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Distribution of neutral amino acid transporter ASCT1 in the non-neuronal tissues of mice

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Abstract

Distribution of ASCT1, a neutral amino acid transporter, in non-neuronal peripheral tissues of adult and developing mice was examined by immunohistochemistry and immunoelectron microscopy. Immunoreactivity for ASCT1 in the digestive system was localized in basal cells of stratified squamous epithelia from oral parietes to nonglandular region of the stomach, chief cells of the glandular stomach, acinar cells of the salivary gland and exocrine pancreas, and Paneth's cells of the small intestine, in all of which the basolateral membrane was selectively immuno-labeled. In the liver of adult mice, ASCT1 immunoreactivity was detected on the plasma membrane of hepatocytes surrounding central veins, and a temporal expansion of immunoreactive hepatocytes was observed in the embryonic and CCl₄-treated adult livers. ASCT1 was also localized on the plasma membranes of proximal uriniferous tubule epithelial cells in the kidney of adult mice, and those of supporting cells in the medulla of adrenal gland. These results suggest that ASCT1 is expressed in various non-neuronal peripheral tissues in mice, and it contributes to the amino acid transport throughout non-neuronal tissues.

Key words : Amino acid transport, ASCT1, Mouse, Neutral amino acid transporter, Non-neuronal tissues

Introduction

Membrane-associated transport of sub-

stances in a cell is, in general, regulated by transporters expressing on plasma membrane. Such transporters are involved in transport of

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hydrophilic chemical compounds which are unable to cross the lipid bilayer of plasma membrane by passive transport. In the nervous system, for example, L-serine synthesized in glial cells is conveyed by a membrane-associated neutral amino acid transporter system to the extracellular milieu, and provided to neurons¹⁴. At the plasma membrane of hepatocytes, considerable kinds of transporters are responsible for the selective transport of glucose, lipid, amino acids, organic anions, and cations on a large scale. Although there are detailed molecular biological analyses¹³, substantial functions of amino acid transporters remain still unclear due to their functional complexities in the transport mechanism.

Amino acid uptake in cells is controlled by many kinds of transporters which are classified into several types according to their specificity for substrates and the requirement for ions⁴. Individual amino acids possess their own transport system, however, some groups of amino acids with similar lateral chains are transported by corresponding transport system. There are seven predominant amino acid transporter systems as follows: 1) System A contains glutamic acid transporter as a member, and transports L-alanine selectively in a Na⁺-dependent manner, 2) System ASC transports neutral amino acids with lateral chains consisting mainly of alanine and serine, and the localization of the system in the non-neuronal peripheral tissues was demonstrated in the present study, 3) System N transports glutamine and histidine selectively, 4) System B transports basic amino acids, 5) Na⁺-independent system L transports large-molecular neutral amino acids and L-DOPA with a wide range of substrate selectivity, 6) System asc, and 7) System C are similar to the system ASC in the affinity to amino acids for transport. Neutral amino acid transport sys-

tem ASC1 (ASCT1) also belongs to a family of excitatory amino acid transporter system (EAAT), and it is reported that the amino acid sequences of transporters within EAAT family resulted in 39-44% homology²⁰. ASCT1 transports neutral amino acids such as alanine, serine, cysteine or threonine depending on the extracellular concentration of Na⁺ in physiological pH^{4, 5, 19, 22}.

ASCT1 cDNA has been cloned in human brain and is located on chromosome 2 p13-p15^{1, 12}. ASCT1 in mice consists of 532 amino acids, and has a homology of 89.85% to human ASCT1 with 524 amino acids. It has been reported in mice that ASCT1 is expressed in glial cells in the brain¹⁸, and plays an important role in secretion of L-serine, a nutritional factor for neurons. In the non-neuronal tissues, however, the cell types expressing ASCT1 and its functions remain still unclear. The present study examined cellular distribution of ASCT1 in mouse non-neuronal peripheral tissues, and discussed its possible functions in neutral amino acid transport.

Materials and Methods

1. Animals and their treatment : Male and female ddY mice (Japan SLC, Hamamatsu, Japan) were used in this study. The study was carried out under the "Guideline for experiments by using experimental animals in Hokkaido University". Tissue samples were obtained from each three male and female mice aged 0, 1, 2, 3 and 8 weeks.

Fifteen hyperovulated mice were obtained by an intraperitoneal injection with 5 units of pregnant mare serum gonadotropin (Serotropin, Teikokuzoki Pharmaceutical Co., Tokyo, Japan) at 4:00 PM, and by an additional injection with 5 units of human chorionic gonadotropin (HCG ; Gonadotropin, Teikokuzoki Pharmaceutical Co., Tokyo, Japan) at 48 hours later. They were then mated with

adult male mice. Five hours after mating, it was estimated as 0 day embryo (E0). The embryos of E11, E17 and E19 were collected from the pregnant mice euthanized with an excess dose of pentobarbital sodium for investigation.

Mice aged 8 weeks were orally treated with 0.3ml of 1% carbon tetrachloride (CCl_4) to examine the change of ASCT1 expression. The hepatic tissues were collected from mice after 3 days, 1 week, and 2 weeks after the treatment.

2. Immunohistochemical and immunoelectron microscopical analyses.

Adult mice deeply anesthetized with pentobarbital sodium were perfused with physiological saline followed by 4% paraformaldehyde fixative in 0.1M phosphate buffer, pH7.4. Almost abdominal organs, including lung, heart, tongue, salivary glands, thyroid gland, and parathyroid gland were removed and then immersed with the same fixative at 4°C for 12 hours. All tissues immersed in 30% sucrose solution for overnight were embedded in Tissue-Tec O.C.T. compound, and quickly frozen in liquid nitrogen. Cryosections, 10µm thick, were pretreated with 0.3% Triton-X in 0.01M phosphate buffered saline (PBS) (pH 7.4) for 1 hour, and with 0.03% H_2O_2 in methanol for inactivation of endogenous peroxidase. Immunohistochemical staining was performed by using Histofine Kit (Nichirei, Tokyo, Japan). Briefly, after treatment with 10% normal goat serum for 30min, the sections were incubated with rabbit polyclonal antibody against ASCT1 diluted in 1/4,000 for overnight at room temperature. The sections were then incubated with biotinylated goat anti-rabbit IgG antibody and peroxidase labeled streptavidin for 1 hour, respectively. The reaction product was visualized in 0.05M Tris-HCl buffer (pH7.6) containing 0.01%

3,3'-diaminobenzidine and 0.001% H_2O_2 . Specimens for negative control were incubated with normal rabbit serum alone as a primary reacting medium. For conventional histological observation, some sections were stained with hematoxylin and eosin. Some cryosections of adrenal gland mounted with glycerin were observed directly with a fluorescent microscope (DMLB 100, LEICA, U.S.A.)⁷⁾, and then processed for the immunohistochemical staining as described above.

For immunoelectron microscopic observation, cryosections of the liver and small intestine were reacted with anti-ASCT1 antibody diluted 1/1,000 for overnight, and then with colloidal gold-labeled anti-rabbit IgG (BBInternational, Golden Gate, UK) (1/200) for overnight. After a slight refixing with 1% glutaraldehyde in PBS for 10 min and immersing in 0.1M acetic acid in PBS (pH 7.0) twice for 5 min, ASCT1 reaction was amplified with silver intensified Kit (HQ silver, Nanoprobe, Astony Brook, Neoprobe Co., U.S.A.) for 5 min in a dark room. The silver-intensified sections were dehydrated and embedded in Quetol 812 (Nisshin EM, Tokyo, Japan). Ultrathin sections were double-stained with uranium acetate and lead citrate, and observed under a transmission electron microscope (JEM 1210, JEOL, Tokyo, Japan).

The anti-ASCT1 antibody (kindly gift from Prof. Watanabe M., Graduate School of Medicine, Hokkaido University) is a rabbit polyclonal polypeptide antibody which was raised against 478-532 amino acid residues of mouse ASCT1. A previous Western blot confirmed that the antibody detected a single band of 62-65 kDa corresponding to the expecting molecular weight of ASCT1 in the mouse brain¹⁸⁾.

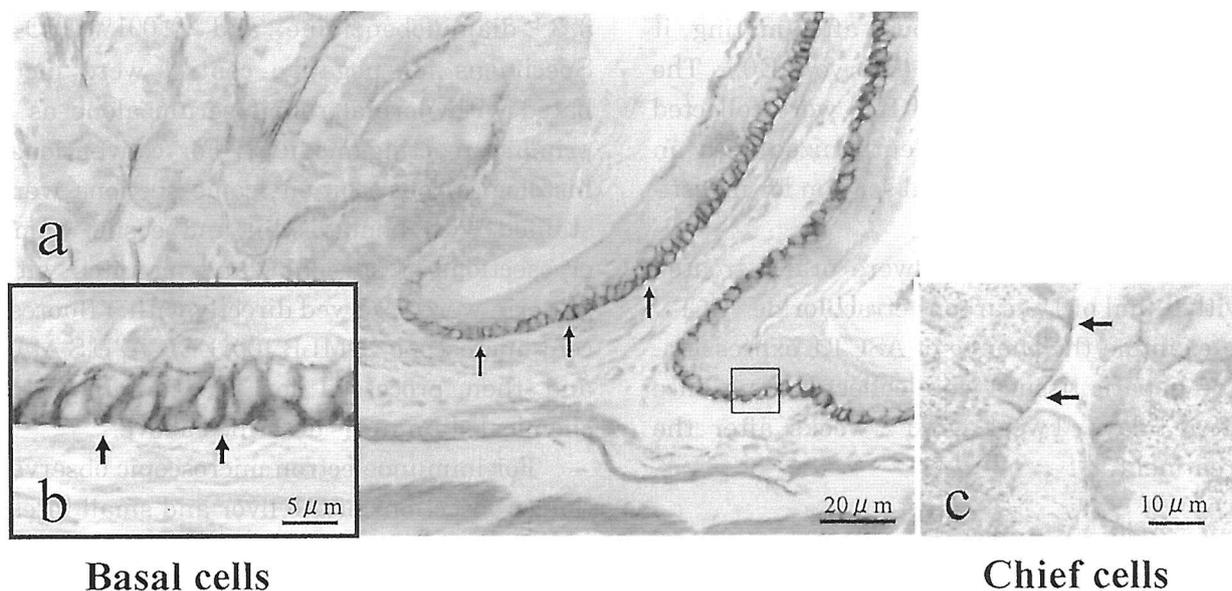


Figure 1. Immunostaining for ASCT1 in the stomach. a) Basal cells of stratified epithelium in non-glandular region are positive for ASCT1 (arrows). b) Higher magnification of basal cells of the stratified epithelium. Intense immunoreactivity is seen at the basolateral plasma membrane of the basal cells (arrows). c) Basolateral plasma membrane of chief cells in fundic glands are reactive to ASCT1 antibody (arrows).

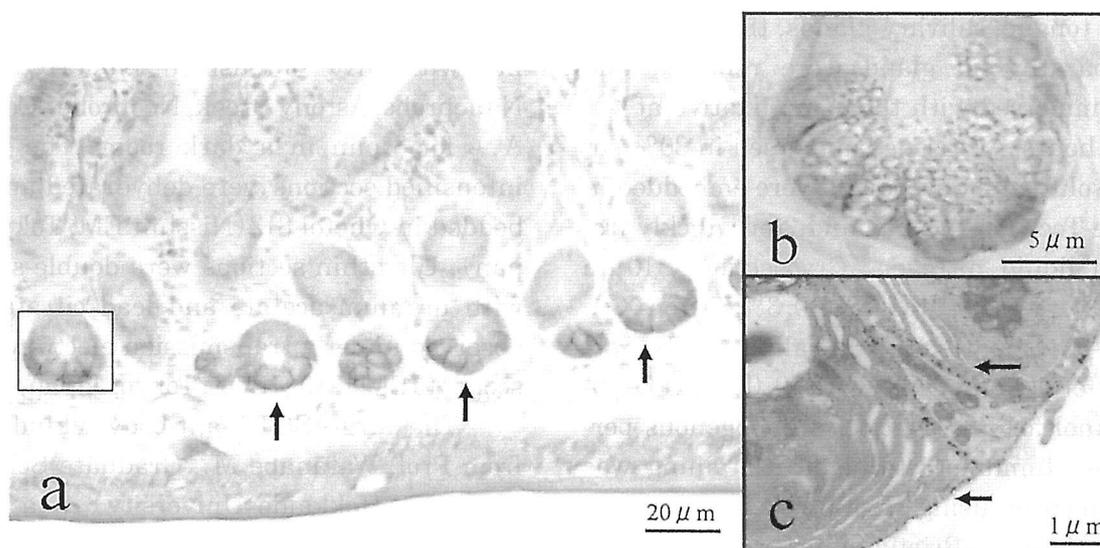


Figure 2. Immunostaining for ASCT1 in the small intestine. a, b) Immunoreactivity is restricted to the basolateral plasma membrane of Paneth's cells present at the bottom of intestinal crypts. c) Silver-intensified particles for ASCT1 can be seen on the plasma membrane of basolateral sides (arrows).

3. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNAs of several tissues were isolated by acid guanidium thiocyanate-phenol-chloroform extraction (ISOGEN, Nippon gene,

Tokyo, Japan) and subjected to reverse transcriptase (Reverta Ace, Toyobo, Osaka, Japan) according to the manufacturer's instructions.

Primer pairs for mouse ASCT1 (forward

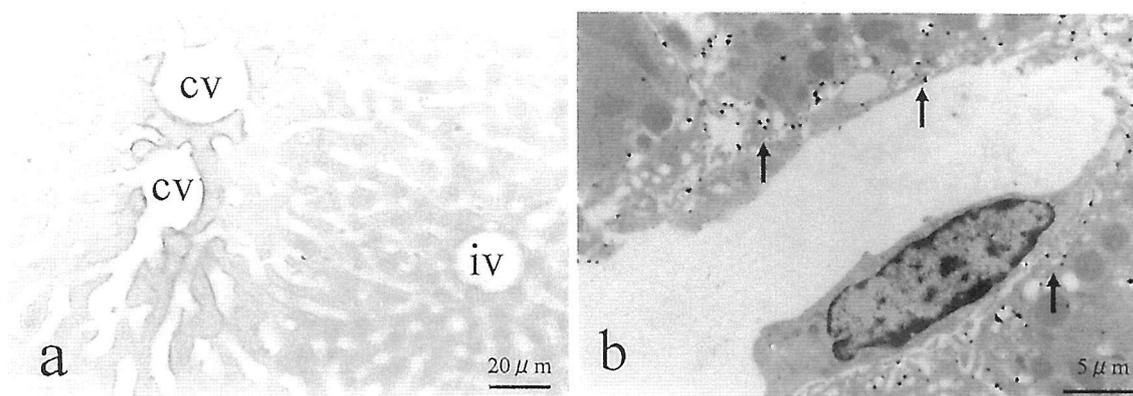


Figure 3. Immunostaining for ASCT1 in the liver. a) The immunoreactivity is seen in hepatocytes located in the vicinity of central veins (cv) (iv: interlobular vein). b) Silver-intensified immunogold particles are seen on the microvillous plasma membrane of hepatocytes facing to the sinusoidal capillary (arrows).

primer 5'-ATTCACTACGTCTGCAACCG-3' and reverse primer 5'-ATTCAGGTGGTTGAGGATCC-3') were constructed. PCR was carried out on a Promega PCR thermal cycler (iCycler, Madison, WI, U.S.A.) with the cycling sequence of 94°C for 5 min (one cycle), followed by 35 cycles consisting of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and extension at 72°C for 1 min. PCR mixture and enzymes were purchased from commercial company (Biolase, Boline, U.S.A.). The amplified samples were electrophoresed with 1% agarose gel, stained with ethidium bromide, and finally photographed under an ultraviolet lamp.

Results

1. Distribution of ASCT1 in adult mice

ASCT1 was broadly distributed in the digestive system. ASCT1 immunoreactivity was found in stratified squamous epithelia from tongue to non-glandular stomach, where the basolateral plasma membrane of basal cells were intensely immunolabeled. In the fundic gland, basolateral plasma membrane of chief cells were ASCT1 positive (Fig. 1).

Paneth's cells in the intestinal glands exhibited the most intense positive reactions in

the small intestine. By immunoelectron microscopy, silver intensified particles showing existing of ASCT1 accumulated on the basolateral plasma membrane of Paneth's cells (Fig. 2). Epithelial cells in the cecum, but not in the colon, showed an immunoreactivity on their basolateral plasma membrane (data not shown). The bundles of smooth muscle cells throughout muscular layers of the stomach and intestine were weakly to moderately ASCT1 positive, and stallate cells in submucosal nerve plexus were immunolabeled, while no reactivity was found in the myenteric nerve plexus (data not shown).

In the liver, positive reaction was found at the plasma membrane of hepatocytes surrounding the central vein in each hepatic lobule, but not at hepatocytes in the peripheral and intermediate regions of the lobules (Fig. 3). Immunoelectron microscopic analysis demonstrated the restricted localization of immunogold particles on the microvillous plasma membrane of hepatocytes facing to the Disse's space (Fig. 3). Acinar cells in exocrine pancreas were immunopositive, while no positive cells were found in endocrine islets (Fig. 4).

In the kidney, basal plasma membrane of

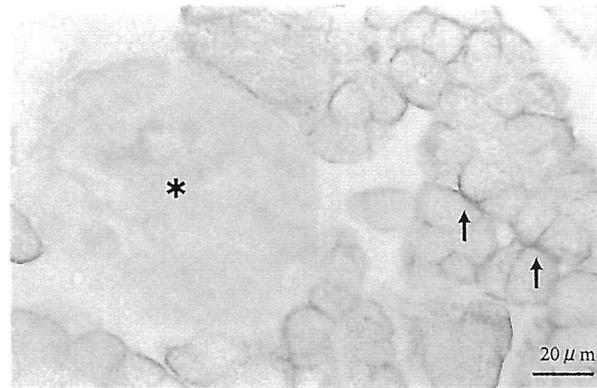


Figure 4. Immunostaining for ASCT1 in the pancreas. The plasma membrane of acinar cells (arrows) shows the immunoreactivity, while no reactivity is seen in the islet (*).

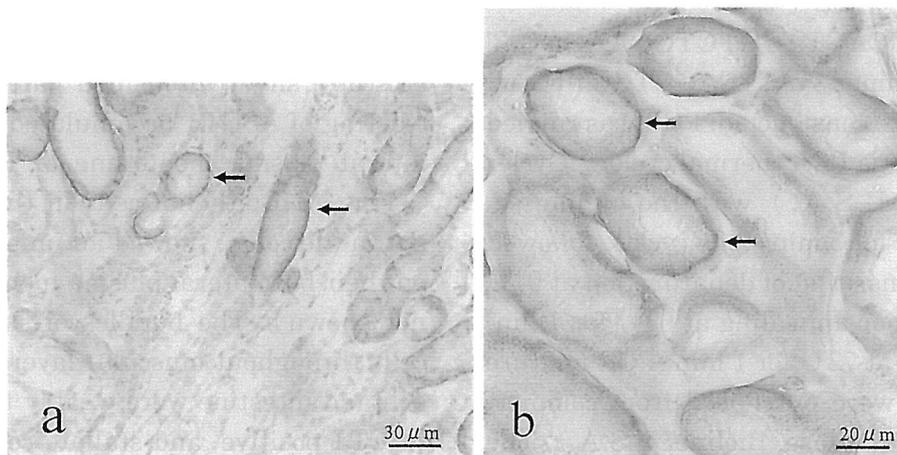


Figure 5. Immunostaining for ASCT1 in the kidney. a) Immunoreactivity is seen in the epithelia of proximal convoluted tubules in the cortex (arrows). b) Higher magnification of the renal tubules. The immunoreactivity is seen on the basal plasma membrane of the proximal tubule epithelial cells (arrows).

epithelial cells at the proximal convoluted tubules and papillary ducts showed positive reactions (Fig. 5). No significant immunoreactivity was recognized in other parts of urinary tubules, collecting ducts and glomerular cells.

Only adrenal medulla among endocrine organs contained ASCT1-immunoreactive cells, which were stellate-shaped supporting cells (Fig. 6). The immunoreactive supporting cells intervened among noradrenaline cells which emitted green fluorescence under a

fluorescent microscope (Fig. 6). In reproductive organs, plasma membrane of almost luteal cells in ovaries of pregnant mice raised an intense reactivity, although there was weak immunoreactivity in cyclic corpus luteal cells (data not shown). These immunohistochemical results are summarized in the Table.

RT-PCR analysis applying specific primer prepared for ASCT1 was carried out, and the ASCT1 mRNA expressions were detected in all peripheral tissues examined in addition to

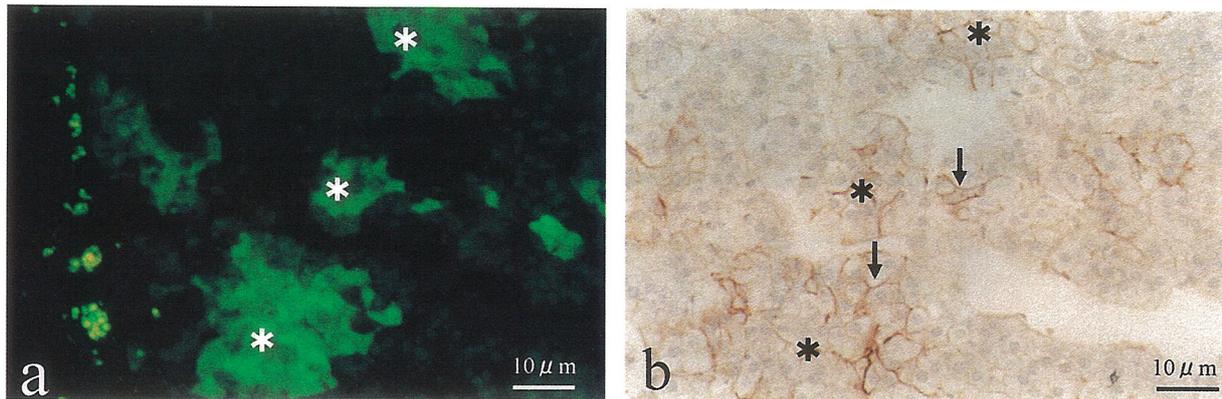


Figure 6 . Double detection of fluorescence for noradrenaline (a) and immunoreactivity for ASCT1 (b) using a single section of the adrenal medulla. ASCT1-immunoreactive stellate cells (arrows) are distributed among fluorescent chromaffin cell groups indicated by asterisks.

Table Immunoreactive cells for ASCT1 in adult mice

Tissues	Reactive cells
Digestive system	
Oral cavity–stomach nonglandular region	basal layer cells of stratified squamous epithelia
Stomach glandular cells	chief cells
Small intestine	Paneth's cells
Cecum	absorptive epithelial cells
Intestinal wall	bundles of smooth muscle cell, satellite cells in the submucosal nerve plexus
Salivary glands	acinar cells
Liver	hepatocytes close to the central vein
Pancreas	exocrine acinar cells
Kidney	epithelial cells of proximal tubules and papillary ducts
Adrenal gland (medulla)	supporting cells among noradrenaline cells
Genital system	
Ovary	luteal cells
Vagina	basal layer cells of stratified squamous epithelia

the central nervous tissues (Fig. 7).

2. Dynamics of ASCT1 in developing and CCl₄-treated mice

In the small intestine of mouse embryos, no Paneth's cells developed until birth. First immunoreactivity in Paneth's cells was found at 1 week of age together with appearance of large eosinophilic granules, and increased in intensity with aging (Fig. 8). ASCT1 reactivity in the intestinal mucosa during developing

stage was confined to the Paneth's cells. Hepatic ASCT1 immunoreactivity during developmental stage was found in E11 embryo, where whole plasma membrane of primordial hepatocytes throughout the liver was immuno-labeled. At E17, however, it was lost in those at the vicinity of interlobular veins in E17 embryo (Fig. 9). In the livers of postnatal mice from 0 to 2 weeks of age, ASCT1 was demonstrated in hepatocytes locating at regions close to the central veins and at the in-

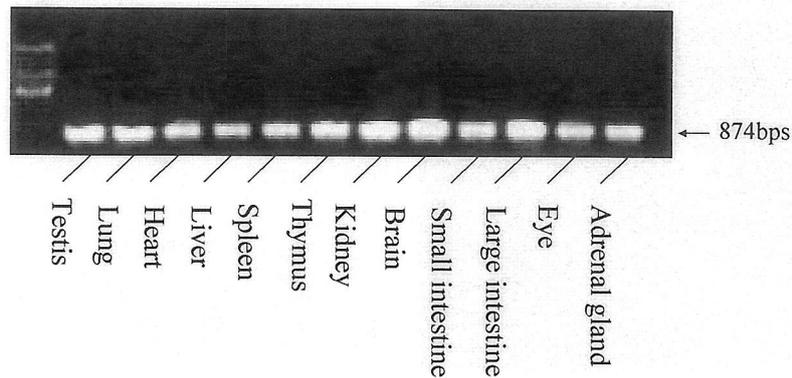


Figure 7. Expression of ASCT1 mRNA in non-neuronal peripheral organs. All tissues examined are positive for ASCT1 gene.

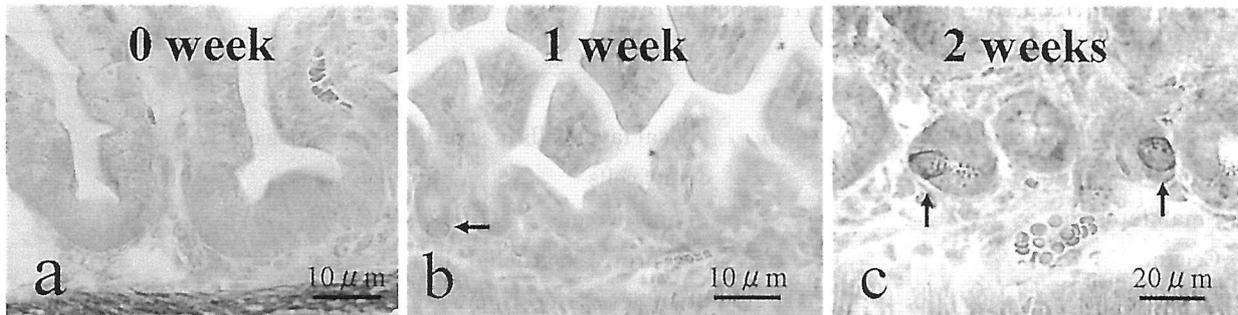


Figure 8. Immunostaining for ASCT1 in the small intestine, aged 0 (a), 1 (b) and 2 weeks (c). Immunopositive Paneth's cells for ASCT1 are seen at the base of intestinal glands from 1 week-old (arrows).

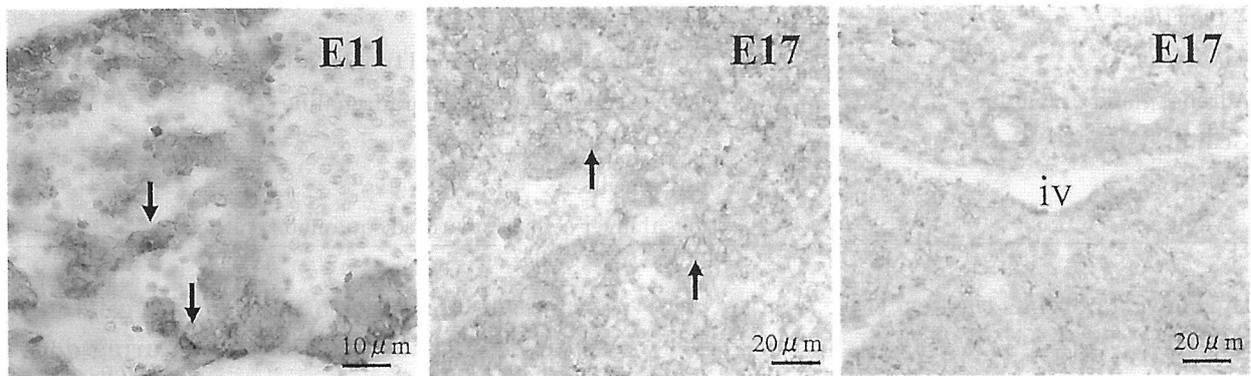


Figure 9. Immunostaining for ASCT1 in the embryonic liver. Immunoreactivity is seen at the plasma membrane in essentially all embryonic hepatocytes at E11 (arrows). However, the immunoreactivity is restricted to the central part of lobules at E17 (arrows). iv: interlobular vein.

intermediate region of a hepatic lobule (Fig. 10).

After 3 days to 1 week of 1% CCl_4 treatment, severe vacuolated changes were observed in hepatocytes throughout hepatic lobules, and then almost disappeared at 2 weeks

after the treatment. The immunoreactivity for ASCT1 in hepatocytes close to the central veins was increased in intensity at 1 week and extended to intermediate region of lobules. After 2 weeks, its distribution was re-

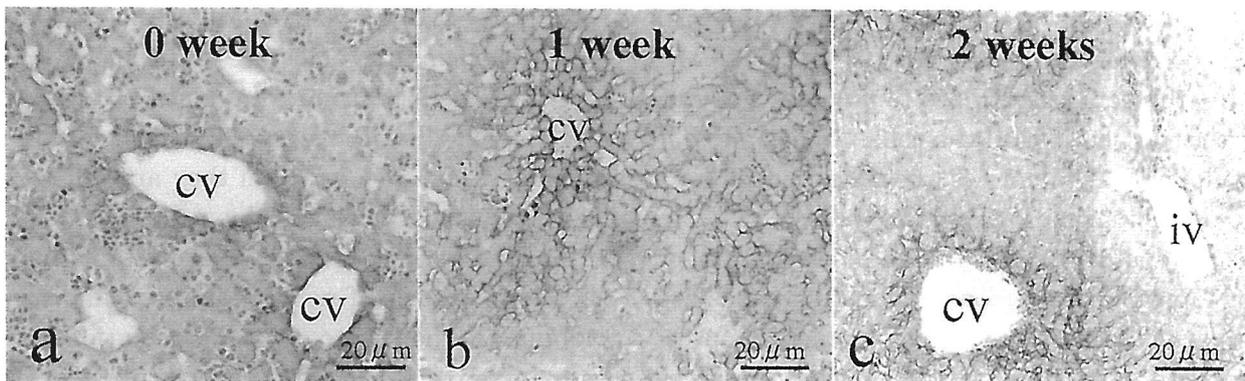


Figure 10. Immunostaining for ASCT1 in the liver aged 0 (a), 1 (b), and 2 weeks (c). It is distributed at the plasma membrane of hepatocytes surrounding central veins (cv), and those of the intermediate region of hepatic lobules (iv : interlobular veins).

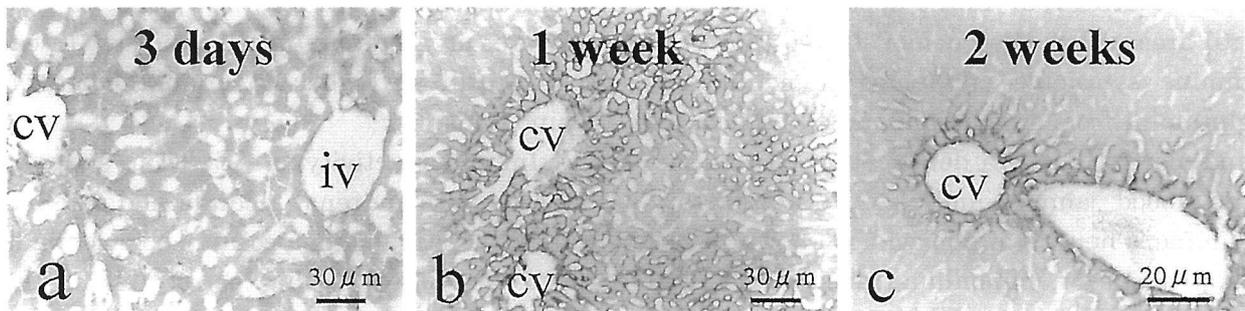


Figure 11. Immunostaining for ASCT1 in the liver from 3 days to 2 weeks after the CCl₄ treatment. a) At 3 days, the immunoreactivity is seen at the plasma membrane of hepatocytes close to the central vein (cv). (iv : interlobular vein)
 b) At 1 week, additional hepatocytes at the intermediate region are reactive to ASCT1 antibody. c) At 2 weeks, the reactivity is restricted again to hepatocytes in the vicinity of the central vein (cv).

stricted to hepatocytes in the vicinity of the central vein (Fig. 11).

Discussion

A wide-range of ASCT1 distribution in non-neuronal peripheral tissues, especially in tissues of epithelial origin was demonstrated in this study. ASCT1 in the brain is localized in glial cells, and functions to transport neutral amino acid, especially L-serine which is a nutritious substance for neuronal cells, from glial cells to the neighboring neurons, suggesting that its direction of transport is from cytoplasm to extracellular space^{9,18}). However, it is suggested that direction of transport by ASCT1 is different among each neuronal cell

type²). Present immunohistochemical results demonstrated the localization of ASCT1 mainly at the basal plasma membrane of epithelial cells adjacent to blood capillaries in the digestive organs, suggesting that amino acid transport by ASCT1 in these cells is directed from extravascular milieu into the cytoplasm, and vice versa.

The cytoplasmic granules in Paneth's cells contain endogenous bacteriocidal peptides such as α -defensin (cryptdin in mouse) and lysozymes^{16,17}). A recent study reported that the deficiency of α -defensin in mice induced a severe microbial infection, suggesting that the Paneth's cells play a role in mucous defense mechanism in small intestine²¹). The

present results assumed us that the neutral amino acids transported by ASCT1 into the Paneth's cells are utilized to produce the anti-bacterial peptides in the cells. Additionally, it is noted that the ASCT1 becomes a useful cytological marker for Paneth's cells.

Hepatocytes distributed at the periphery or intermediate regions of hepatic lobules are known to contribute to blood ammonium processing by the activated urea cycle. An excess amount of the ammonium is combined with pyruvic acid, and is further incorporated into what and processed by the hepatocytes close to the central vein. In the hepatocytes of central area, it has been demonstrated that glutamine synthetase promotes to synthesize glutamine from ammonium and glutamic acid, and also to induce a detoxication process for the blood ammonium^{3,10,11,15}. Additionally, alanine, a neutral amino acid, is known to act as an agent for ammonium transport. The restricted localization of ASCT1 shown in the present study and the active glutamine synthetase in hepatocytes around central vein suggest that ASCT1 plays an important role for ammonia detoxication by alanine uptake and glutamine synthesis in the hepatocytes.

An increased expression of ASCT1 both in developing and CCl₄-recovering hepatocytes may be explained that an enormous amount of energy is needed in hepatocytes under these conditions, and alanine is transported into hepatocytes from blood by system A and system ASC. In the previous report, an increased expression of the system A was observed in regenerating livers after partial surgical hepatectomy⁸. Based on these results, it is suggested that an expression of ASCT1 must be increased in developing and recovering hepatocytes for supplementation of system A, at which the cellular activity is insufficient to such a higher demand of alanine transport.

In the kidney, it is reported that intraluminal basic amino acids are transported into epithelial cells of urinary tubules by a system b⁺0 which distributes at the plasma membrane of the cells, and then they are released toward blood circulation by a system y⁺L⁶. From these viewpoints, it is likely that there is a transcytosis of neutral amino acids from tubular lumen into blood circulation via proximal epithelial cells, acting in concert with basic amino acid transport. In the ovary, an enhanced immunoreactivity of ASCT1 was demonstrated in corpus luteal cells of pregnant animals. ASCT1 may be up-regulated to activate progesterone production in luteal cells by utilizing higher amount of the neutral amino acids.

Immunoreactivity for ASCT1 in supporting cells of the adrenal medulla and satellite cells of intestinal submucosal plexus reminds us of ASCT1-expressing astrocytes in the brain. According to the functions of ASCT1 in the brain mentioned above, a similar contribution of supporting cells to serine transport is suggested to be conserved in adrenal medulla and peripheral nervous system.

In conclusion, ASCT1, a neutral amino acid transporter, is widely distributed in non-neuronal peripheral tissues throughout the body, and may function in uptake of neutral amino acids from the blood circulation or transcellular transport. It is strongly expected that the present fundamental data concerning ASCT1 is helpful for further studies, clarifying the cause of diseases associated with amino acid transport system.

Acknowledgments

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