NOTE *Internal Medicine*

Evidence of LAT1 expression in canine caput epididymis

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ABSTRACT. L-type amino acid transporter 1 (LAT1), the first isotype of amino acid transport system L, transports aromatic and branched amino acids pivotal for fundamental cellular activities such cellular growth and proliferation. LAT1 expression was high only in the brain in contrast to its limited distribution and low level of expression in normal tissues. We found potent LAT1 expression in canine caput epididymis by quantitative RT-PCR and Western blotting analysis. Immnuno-histochemical examination revealed observable LAT1 in microvillous epithelial cells.

KEY WORDS: distribution, epididymitis, LAT1, sperm maturation

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System L is an amino acid transport system that mediates the Na-independent transport of large neural amino acids as a Na-independent system for neutral amino acids, which are inhibited by 2-aminobicyclo [2.2.1] heptane-2-carboxylic acid (BCH) [2, 20]. It also plays a pivotal role in the permeation of amino acids through blood-tissue barriers, such as the blood-brain and placenta barriers [3]. L-type amino acid transporter 1 (LAT1), an isoform of amino acid transport system L, transports branched or aromatic amino acids essential for fundamental cellular activities, such as cellular growth, proliferation and maintenance. LAT1 has recently received a considerable amount of attention owing to observations that many tumor cell lines and primary human tumors highly and preferentially express LAT1, in contrast to its limited distribution and low-level expression in normal tissues [6, 7, 9–11, 14, 16, 21].

Spermatozoa produced in the testis pass through the epididymis, where post testicular maturation of the spermatozoa occurs. During the epididymis transition, spermatozoa become fully motile and capable of fertilizing the oocyte. The epididymis is structurally organized into three major distinctive segments: caput, corpus and cauda. In all segments, the epididymal duct consists of epithelial cells attached to a basal lamina, which is surrounded by contractile cells. The duct is coiled and encapsulated within a sheath of a connective tissue of the tunica vaginalis and has 3 distinct functions. Fisrt is sperm transport which is achieved by contraction and movement of fluid from the testis. Second is maturation of spermatozoa, as during their epididymal transit, the spermatozoa acquire a change in their motility pattern and become able to bind to and penetrate the oocyte. Third is the storage of sperm, which occurs in the caudal region. Previously, we reported the cDNA sequence and distribution of canine LAT1 and confirmed its limited distribution [12]. In this study, we found potent LAT1 expression in caput epididymidis and investigated its detailed expression using anti-canine LAT1 polypeptide antiserum.

All experiments were performed according to the guidelines of The Laboratory Animal Care Committee of Azabu University and were in compliance with the Fundamental Guideline for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions. All canine tissues were obtained from a healthy male beagle dog (2 years old). To investigate LAT1 protein expression in various tissues, anti-canine LAT1 serum was prepared with the synthetic peptide antigen designed according to the C-terminus amino acid sequence of canine LAT1 (ref. 12: C-QKLMQVVPQET). The cell membrane of brain tissue for Western blotting analysis was prepared as reported by Denker *et al*. [4]. The membranes were simply solubilized and electrophoresed on 12% polyacrylamide gels, and the proteins were blotted to PVDF membrane on wet condition (100 V for 2 hr). After blocking (Block Ace, DS Pharma Biomedical, Osaka, Japan), the membrane was then treated with a primary antibody (Rabbit anti-dog LAT1 polyclonal antibody), followed by a secondary antibody (anti-rabbit IgG (H+L) goat IgG Fab' HRP, × 20,000, Seikagaku Corp., Tokyo, Japan). The LAT1 protein was detected with an ECL plus a chemiluminescence detection system (GE Healthcare Bioscience, Chalfont, U.K.) and exposed to an x-ray film. Figure 1A shows Western blotting analysis of LAT1 expression of various canine tissues. The single band at 40 kDa was observed only in epididymis and cerebrum. These signals disappeared when the antiserum was pre-absorbed with

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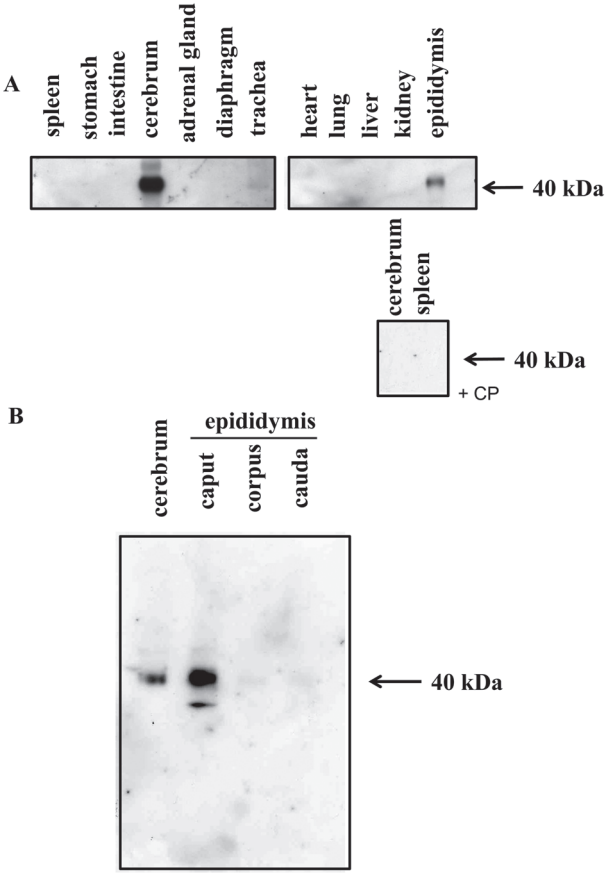

Fig. 1. Western blotting analysis of LAT1 protein expression in cell membranes from various tissues (each lane contains 10 μg protein) (A). Comparison of LAT1 expression between caput, corpus and cauda of canine epididymis. Cerebrum (10 μg) and each part of the epididymis (each 20 μg) were loaded on 15% SDS-PAGE gel (B). +CP represents the using pre-absorbed antiserum with corresponding synthetic peptide.

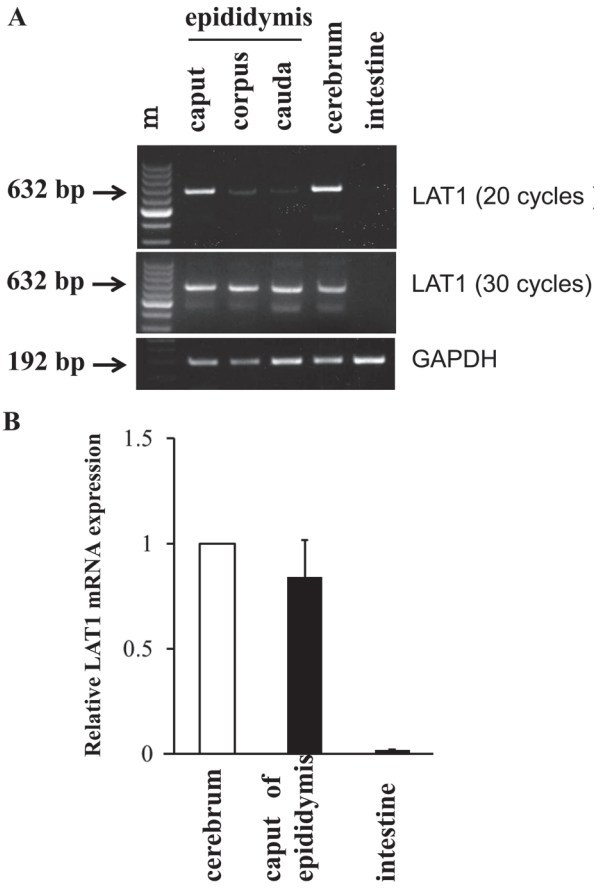

Fig. 2. RT-PCR analysis of LAT1 mRNA expression in epididymis, cerebrum and intestine. At 20 and 30 cycles, the PCR samples were loaded on 2% agarose gel. m: 100 bp ladder (A). Relative mRNA abundance of LAT1 was quantified by qRT PCR by calculating the abundance of LAT1 relative to RP19 (internal control). Three independent experiments were performed. Means and SD were indicated (B).

corresponding synthetic peptide. Figure 1B revealed that LAT1 protein was detected only in the caput epididymidis and was not observed in the corpus and cauda. To confirm the LAT1 expression in mRNA level, RT-PCR analysis was performed. Simply, total RNA was isolated from canine tissues [13] using the RNA extraction solution (Isogen, Nippon Gene, Tokyo, Japan). The RNA was treated with DNase I (Invitrogen, Carlsbad, CA, U.S.A.). The primers used in this study were following; LAT1 (sense: 5′-TGT ACG GGC CCA CGA AGA CAG GGA T-3′, antisense: 5′-CAG GAC AGG CCC ACG AAG ACA GGG AT-3′, expected size: 632 bp), GAPDH (sense: 5′-ATC ACC ATC TTC CAG GAG CGA GA-3′, antisense: 5′-GTC TTC TGG GTG GCA GTG ATG G-3′, expected size: 192 bp). RT-PCR amplification was performed using the Superscript III first cDNA System Kit (Invitrogen) and Hot start Ex Taq DNA polymerase (Takara Bio, Kyoto, Japan). PCR conditions were as follows: 20 or 30 cycles of 3 steps: 94°C for 15 sec, 65°C for 15 sec and 72°C for 45 sec. Figure 2A showed RT-PCR analysis of the canine LAT1 mRNA in the 3 parts of epididymis. Discrete bands 632 bp in length, derived from canine mRNA, were observed in the cerebrum and caput epididymidis at 20 cycles, while LAT1 was observed in all parts of the epididymis at 30 cycles but not in the intestine. To evaluate the detailed mRNA expression of LAT1, quantitative real-time PCR evaluation of LAT1 in canine epididymis was investigated. The primer set used for qRT PCR was following; LAT1 (sense: 5′-CCT GGT GTA CGT GCT GAC GAA-3′, antisense: 5′-TCC CAG GTG ATA GGT CCC AAA G-3′) and RP19 (sense: 5′-CCT TCC TCA AAA AGT CTG CG-3′, antisense: 5′-GTT CTC ATC GTA GGG AGC-3′). qRT PCR was performed using the Thermal Cycler Dice® Real Time System II (Takara Bio). The relative standard curve method was used to analyze the data, with relative amounts of unknown samples being calculated using linear regression analysis. The qRT-PCR results are presented as the gene expression of the target gene (LAT1) relative to that of the

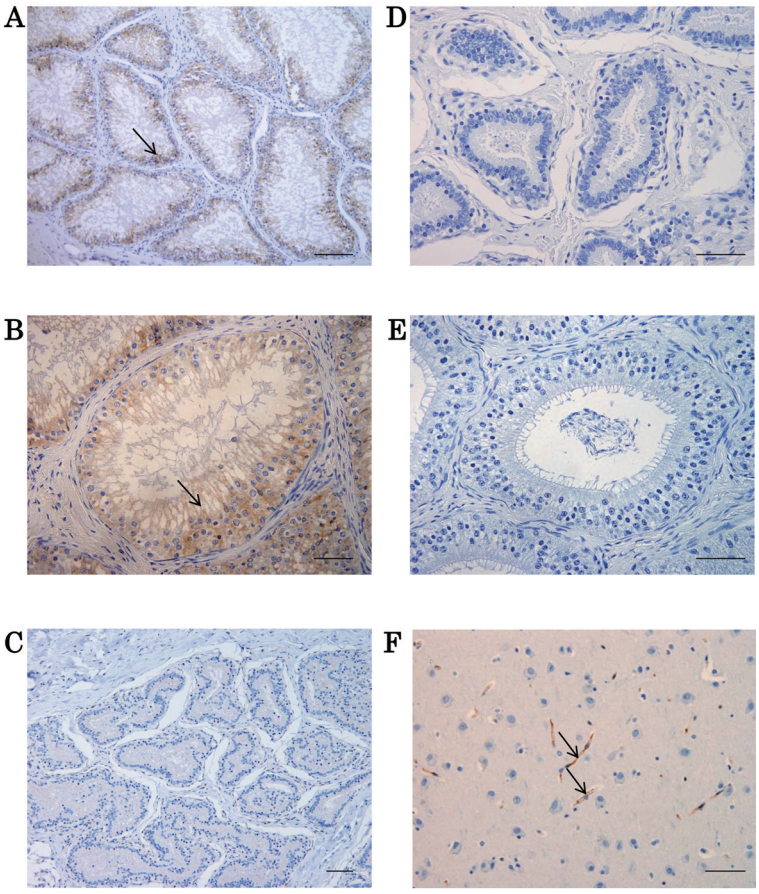

Fig. 3. Immuno-histochemistry of LAT1 localization using anti-LAT1 serum prepared in this study (A, B, D, E and F) or anti-LAT1 serum which was pre-absorbed with synthetic LAT1 peptide (C). LAT1 was expressed in the microvillous epithelial cells of caput epididymidis (A, B), while there was no signal in corpus and cauda epididymidis (D, E). The signal of caput epididymidis disappeared when the anti-serum pre-absorbed with LAT1 peptide was used (C). The distinct signal was observed at microvessel endothelial cells in cerebrum (F). Bar=200 μm (A), Bar=50 μm (B, C, D, E, F).

housekeeping gene RP19 and expressed relative to the level in cerebrum. qRT PCR showed that LAT1 expression in the caput epididymidis was the same level (0.85) as cerebrum, which was 50 times higher than that of intestine (Fig. 2B).

Immunohistochemistry was performed as described elsewhere [18]. Simply, after deparaffinization, the sections were heated 5 times for 3 min in a microwave oven in 0.01 M citric acid (pH 6.0) and rinsed with Dulbecco's PBS. Rabbit anti-dog LAT1 polyclonal antibody prepared in our laboratory was incubated with the sections after inactivation of endogenous peroxidase with methanol containing 0.3% H_2O_2 at room temperature for 20 min. Immunostaining was carried out using a commercial kit (Histofine Simple Stain MAX PO, Nichirei, Tokyo, Japan). Then, 3,3′-diaminobenzidine H_2O_2 solution (DAB) was applied to induce a color development reaction. After DAB reaction, the specimens were washed in deionized water three to four times followed by staining of the nuclei with hematoxylin for observation. Immnuno-histochemical examination using the C-terminus canine LAT1 antiserum revealed that LAT1 was observed in microvillous epithelial cells (Fig. 3A and 3B), while no signal was observed in corpus and cauda epididymidis (Fig. 3D and 3E). LAT1 expression in cerebrum was observed in capillary endothelium (Fig. 3F). These signals in caput epididymidis disappeared when the antiserum pre-absorbed with corresponding LAT1 peptide was used (Fig. 3C).

LAT1 is vital, because of its ability to transport essential amino acids. LAT1 is mainly expressed in fetal tissues and cancer cells, where it endows a growth advantage [15, 21]. The LAT1 expression was high only in the brain, in contrast to its limited distribution and low level of expression in normal adult tissues. In this study, we found potent LAT1 expression in canine epididymis by RT-PCR and Western bolt analysis. A detailed examination revealed that the microvillous epithelial cells exhibited LAT1 expression. Through immnuno-histochemical examinations, LAT1 was observed

in microvillous epithelial cells of caput epididymidis and microvessel endothelial cells in brain. The maturation of spermatozoa and the acquisition of motility and fertilizing ability occur as a consequence of exposure to the luminal environment of the epididymis. The composition of the luminal fluid that bathes spermatozoa as they transit through the epididymis is highly complex and changes progressively along the tissue [5, 19]. The secretory and absorptive activities of the epididymal epithelium mediate the changes in luminal fluid and thus determine the microenvironment in which spermatozoa are able to become fully mature. The maturation of spermatozoa is connected to a myriad of biochemical and molecular changes as they traverse the epididymis. These changes are considered to be caused by ion transport and attachment of proteins secreted in the lumen of the epididymal duct. Sperm maturation is well orchestrated along the epididymis and depends on highly regionalized gene expression patterns [8]. For example, it was reported that lack of group III secreted phospholipase A_2 (sPLA$_2$-III) which is highly expressed in caput epididymidis leads to defects in sperm maturation and fertility [17].

Interestingly, it was reported that leucine transport activity was increased in developing mouse ovarian follicles and this activity was reduced by BCH [1]. While iso-type of system L was not identified in follicles, system L may participate in follicles maturation. The importance of LAT1 expression in sperm maturation remains unknown. LAT1 may contribute to the maturation of spermatozoa by providing aromatic and branched amino acids. To our knowledge, this is the first report to address LAT1 expression in the caput epididymidis, and we will next investigate the relationship between sperm maturation and amino acid concentration in epididymis.

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