.8 µM
Vₘₐₓ = 156 ± 43 pmol mg protein⁻¹ min⁻¹

**OATP1A2*2 (T38C)**

Estrone Sulfate (µM) Uptake, pmol mg protein⁻¹ min⁻¹

Kₘ = 15.2 ± 8.6 µM
Vₘₐₓ = 150 ± 31 pmol mg protein⁻¹ min⁻¹

**OATP1A2*3 (A516C)**

Estrone Sulfate (µM) Uptake, pmol mg protein⁻¹ min⁻¹

Kₘ = 4.5 ± 4.1 µM
Vₘₐₓ = 14.2 ± 3.6 pmol mg protein⁻¹ min⁻¹

**OATP1A2*4 (G559A)**

Estrone Sulfate (µM) Uptake, pmol mg protein⁻¹ min⁻¹

Kₘ = 5.8 ± 3.7 µM
Vₘₐₓ = 13.6 ± 2.8 pmol mg protein⁻¹ min⁻¹

**OATP1A2*6 (A404T)**

Estrone Sulfate (µM) Uptake, pmol mg protein⁻¹ min⁻¹

Kₘ = 33.1 ± 13.9 µM
Vₘₐₓ = 63.3 ± 14.6 pmol mg protein⁻¹ min⁻¹
FIG. 4

**OATP1A2*1**

\[ V_{\text{max}} = 15.9 \pm 5.5 \text{ pmol mg protein}^{-1} \text{ min}^{-1} \]

**OATP1A2*2 (T38C)**

\[ V_{\text{max}} = 51.3 \pm 38.1 \text{ pmol mg protein}^{-1} \text{ min}^{-1} \]

**OATP1A2*4 (G559A)**

\[ V_{\text{max}} = 17.9 \pm 8.0 \text{ pmol mg protein}^{-1} \text{ min}^{-1} \]

**OATP1A2*5 (A382T)**

\[ V_{\text{max}} = 2.8 \pm 0.7 \text{ pmol mg protein}^{-1} \text{ min}^{-1} \]
FIG. 6

A

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B

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FIG. 7

A

HepG2

B

C

HeLa

*1

*2

*3

*4

*5

*6

*7

vector control
FIG. 8

*5
A382T
Asn\textsubscript{128}\text{Ty}r

*6
A404T
Asn\textsubscript{135}\text{Ile}

*2
T38C
Ile\textsubscript{13}\text{Thr}

A516C
*3

Glu\textsubscript{172}\text{Asp}

*3

A516C

*4
G559A

*4

T38C

*5

A382T

*6
A404T

*7
C2003G
Thr\textsubscript{688}\text{Ser}

Extracellular

Intracellular
Polymorphisms in human organic anion transporting polypeptide 1A2: Implications for altered drug disposition and CNS drug entry


J. Biol. Chem. published online January 4, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M411092200

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