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Expression profiles of various transporters for oligopeptides, amino acids and organic ions along the human digestive tract

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Abstract

Various transporters such as H^+/peptide cotransporter PEPT1 are expressed in the intestine, and play important physiological and pharmacological roles in the body. Present study was performed to examine the expression profile of 20 kinds of transporters (PEPT1 and 2, P-glycoprotein, amino acid transporters and organic ion transporters) along the human digestive tract, especially focusing on PEPT1. Using normal mucosal specimens, real-time polymerase chain reactions were carried out. Immunoblot analyses were also performed for PEPT1 expression. PEPT1 mRNA was highly expressed in the small intestine (duodenum > jejunum > ileum) compared to other tissues, and some patients showed a significant level of expression in the stomach. The expressional pattern of PEPT1 in the stomach and histological diagnosis indicated that gastric PEPT1 originated from the intestinal metaplasia. The amino acid transporters showed unique mRNA expression levels and distributions in the digestive tract. For example, the expression levels of B^0AT1, a Na^+-dependent and chloride-independent neutral amino acid transporter, were increased from the duodenum to ileum, which pattern is completely inverted to that for PEPT1. There is little expression of organic ion transporters except for organic cation/carnitine transporter OCTN2. In conclusion, PEPT1 was abundantly expressed in the small intestine, and the reciprocal expression of PEPT1 and B^0AT1 may serve for the efficient absorption of protein digestive products.

Keywords: Protein absorption; Digestive tract; Transporter; PEPT1; B^0AT1; OCTN2; Organic ion transporter

1. Introduction

Cellular uptake of small peptides (di- and tri-peptides) is mediated by a H^+/peptide cotransporter at the brush-border membranes of intestinal absorptive epithelial cells [1,2]. Cloning studies have clarified the molecular nature of the intestinal H^+/peptide cotransporter (PEPT1) in various species [2,3]. Another H^+/peptide cotransporter, human PEPT2 is expressed mainly in the kidney, but not in the small intestine, and has a higher affinity for most substrates [3]. As PEPT1 has broad substrate specificity, the intestinal absorption of several pharmacologically active drugs, such as oral β-lactam antibiotics and the anti-viral agent valacyclovir are mediated by this transporter, and therefore, PEPT1 also plays important roles as a drug transporter [3].

In addition to PEPT1, various amino acid transport systems, such as L, y^+, y^+L, $b^{0,+}$, A, ASC, B^0, $B^{0,+}$ and X_{AG}^- are expressed in the small intestinal epithelial cells. These systems have been classified by their ion-dependence and substrate specificity [4], and the molecular nature of each system has been identified [5,6]. For example, system B^0, which is a Na^+-dependent and chloride-independent transporter, is responsible for the uptake of most neutral amino acids at the brush-border membranes of intestinal epithelial cells [7,8]. Two independent groups have recently isolated and characterized the transporter cDNA corresponding to system B^0 (B^0AT1), and demonstrated that a mutation of B^0AT1 results in the Hartnup disorder [9,10].

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Although the number of peptide transporters is smaller than that of amino acid transporters, numerous investigations have shown that the peptide transporters play a major role in the completion of the final step of protein digestion in the human intestine [1]. These findings led us to examine the expression levels of each transporter along the human digestive tract. Thus, in the present study, using normal portions of mucosal samples from cancer patients treated surgically, mRNA expression levels of PEPT1 and seven amino acid transporters were qualified by real-time PCR techniques. Furthermore, to investigate the pharmacokinetic aspects of PEPT1, we compared mRNA expression level of PEPT1 with those of other drug transporters, i.e., organic ion transporters. We previously examined the expression profiles of organic ion transporters in the kidney [11], but there is little information about their expression in the human gut.

2. Material and methods

2.1. Patients and tissue sampling

The mucosal samples from normal tissues along the digestive tract and normal pancreatic tissue were obtained from cancer patients during surgery at the First Department of Surgery, Kyoto University Hospital. Normal mucosal samples were resected at the site most distant from the affected portions. Table 1 shows the number, sex and age of patients for each tissue sample. No patients underwent preoperative chemotherapy and/or radiation therapy. The samples were frozen in liquid nitrogen and stored at −80 °C until RNA extraction and membrane preparation. This study was conducted in accordance with the Declaration of Helsinki and its amendments, and was approved by the Ethics Committee of Kyoto University (G-39). Written informed consent was obtained from all patients for surgery and use of their resected samples.

2.2. Isolation of total RNA

Total cellular RNA was isolated from the tissue samples using a MagNA Pure LC RNA isolation Kit II (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's instructions, and the concentrations of total cellular RNA were measured by spectrophotometry. Isolated total RNA was reverse-transcribed and the reaction mixtures were used for real-time PCR.

Table 1
The number, sex and age of patients for each tissue sample

	n	Male	Female	Age (years)
Esophagus	16	13	3	59.3 ± 1.6
Stomach	31	20	11	64.4 ± 2.3
Duodenum	13	7	6	63.8 ± 2.2
Jejunum	15	13	2	66.6 ± 3.7
Ileum	10	4	6	65.3 ± 5.7
Colon	15	8	7	71.6 ± 3.1
Rectum	15	11	4	64.6 ± 3.0
Pancreas	9	3	6	61.8 ± 2.8

2.3. Real-time PCR

Primers and probes for PEPT1 and 2, seven amino acid transporters and urate transproter (URAT1) were designed using the Primer Express® software program (Table 2). Those for multidrug resistance 1 (MDR1) [12] and organic ion transporters [11] were previously designed. Oligonucleotide probes were labelled with a fluorogenic dye, 6 carboxyfluorescein (Fam) and quenched with 6 carboxy-tetramethylrhodamine (Tamra) (Table 2). Real-time PCR was performed in an ABI PRISM 7700 (Applied Biosystems, Foster, CA) and quantification by use of standard plasmid DNA was performed as described previously [11]. For each reaction, the assay was carried out in duplicate. Glyceroladehyde 3-phosphate denydrogenase (GAPDH) mRNA was also measured as an internal control with GAPDH control reagent (Applied Biosystems).

2.4. Antibodies

Rabbit anti-human PEPT1 antibody was prepared previously, and the specificity was already confirmed [13]. Mouse anti-Na^+/K^+-ATPase monoclonal antibody was purchased from Upstate Biotechnology (Lake Placid, NY). For immunohistochemistry, we used anti-CD10 antibody (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK).

2.5. Western blot analysis

Crude plasma membranes were prepared from tissue samples taken along the digestive tract as described previously [11]. Western blot analyses using affinity-purified anti-PEPT1 antibody [13] and mouse monoclonal Na^+/K^+-ATPase antibody [11] were carried out based on our previous reports.

2.6. Immunohistochemistry

Paraffin-embeded sections were stained for CD10 antibody. The sections were deparaffinized, and antigens were retrieved by autoclave in 10 mM EDTA buffer (pH 8.0) for 15 min. Non-specific antigen was blocked by 10% normal horse serum for 30 min at room temperature. Anti-CD10 antibody (×50) in PBS containing 1% bovine serum albumin was applied and sections were incubated at 37 °C for 32 min. Slides were washed six times in PBS, and then incubated with biotinylated horse anti-mouse antibody for 40 min at room temperature. The slides were then counterstained with hematoxylin.

Table 2
Primer sets and probes used for real-time PCR

	Sequence	Nucleotide numbers	Accession number
PEPT1			NM_005073
Forward primer	attgtgtcgctctccattgtctac	306–329	
Reverse primer	atgacctcacagaccacaaccat	389–367	
TaqMan probe	ttggacaagcagtcacctcagtaagctcca	334–363	
PEPT2			NM_021082
Forward primer	ttaaacaaggccccagagactct	2382–2404	
Reverse primer	cccacttagttctggacctgctt	2463–2445	
TaqMan probe	tgccccaaccagtcttcaggaggaag	2410–2439	
B^0AT1			XM_291120
Forward primer	gtgtggacaggttcaataaggacat	1646–1670	
Reverse primer	ccacgtgacttgccagaagat	1719–1699	
TaqMan probe	tcatgatcggccacaagcccaa	1676–1697	
ASCT2			U53347
Forward primer	gcgagaaatatcttcccttcca	1152–1202	
Reverse primer	gttccggtgatattcctctcttca	1266–1243	
TaqMan probe	tgtcagcagcctttcgctcatactctacca	1209–1238	
$b^{0,+}AT$			AF141289
Forward primer	ggcctgacgattctaggactca	1344–1365	
Reverse primer	ggagccagaaccaaaaacaca	1468–1448	
TaqMan probe	atcaaggtgcccgtagtcattcccgt	1404–1429	
LAT1			AF104032
Forward primer	ggaagacacccgtggagtgt	1421–1440	
Reverse primer	acaggacggtcgtggagaag	1552–1533	
TaqMan probe	tggaaaaacaagcccaagtggctcc	1498–1522	
LAT2			AF171669
Forward primer	tgaggagcttgttgatccctaca	988–1010	
Reverse primer	gcgacattggcaaagacataca	1080–1059	
TaqMan probe	tcccagagccatcttcatctccatcc	1018–1043	
y^+LAT1			AF092032
Forward primer	gatccatgttgagcggttcac	1336–1356	
Reverse primer	ccacgcacaagtagatcaatgc	1412–1391	
TaqMan probe	ccagtgccttctctgctcttcaatggtatc	1358–1387	
ATA2			AF259799
Forward primer	gacagcagcagctacagttcca	66–87	
Reverse primer	cataatggcttttcagagcagctt	144–121	
TaqMan probe	agcgacttcaactactcctaccccaccaag	90–119	
URAT1			AB071863
Forward primer	agctcttggaccccaatgc	437–455	
Reverse primer	cttcagagcgtgagagtcacaca	560–582	
TaqMan probe	cgcagcatcttcacctccacaatcgt	517–542	

Primers and probes used for real-time PCR. All primers and probes were designed using Primer Express software from Applied Biosystems. Primers were tested using RT-PCR and only those yielding a single band of the expected size were used for subsequent real-time PCR experiments.

3. Results

3.1. Quantification of PEPT1 and PEPT2 mRNA in tissues along the digestive tract and pancreas

First, to verify the conditions and experimental techniques, MDR1 mRNA expression levels were measured (Fig. 1 right). We previously found that the level of MDR1 mRNA in the jejunum from living-donor liver transplantation patients was 0.41 ± 1.19 amol/μg of total RNA (mean ± S.E., $n = 48$) using a competitive PCR-based method, and that the level of MDR1 mRNA correlated well with protein level [14]. In the present study, the MDR1 mRNA level in the jejunum was 2.73 ± 0.51 amol/μg of total RNA (mean ± S.E., $n = 16$). This value was similar to previously reported ones [14]. Furthermore, MDR1 mRNA levels gradually increased from the duodenum to ileum, well consistent with previous findings on the distribution of MDR1 mRNA [15] and P-glycoprotein [16] in the human small intestine (Fig. 1 right). The expression of MDR1 mRNA in the colon and rectum (Fig. 1 right) also corresponded to a previous report [17]. All these findings suggested that the conditions and experimental techniques were reliable.

We then examined the mRNA expression of PEPT1 and PEPT2 along the digestive tract (esophagus, stomach,

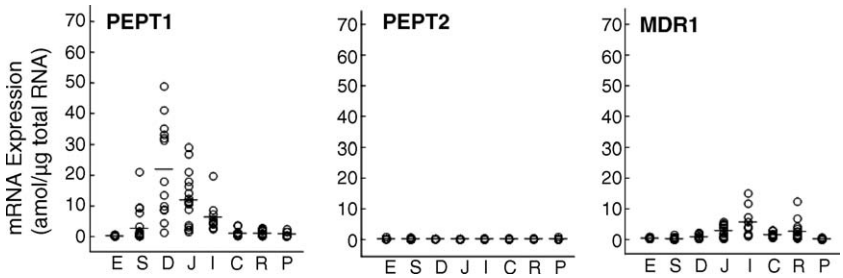

Fig. 1. mRNA expression of PEPT1, PEPT2 and MDR1 along the digestive tract (esophagus (E), stomach (S), duodenum (D), jejunum (J), ileum (I), colon (C), rectum (R)) and pancreas (P). Total cellular RNA was extracted from each tissue sample, and reverse-transcribed. The mRNA levels were determined by real-time PCR using an ABI prism 7700 sequence detector.

duodenum, jejunum, ileum, colon and rectum) and pancreas. As shown in Fig. 1 left, PEPT1 mRNA is highly expressed in the small intestine (duodenum > jejunum > ileum), but is not expressed or only slightly expressed in the esophagus, colon, rectum and pancreas. In the stomach, some patients showed a high level of PEPT1 mRNA. The tissue which showed the highest expression level was the duodenum (22.0 ± 4.23 amol/μg RNA). In contrast to PEPT1 mRNA, PEPT2 mRNA was not expressed in all tissues examined (Fig. 1 middle).

3.2. Western blot analysis

Fig. 2 shows Western blot analyses using crude membranes from each tissue. A primary band of about 80 kDa of PEPT1 protein was detected in the stomach, duodenum, jejunum and ileum, as observed in the mRNA analyses. In other tissues, no PEPT1 protein was detected. In the case of PEPT2 protein, there were no detectable bands in the tissue samples from along the digestive tracts (data not shown). The protein band of Na^+/K^+-ATPase was detected in all specimens (Fig. 2).

3.3. PEPT1 in the stomach

There is no expression of PEPT1 protein in the rat stomach [18]. On the other hand, the present study has demonstrated that some patients had a relatively high level of PEPT1 in the stomach, although there were interindividual differences. To clarify the distribution of PEPT1 in the gastric mucosa, fundus (the upper-third: U), body (the middle-third: M) and antrum (the lower-third: L) mucosa were assessed for PEPT1 protein expression when sections were available from one patient. As shown in Fig. 3,

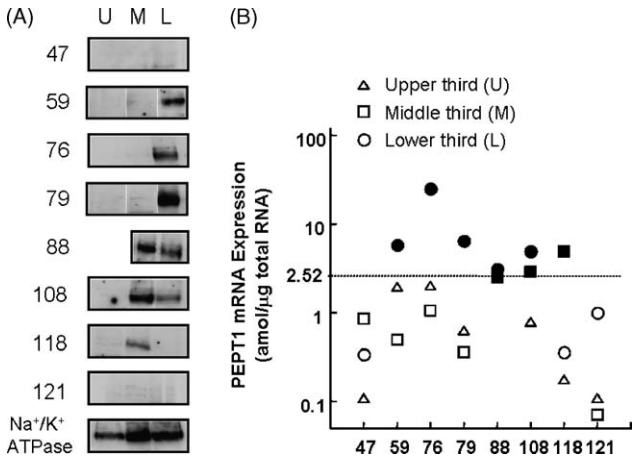

Fig. 3. PEPT1 expression in the gastric mucosa. (A) Crude membranes were isolated from the upper-third (U), middle-third (M) and lower-third (L) of the gastric mucosa from eight patients and subjected to Western blot analyses (50 μg protein/lane) for PEPT1 and Na^+/K^+-ATPase. Conditions for Western blotting were identical to those in Fig. 2. For Na^+/K^+-ATPase, representative data are shown. A sample for the upper-third of patient 88 was not obtained. (B) Relationship between mRNA and protein expression levels of PEPT1 in gastric mucosa. Closed symbols represent the samples with PEPT1 protein expression. When the mRNA level was more than 2.52 amol/μg of total RNA, the band for PEPT1 protein was detected.

PEPT1 protein was expressed in the antral mucosa in most cases, and well correlated with mRNA expression level. When the mRNA level was more than 2.52 amol/μg of total RNA, the band for PEPT1 protein was detected.

These expressional patterns and the histological diagnosis suggested that intestinal metaplasia may induce the expression of PEPT1 in the stomach. Intestinal metaplasia primarily affects the antrum in a patchy fashion, and the superficial gastric epithelium is replaced by intestinal goblet and absorptive cells [19]. Using paraffin sections

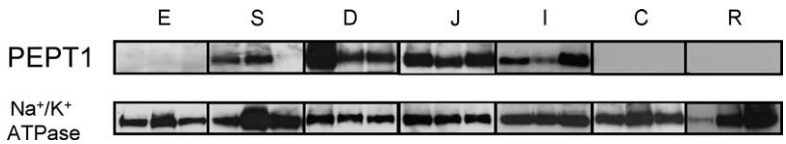

Fig. 2. Western blot analyses of crude membranes isolated from human tissues along the digestive tract for PEPT1 and Na^+/K^+ ATPase. Crude membranes were isolated from each tissue and subjected to Western blotting (50 μg protein/lane). Abbreviations for each tissue are identical to those in Fig. 1. PEPT1 protein was identified using affinity-purified anti-PEPT1 antibody (1:500 dilution). After the membranes were deprobed, Na^+/K^+-ATPase protein was detected by the mouse monoclonal anti Na^+/K^+-ATPase antibody (1:10,000).

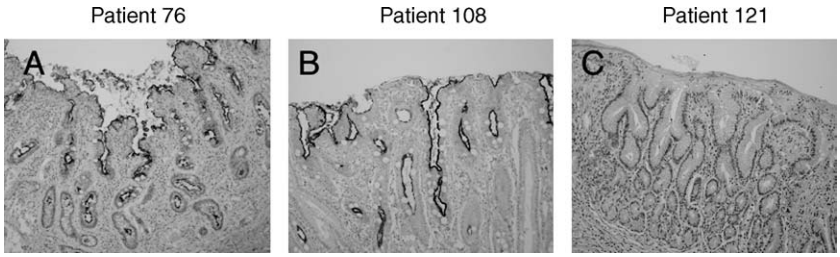

Fig. 4. Localization of CD10 in the intestinal metaplasia. (A and B) CD10 can be seen along the brush-border membranes of the luminal surface of the epithelium in antral mucosa from patients 76 and 108 (original magnification ×50). (C) Hematoxilin–eosin staining of antral mucosa from patient 121.

of antral mucosa from patients 76, 108 and 121, immunostaining for CD10 was carried out. CD10, also known as enkephalinase, has been shown to localize to the brush-border membranes of absorptive small intestinal enterocytes [20]. CD10 was positively expressed along the luminal surface of the epithelium of patients 76 and 108 (Fig. 4A and B), but not patient 121 (data not shown). Hematoxilin–eosin staining revealed that intestinal metaplasia did not occurr in patient 121 (Fig. 4C).

3.4. Comparison of mRNA expression levels of PEPT1 with those of amino acid transporters in the digestive tract

We next examined the expression profile of amino acid transporters in the digestive tract to compare with PEPT1. The human amino acid transporters examined here were mainly neutral amino acid transporters and reported to be important for intestinal epithelial transport, i.e., system B^0 (B^0AT1) [9,10], system ASC (ASCT2) [21], system $b^{0,+}$ ($b^{0,+}AT$) [22], system L (LAT1 [23,24] and LAT2 [25–27]), system y^+L, (y^+LAT1) [28] and system A (ATA2) [29]. It has been demonstrated that B^0AT1, ASCT2 and $b^{0,+}AT$ localized at the brush-border membranes of epithelial cells, whereas LAT1, LAT2, y^+LAT1 and ATA2 localized at the basolateral membranes.

Fig. 5 shows the mRNA expression levels along the digestive tract for the amino acid transporters localized to brush-border membranes: B^0AT1, ASCT2 and $b^{0,+}AT$. These three transporters exhibited distinguishable expression patterns. B^0AT1 mRNA levels were increased from duodenum to ileum like MDR1 mRNA levels. ASCT2 showed little expression in the small intestine, but significant expression in the large intestine (colon and rectum). In contrast, $b^{0,+}AT$ exhibited little expression in the digestive tract except for the stomach.

Fig. 6 shows the mRNA expression levels along the digestive tract for the amino acid transporters localized to basolateral membranes: LAT1, LAT2, y^+LAT1 and ATA2. ATA2 showed a strong expression in the tissues tested. LAT1 was preferentially expressed in the esophagus, stomach and pancreas, as compared with the small and large intestine. The expression of LAT2 was abundant in the stomach and rectum, and modest in the small intestine

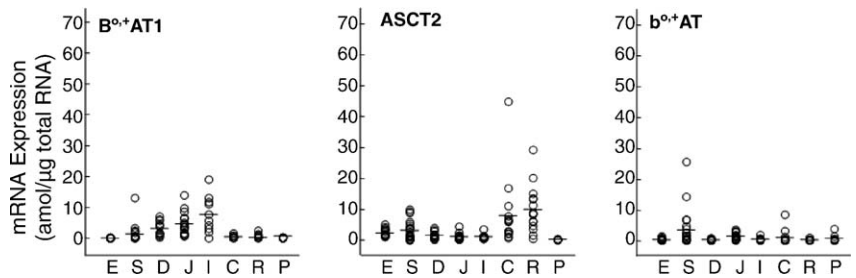

Fig. 5. mRNA expression of brush-border membrane-localized amino acid transporters B^0AT1, ASCT2 and $b^{0,+}AT$ along the digestive tract and pancreas. Abbreviations for each tissue and experimental methods are identical to those in Fig. 1.

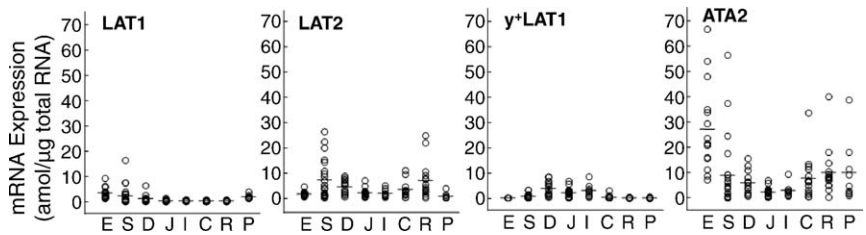

Fig. 6. mRNA expression of basolateral membrane-localized amino acid transporters LAT1, LAT2, y^+LAT1 and ATA2 along the digestive tract and pancreas. Abbreviations for each tissue and experimental methods are identical to those in Fig. 1.

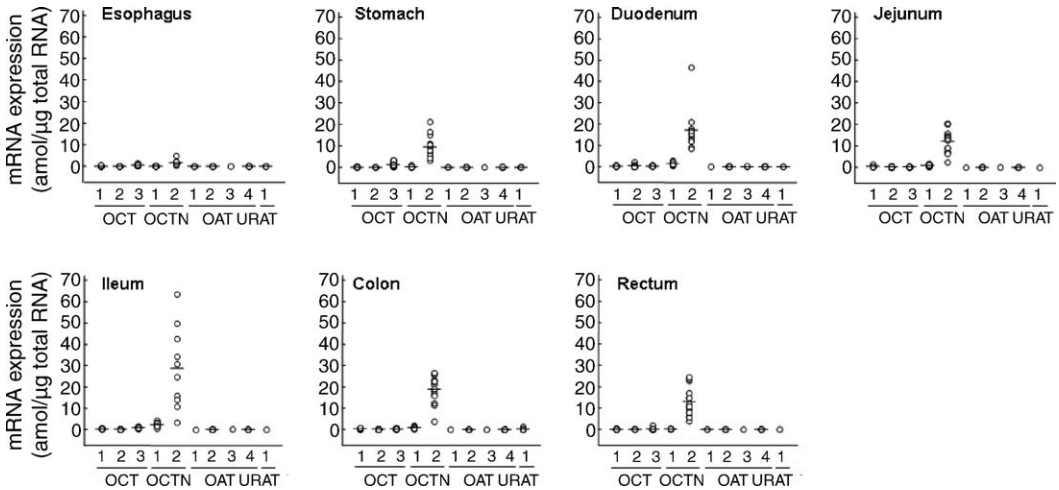

Fig. 7. mRNA expression of organic ion transporters along the digestive tract. Tested transporters are as follows: OCT1–3, OCTN1 and 2, OAT1–4 and URAT1. Experimental methods are identical to those in Fig. 1.

(duodenum, jejunum and ileum). In contrast, y^+LAT1 was predominantly expressed in the small intestine (duodenum, jejunum and ileum) with little expression in other tissues.

PEPT1 also works as a drug transporter. To compare the mRNA expression level of PEPT1 with other drug transporters, we selected organic ion transporter family including organic cation transporters (OCT1–3), organic cation/carnitine transporters (OCTN1 and 2), organic anion transporters (OAT1–4) and urate transporter (URAT1). These transporters are mainly expressed in the kidney [11] and play important roles for renal secretion of ionic drugs [30], but some transporters were suggested to mediate the intestinal secretion of drugs [31]. As shown in Fig. 7, there was little expression for organic ion transporters except for OCTN2. OCTN2 exhibited a similar expression level of PEPT1 in the intestine. In addition to intestine, OCTN2 exhibited abundant expression in the stomach, colon and rectum.

4. Discussion

In the present study, we have demonstrated that the mRNA expression level of PEPT1 is higher in the duodenum and jejunum than that of B^0AT1, ASCT2 and $b^{0,+}$AT, which are localized at the brush-border membranes, and is equal to that of B^0AT1 in the ileum. Previous works showed that luminal contents after protein ingestion mostly consist of two to six amino acids [32], and that most of the dipeptidase activity is located in the cytoplasm of mucosal cells [33]. Thus, higher expression levels of PEPT1 than amino acid transporters at brush-border membranes should support the nutritional importance of the intestinal absorption of small peptides for protein nutrition. The high expression levels of intestinal PEPT1 also emphasize the pharmacological implication of peptide transporters. The peptide-like drugs transported by PEPT1 showed good oral bioavailability [3]. Transport abilities of PEPT1, with great flexibility for structural modification, and high expression of PEPT1 in the small intestine should be helpful for drug development to improve the intestinal absorption of poorly absorbed drugs.

The expression level of PEPT1 mRNA showed a peak in the duodenum, and gradually decreased to the ileum. Interestingly, the expression gradient of B^0AT1 mRNA is completely inversed to that of PEPT1. These reciprocal axial gradients in the mRNA expression of PEPT1 and B^0AT1 well correspond to the transport activities of small peptides [34,35] and amino acids for system B [36], and may have physiological relevance and importance to the maintenance of optimal protein nutrition [37]. Namely, the ingested proteins are digested by the membrane-bound peptidases to generate a major portion of the absorbable products, namely, amino acids and small peptides. Though these peptidases are present throughout the small intestine, levels of activity are much higher in the ileum than in the jejunum [38], indicating that the concentrations of free amino acids in the lumen are gradually increased, while the luminal concentration of small peptides are gradually decreased as the luminal contents move along the intestine. Furthermore, K_m values of typical substrates for PEPT1 (glycylsarcosine) [39] and B^0AT1 (leucine) [10] were about 1 mM, suggesting that both transporters have similar kinetic parameters. It is, therefore, suggested that the efficient absorption of digestive products of proteins may be achieved by a good correlation between the expression profiles for PEPT1 and B^0AT1 and the luminal concentrations of the corresponding substrates along the intestine.

Using tissue biopsy samples, Gutmann et al. [40] demonstrated that the mRNA expression of human breast cancer resistance protein (BCRP) was maximal in the duodenum and decreased continuously down to the rectum [40]. This expression profile is contrast to that of MDR1 mRNA expression (Fig. 1 right), suggesting that BCRP and

MDR1 also complement the transport function each other along the digestive tract as the substrate specificity of both transporters is partially overlapping [41]. Taken together, reciprocal expression of functionally related membrane transporters along the digestive tract should be responsible for the efficient biological systems in physiology and pharmacology.

Recently, Dave et al. [42] examined the expression of heteromeric amino acid transporters along the murine intestine. Their major findings related to our study were as follows: (i) the main sites of mRNA expression of absorptive amino acid transporters ($b^{0,+}$AT, LAT2 and y^+LAT1) were the jejunum and ileum; (ii) a substantial level of LAT2 mRNA was found in the stomach, an organ not previously recognized as a major site of amino acid absorption and (iii) LAT1 was most abundant in brain and weakly expressed in the intestine except for the stomach. Most of these findings were also confirmed in the human intestine, although the expression levels of $b^{0,+}$AT, LAT2 and y^+LAT1were not so high in the human intestine as compared to mouse intestine. Mutations in $b^{0,+}$AT lead to the hereditary disease cystinuria [22], and characterized by renal loss and altered intestinal absorption of cationic amino acids and cystine. Interestingly, patients with cystinuria do not exhibit obvious symptoms of protein malabsorption because the affected amino acids are absorbed adequately in the form of small peptides [43]. The much higher levels of PEPT1 than $b^{0,+}$AT explain this compensation.

In the present study, PEPT1 was found to be expressed in the stomach, induced by intestinal metaplasia. Intestinal metaplasia is characterized by the transdifferentiation of gastric epithelial cells to an intestinal phenotype [19]. Several intestinal-specific gene products such as trefoil peptides [44] were reported to be expressed in the stomach after intestinal metaplasia. *CDX2*, an intestine-specific transcription factor belonging to the *caudal*-related homeobox gene family, was involved in the induction of intestinal metaplasia of the stomach [45]. We have recently clarified the significant role of Sp1 in the basal transcriptional regulation of PEPT1 [46], but the mechanisms of intestine-specific expression of PEPT1 have not been clarified yet. CDX2 may also be responsible for the small intestinal and gastric expression of PEPT1.

Among organic ion transporters tested, only OCTN2 showed significant expression in the intestine, as the same expression level of PEPT1. The physiological substrate for OCTN2 is the carnitine, and mutations in this protein cause the autosomal recessive systemic carnitine deficiency [47]. In addition to carnitine, OCTN2 can transport various drugs such as verapamil [48]. This transport activity and abundant expression in the intestine suggested the pharamcokinetic role of OCTN2 for intestinal absorption like PEPT1. Using *Oct1* knockout mice, it was demonstrated that Oct1 plays important roles for intestinal excretion of cationic drugs [49]. But, the present expression analyses revealed that there was little expression of OCT1 along the digestive tract. Further studies are needed to clarify the clinical implication of intestinal OCT1.

In conclusion, we demonstrated the expression profile of PEPT1, amino acid transporters and organic ion transporters along the human digestive tract. The reciprocal distribution of PEPT1 and B^0AT1 may contribute to the efficient absorption of digestive products of ingested proteins. PEPT1 expression in the stomach is caused by intestinal metaplasia. OCTN2 is also abundantly expressed in the intestine. These findings may provide useful information about enteral nutrition, gastric pathology and pharmacology.

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