Molecular characterization of human and rat organic anion transporter OATP-D


Molecular characterization of human and rat organic anion transporter OATP-D. Am J Physiol Renal Physiol 285: F1188–F1197, 2003; 10.1152/ajprenal.00402.2002.—We have isolated and characterized a novel human and rat organic anion transporter subtype, OATP-D. The isolated cDNA from human brain encodes a polypeptide of 710 amino acids (M, 76,534) with 12 predicted transmembrane domains. The rat clone encodes 710 amino acids (M, 76,821) with 97.6% amino acid sequence homology with human OATP-D.

Molecular characterization of human and rat OATP-D have moderate amino acid sequence homology with LST-1/rlst-1, the rat oatp family, the prostaglandin transporter, and moat1/moat1 of the OATP/LST family. The other members of the OATP/LST family are apt to carry organic anions, whereas PGTs carry PGs exclusively.

Here, we report the isolation, functional expression, and pharmacological characterization of a novel organic anion transporter subtype, OATP-D, from human and rat brain, which showed pharmacological characteristics and protein expression similar to those of PGT.

MATERIALS AND METHODS

Isolation of human and rat OATP-D cDNAs. A human hippocampal cDNA library was constructed, and 8 × 10^5

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independent clones were hybridized with an EcoRI-HindII 720-bp fragment of human LST-1 in a formamide (25%) solution at 42°C as described elsewhere (1–3, 39). Filters were washed in 2× SSC and 0.1% SDS at 50°C for 1 h. Two positive clones were isolated and rescued into pHBlueprint SK+ (2, 30). The cDNA inserts of these clones showed an identical restriction enzyme digestion pattern except for some length differences in their 5’-portion. A clone containing the largest cDNA insert (pHPG5-2) was chosen for further analysis. A rat brain cDNA library (2×10⁶ independent clones) was also screened with the fragment of pHPG5-2 (NcoI-NcoI, 765 bp). Among 10 isolated clones, a representative clone (pPG2-1) was further analyzed. The sequence was determined using an ABI Prism 377 DNA sequencer (PerkinElmer, Foster City, CA).

The experiments were carried out according to the Declaration of Helsinki and the Animal Care Committee of Tohoku University Graduate School of Medicine (based on Title 45, U.S. Code, Part 46, Protection of Human Subjects, Rev. November 2001).

The sequences of human and rat OATP-D have been deposited in the GenBank accession numbers AF239219 and AF187816, respectively.

Homology analysis. The hydropathy profile analysis was performed according to Kyte and Doolittle (30). Multiple sequence alignments of amino acid sequences were carried out using CLUSTAL W (48). The phylogenetic tree was described by TREEVIEW (42).

Northern blot analysis. Human and rat multiple tissue Northern blots and human cancer cell line blots containing 2 μg of poly (A)-RNA were purchased (Clontech Laboratories, Palo Alto, CA). The latter half of the coding region of pHPG5-2 (NcoI-NcoI, 765 bp) was used as a probe because the 3’-untranslated region contains repetitive sequences. Filters were hybridized with a 32P-labeled fragment in a buffer containing 50% formamide, 5× SSC, 5× Denhardt’s solution, and 1% SDS overnight at 42°C, washed in 0.2× SSC, 1% SDS at 65°C for 1 h, and exposed to film at −80°C overnight. The rat filter was also hybridized with the full coding region of pPG2-1.

Functional expression in Xenopus laevis oocytes. The capped RNA of pHPG-2 or pPG2-1 was transcribed in vitro. X. laevis oocytes were prepared as described previously (1, 2, 39). Defolliculated oocytes were microinjected with 10 ng of transcribed RNA and cultured for 72 h in a modified Barth’s medium at 18°C. The uptake of radiolabeled chemicals was measured at room temperature in a medium containing (in mM) 100 NaCl, 2 KCl, 1 CaCl2, 1 MgCl2, and 10 HEPES, pH 7.5. After being washed with the same buffer, each oocyte was dissolved in 500 μl of 10% SDS and 4 ml of scintillation fluid and the radioactivity was counted in a liquid scintillation counter (Packard, Downers Grove, IL). Water-injected oocytes were used as controls. To evaluate the substrate specificity, the uptake rate of [3H]PGE2 (15 nM) by human OATP-D-expressing oocytes was determined in the presence of 1.5, 15, and 150 nM of inhibitors. Statistical significance was analyzed by unpaired t-test.

Preparation of antibodies. Peptides containing 13 amino acids (NYKRYKINHEGGL, position 650–662) at the COOH terminus of rat OATP-D and 14 amino acids (SYTAAET-MQFSEEDK, position 289–302) at the COOH terminus of rat PGT (15, 26) were synthesized. These peptides were linked to the maleimide-activated key hole limpet hemocyanin (KLH; Pierce, IL). The KHL-linked peptide (1 mg/injection) was emulsified by mixing with an equal volume of Freund’s complete adjuvant and injected into female rabbits. After a booster injection, rabbits were killed at 10 wk. The antibodies were affinity-purified using CNBr-activated Sepharose CL-4B (Amersham Pharmacia Biotech, Piscataway, NJ) coupled with the synthetic peptides according to standard procedures (5, 24).

Immunohistochemistry. Adult Wister rats weighing 250–300 g were killed as described above, and the systemic circulation was perfused with intra-aortic administration of 4% periodate-lysine–4% paraformaldehyde for 20 min. Each frozen block was sectioned at a thickness of 3 μm (24). After incubation in PBS containing 1% bovine serum albumin for 10 min, the sections were incubated with the affinity-purified primary antibody against rat OATP-D or rat PGT, at a final concentration of 2 μg/ml at 4°C for overnight. The sections were then incubated in 0.3% H2O2 in methanol for inhibition of endogenous peroxidase activities. Subsequently, the sections were incubated with an Envision+ peroxidase rabbit kit (Vector Laboratories, Burlingame, CA) for 40 min. The sections were then washed three times with PBS and treated with DAB solution (0.01% 3,3-diaminobenzamide tetrahydrochloride, Tris-HCl, pH 7.5, and 0.002% H2O2). To identify the specificity of the antibodies, the primary antibody against rat OATP-D or rat PGT was preabsorbed with 8 mg each of pure polypeptide or exchanged polypeptide overnight before use. No cross-reactivities between these antibodies were verified (data not shown).

RESULTS

Isolation and structural analysis of human and rat OATP-D. The isolated cDNA encodes a novel human PGT subtype, human OATP-D, consisting of 710 amino acids (Ms, 76,534). Hydrophobicity analysis of the predicted human OATP-D protein suggested the presence of 12 transmembrane domains (TM) (Fig. 1A). There are seven putative N-glycosylation sites in the predicted extracellular loops, three potential phosphorylation sites for cAMP-dependent protein kinase, and two potential phosphorylation sites for protein kinase C in the intracellular portions (27, 28) (Fig. 1A). Sequence homology analysis revealed a moderate sequence similarity to the oatp/LST family (1, 2, 5, 25, 29, 32, 34, 39, 40, 43) and PGT (26, 33). Recently, Tamai et al. (46) isolated OATP-D from adult human brain. The human sequence is 100% identical to human OATP-D. Compared with the OATP/LST family, the overall amino acid sequence identities were 36.2% to human PGT (33), 32.1% to rat pgt (26), 33.8% to oatp1 (23), 35.2% to oatp2 (3, 40), 35.4% to oatp3 (1), 32.9% to OAT-K1 (43), 32.7% to OAT-K2 (34), 33.7% to human OATP (29), 32.0% to human LST-1 (2), 33.3% to human LST-2 (5), 31.7% to rat rlst-1 (25), 33.0% to rat moat1 (39), and 32.0% to human MOAT1/KIAA0880/OAPT-B (36, 46).

A rat counterpart cDNA, pPG2-1, was also isolated from the brain. The isolated clone encoded 710 amino acids (Ms, 76,821) with 97.6% amino acid sequence homology with human OATP-D. The high structural similarity to human OATP-D and the function analysis (discussed below) revealed that the pPG2-1 clone encodes a rat counterpart. All the motifs except one potential protein kinase C phosphorylation site at the third intracellular loop between TM VI and VII were conserved. The phylogenetic tree analysis showed that human OATP-D and rat OATP-D can be localized differentially from the oatp family, LST-1/rlst-1, the PGT, \(\text{AJP-Renal Physiol} \bullet \text{VOL 285} \bullet \text{DECEMBER} 2003 \bullet \text{www.ajprenal.org}\)
and moat1/KIAA0880/OATP-B (Fig. 1B). These data suggest that OATP-D can be categorized as a new subtype of organic anion transporter.

**Northern blot analysis.** Northern blot analysis of the human OATP-D showed two bands (1 major band at 3.0 knt and another minor band at 5.0 knt) in the heart, brain, and testis (Fig. 2A). Moderate signals of the same size were also detected in the lung, kidney, pancreas, and ovary. In the rat, the expression pattern of rat OATP-D was slightly different from that in humans. Positive bands were detected in the heart, brain, lung, and kidney (Fig. 2B).

Distribution of human OATP-D mRNAs in cancer cells. According to the GenBank dbEST search, many sequences identical to the OATP-D were found in several cancer tissues or cancer cell lines [GenBank accession nos. AA075159 (ovarian cancer), AA72990 (germ cell cancer), AA843188 (parathyroid tumor), AI082669 (human melanocyte)]. Northern blot analysis using the specific OATP-D probe gave rise to significant hybridization bands in promyelocytic leukemia HL-60, cervical cancer (HeLa S3), chronic myelogenous leukemia K-562, lymphoblastic leukemia MOLT-4, Burkitt’s lymphoma (Raji), colorectal adenocarcinoma

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Fig. 1. A: alignment of deduced amino acid sequences of rat and human organic anion transporter subtype (OATP-D). The sequences are aligned with single-letter notation by inserting gaps (-) to achieve maximum homology. The 12 putative transmembrane segments (1–12) were assigned on the basis of hydrophobicity analysis. Sequence motifs for potential N-glycosylation sites (*) and possible phosphorylation sites (•) are indicated. PGT, PG transporter; TM, transmembrane domain. B: phylogenetic relationship among OATP-D, LST-1/rlst-1, the oatp family, PGT, and moat1. Branch lengths are drawn to scale.
Pharmacological characterization. Among the putative substrates tested, the oocytes injected with human OATP-D cRNA transported PGE1 and PGE2 (Table 1). PGF2α was also weakly but significantly transported. On the other hand, other eicosanoids [PGD2, iloprost, thromboxane B2 (TBX2)] were not transported. These human OATP-D-mediated PGE1 and PGE2 uptakes were saturable with increasing substrate concentrations. The apparent $K_m$ values for PGE1 and PGE2 of human OATP-D were 48.5 ± 1100612.2 and 55.5 ± 110066.7 nM, respectively (Fig. 3, A and B). These human OATP-D-mediated PGE1 and PGE2 uptakes were not inhibited by replacing the extracellular sodium with choline (data not shown). Neither taurocholate nor methotrexate, both of which are preferable substrates for the oatp/LST family, was transported (Table 1). Despite the structural difference between human OATP-D and human PGT, the preferable substrate for human OATP-D was similar to that of the PGT. To further characterize the substrate specificity of human OATP-D, a cis-inhibitory experiment was performed. As in Fig. 4, unlabeled PGE1, PGE2, and PGF2α (1.5, 15, and 150 nM) showed dose-dependent inhibitory effects on human OATP-D-mediated PGE2 uptake (15 nM). At the highest concentration (150 nM), PGE2

Table 1. Uptake of various [3H]-labeled compounds by human OATP-D-expressing oocytes

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Uptake</th>
<th>Uptake Ratio, HPGT2/HPGT2/H2O</th>
<th>HPGT2/H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostaglandins, fmol-oocyte$^{-1}$-60 min$^{-1}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prostaglandin D$_2$ (18 nM)</td>
<td>1.56 ± 0.13</td>
<td>1.39 ± 0.03</td>
<td>0.89</td>
</tr>
<tr>
<td>Prostaglandin E$_2$ (50 nM)</td>
<td>1.24 ± 0.13</td>
<td>2.53 ± 0.28</td>
<td>2.03†</td>
</tr>
<tr>
<td>Prostaglandin E$_2$ (15 nM)</td>
<td>2.01 ± 0.07</td>
<td>7.22 ± 1.88</td>
<td>3.61†</td>
</tr>
<tr>
<td>Prostaglandin F$_2$α (13.7 nM)</td>
<td>0.73 ± 0.05</td>
<td>1.15 ± 0.02</td>
<td>1.57#$^*$</td>
</tr>
<tr>
<td>Thromboxane B$_2$ (36 nM)</td>
<td>2.52 ± 0.97</td>
<td>2.81 ± 0.26</td>
<td>1.11</td>
</tr>
</tbody>
</table>

Organic anions, pmol-oocyte$^{-1}$-60 min$^{-1}$

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Uptake</th>
<th>Uptake Ratio, HPGT2/HPGT2/H2O</th>
<th>HPGT2/H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iloprost (2.6 µM)</td>
<td>0.77 ± 0.03</td>
<td>1.03 ± 0.06</td>
<td>1.33</td>
</tr>
<tr>
<td>Taurocholate (15 µM)</td>
<td>0.15 ± 0.01</td>
<td>0.12 ± 0.008</td>
<td>0.80</td>
</tr>
<tr>
<td>Methotrexate (3 µM)</td>
<td>0.03 ± 0.002</td>
<td>0.03 ± 0.002</td>
<td>1.00</td>
</tr>
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Values are means ± SE of 8–15 oocytes determinations. OATP-D, organic anion transporter subtype. The uptake experiments were performed at the concentration indicated for 60 min. Significance between water-injected- and OATP-D cRNA-injected oocytes was determined by unpaired $t$-test ($^*P < 0.05$, $^†P < 0.01$).

Fig. 2. A: human multiple-tissue Northern blots [2 µg poly(A)$^+$ RNAs] were hybridized with the OATP-D probe. The size marker (knt) used was the RNA ladder. B: rat multiple tissue Northern blots were also hybridized with the rat ATP-D probe. C: localization of OATP-D mRNA in human cancer cell lines by RNA blot analysis. Human multiple cancer tissue Northern blots were hybridized with the OATP-D probe.
uptake was completely inhibited by PGE₁, PGE₂, and PGF₂α. On the other hand, PGD₂ and PAH, which were not transported by human OATP-D, did not show a significant inhibitory effect at the concentrations of 1.5 and 15 nM. Even at the highest concentration tested (150 nM), PGD₂ and PAH did not abolish the uptake of PGE₂. Furthermore, taurocholate did not significantly inhibit human OATP-D-mediated [³H]PGE₂ uptake at any concentration.

We also examined the transport activity of the rat counterpart. The oocytes injected with transcribed rat OATP-D RNA also transported PGE₁, PGE₂, and PGF₂α significantly (Fig. 5, *P < 0.05). These data demonstrated that OATP-D encodes a functional organic anion transporter subtype.

Immunohistochemistry. Northern blot analysis of rat OATP-D indicated that rat OATP-D is expressed widely. To elucidate differences in the tissue distribution of rat PGT and rat OATP-D, immunohistochemical analysis was performed in cardiorespiratory systems (Fig. 6A) and in reproductive systems (Fig. 6B). Previous work showed that the rat PGT was expressed in arterial endothelial cells (49). Rat PGT and rat OATP-D immunostaining was detected on endothelial cells of the aorta (Fig. 6A). In the heart, significant rat PGT and rat OATP-D immunostaining was detected on cardiac muscle cells, endothelial cells of left ventricular endocardium, and endothelial cells of the coronary artery. With reference to cardiac muscle cells, rat OATP-D was expressed mainly on endothelial cells of the coronary artery. In the lung, rat PGT and rat OATP-D immunostaining was detected on alveolar epithelial cells. In the trachea, rat PGT and rat OATP-D immunostaining was detected in the epithelium of the mucosa of the trachea. Rat OATP-D immunostaining was more significant than rat PGT in the lung and trachea. In the testis, rat PGT and rat OATP-D immunostaining was detected on spermatozoa (Fig. 6B) and, at a higher magnification, signals were detected at the tails of spermatozoa (data not shown). In the epididymis, signals of rat PGT were detected on the epithelium of the ductus epididymis, and rat OATP-D immunostaining was not detected. In the ovary, rat PGT and rat OATP-D immunostaining was detected on oocytes and smooth muscle cells of the ovary.

In the uterus, rat PGT and rat OATP-D immunostaining was detected in the epithelium of the glandula uterina and on a part of the smooth muscle cells of the myometrium and surface epithelium of the endometrium.
In the kidney, both rat PGT and rat OATP-D immunostaining was detected in afferent arterioles, efferent arterioles, and the epithelium of distal tubules and collecting tubules (Fig. 7). Particularly, PGT and OATP-D staining was detected on epithelial cells of collecting tubules throughout the cortex and medulla. The staining pattern was fine in the cortex and rough in the inner medulla, suggesting the localization of...
PGT and OATP-D on intercalated cells. While rat OATP-D staining of tubules and arterioles was similar to that for rat PGT, Bowman’s capsule of the glomerulus was stained by rat PGT but not by rat OATP-D.

In the brain, rat PGT staining was detected around the periventricular thalamic nucleus, whereas rat OATP-D stained at the arcuate nucleus (Fig. 8). Both rat PGT and OATP-D were detected in the choroid plexus of the third ventricle.

DISCUSSION

We have identified the human and rat transporter OATP-D. Isolated human and rat cDNAs were highly conserved and belong to the organic anion transporter OATP/LST family. The oatp/LST family recognizes various compounds and transports bile acid (taurocholate, cholate, bromosulfophthalein); thyroid hormones [l-thyroxine (T4), 3,3',5-triiodo-l-thyronine (T3)],
l-3,3',5'-triiodothyronine (rT3)); conjugated steroid hormones (estradiol-17β sulfate, dehydroepiandrosterone sulfate); eicosanoids (PGD$_2$, PGE$_1$, PGE$_2$, PGF$_2\alpha$, TXB$_2$); and many xenobiotics (pravastatin, a potent hydroxymethylglutaryl-CoA reductase inhibitor, digoxin, methotrexate), etc. Compared with the broad substrate specificity of the oatp/LST family, the prostaglandin transporter PGT transports eicosanoids (PGD$_2$, PGE$_1$, PGE$_2$, PGF$_2\alpha$, TXB$_2$) but does not transport taurocholate. OATP-D transports PGE$_1$, PGE$_2$, and PGF$_2\alpha$ in a sodium-independent manner. Further pharmacological characterization revealed that OATP-D-mediated [$^3$H]PGE$_2$ uptake was markedly inhibited by PGE$_1$, PGE$_2$, and PGF$_2\alpha$, which are preferable substrates for OATP-D. In contrast, taurocholate and PAH, which were not well transported as substrates, showed only slight inhibition even at the highest concentration. These data suggest that PGs are preferential substrates for OATPD, although the overall homology of OATP-D among the oatp/LST family is quite similar in PGT and OATP-D but not similar to that of OATP-D on chromosome 15, which is consistent with the sequence reported by Tamai et al. (46) (GenBank accession no. AB031050).

PGT and OATP-D are expressed in vascular endothelium. It is well known that prostaglandins are local regulators of vascular tone and are produced in the endothelium. Although PGs are produced in the endothelium, PGs are thought to diffuse poorly through the plasma membrane (7, 11), and responsive molecule(s) involved in membrane transport should be addressed. In addition, steady laminar shear stress induced human PGT expression in cultured human vascular endothelium (49). These data suggest that PGT and OATP-D play an important role in the maintenance of cardiovascular homeostasis as regulators of PG transport.

OATP-D is also expressed in the coronary artery and myocardium. PGE$_2$ receptor subtype EP$_3$, which inhibits adenylly cyclase, was upregulated in the ischemic heart of pigs (21). The expression of OATP-D in vascular endothelial cells also suggests a role of transporting PGs in the cells.

PGs are synthesized in the reproducive system. The biosynthesis of PGE$_1$ was demonstrated not in the testis but in the seminal vesicles (18). The expression of OATP-D and PGT in the spermatozoa is in agreement with this finding. Mice deficient in cyclooxygenase (COX)-2 or EP$_2$ receptor showed a failure in fertilization. COX-2 $^{-/-}$ mice showed a disability in uterinal implantation (32). Cumulus expansion became abortive in EP$_2$ $^{-/-}$ mice (20). Milne et al. (35) described that the menstrual cycle is associated with the coexpression of PGE synthase, PGE$_2$, EP$_2$ receptor, and EP$_3$ receptor in the endometrium. They detected the expression of PGE synthase and PGE$_2$ synthesis on glandular epithelial cells, endothelial cells, and stromal cells in the human endometrium. The findings of OATP-D and PGT in the reproductive organs suggest that these transporters play important roles in regulating the local concentration of PGs in the reproductive system, implying their roles in conception and menstruation.

OATP-D is expressed in the distal and collecting tubules of the kidney. PGE$_2$ is the dominant synthesized eicosanoid in the kidney. All types of PGE$_2$ receptors are expressed in the kidney. Because EP$_1$ receptor expression predominates in the collecting ducts and EP$_3$ receptors are expressed in the thick ascending limbs and collecting ducts, PGE$_2$ secreted through OATP-D appears to contribute to body-fluid balance, including both natriuresis and vasopressin-mediated water-salt transport (8, 15). EP$_4$ receptors are expressed in the glomerulus and collecting duct (9). EP$_3$ and EP$_4$ receptors are associated with the regulation of the vasoconstriction of afferent arterioles (47), and PGE$_2$ stimulates renin release via the macula.
densa (22). PGF$_2$α is also synthesized mildly in the glomerulus. Both PGT and OATP-D are able to transport PGE$_2$ and PGF$_2$α. OATP-D and PGT expressed onafferent arterioles are speculated to control tubuloglomerular feedback and the renin release.

It has been recognized that PGE$_2$ and PGF$_2$α are rapidly cleared by a single passage through the pulmonary or other vascular beds in vivo (6, 13). The similar expression of PGT and OATP-D in alveolar cells suggests their involvement in the elimination of PGs.

Because the arcuate nucleus regulates gonadal hormone secretion by PGs, the expression of OATP-D in the arcuate nucleus may have an important role in hormonal regulation. As opposed to OATP-D, PGT is located around the periventricular nucleus but not in the arcuate nucleus. The combined expression of PGT and OATP-D in the choroid plexus and ventricle suggests that PGT may be involved in regulation of cerebrospinal homeostasis.

Northern blot analysis in cancer cell lines showed that expression of OATP-D mRNA could be detected in the all cell lines tested. The higher expression of mRNAs was detected in cell lines of solid-type (or epithelial) cancer than the soluble-type neoplasia like leukemia. Tamai et al. (46) also showed by RT-PCR that mRNA of OATP-D was detected in almost cell lines of solid cancer, whereas PGT mRNA was not detected in cancer cell lines.

One of PGs synthases, COX-2, is not detected in most normal tissues, but it is induced by mitogenic and inflammatory stimuli, which results in enhanced synthesis of PGs in neoplastic and inflamed tissues, and selective COX-2 inhibitors have been reported to reduce the formation of cancers derived from many organs in animals and human (reviewed in Ref. 37). It is interesting that OATP-D and COX-2 are simultaneously upregulated in cancer cells, although it is still unclear whether OATP-D is associated with cell function or PG production.

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