Mouse MCT3 gene is expressed preferentially in retinal pigment and choroid plexus epithelia

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Philp, Nancy J., Heeyong Yoon, and Lorraine Lombardi. Mouse MCT3 gene is expressed preferentially in retinal pigment and choroid plexus epithelia. Am J Physiol Cell Physiol 280: C1319–C1326, 2001.—Monocarboxylate transporters (MCTs) are a family of highly homologous membrane proteins that mediate the 1:1 transport of a proton and a lactate ion. In chicken, MCT3 is preferentially expressed in the retinal pigment epithelium (RPE). We have isolated the mouse MCT3 cDNA and gene and characterized the pattern of tissue expression. MCT3 is a single copy gene with a 1.8-kb transcript that encodes a protein with a predicted molecular mass of 51.5 kDa. Based on Northern hybridization analysis, MCT3 transcript was expressed in only two tissues: RPE and choroid plexus epithelium (CPE). The choroid plexus forms a barrier between the cerebrospinal fluid and fenestrated capillaries, similar to the organization of the RPE and choroidal vessels. Immunohistochemical staining demonstrated that MCT3 was restricted to the basolateral membranes of both epithelia but was more abundant in RPE than CPE. Differences in the level of protein expression were confirmed by Western blot analysis. The cloning of MCT3 identifies a specific transporter that could regulate lactate levels in fluid-bathing neuronal tissues.

THE RETINAL PIGMENT EPITHELIUM (RPE) forms the outer blood-retinal barrier that controls the chemical composition of the subretinal space in much the same way that the choroid plexus epithelium (CPE) maintains the composition of the cerebrospinal fluid (CSF) (18). The basolateral surface of the RPE is in contact with the blood plasma that filters through the fenestrated capillaries in the choroid. The apical surface of the RPE is in intimate contact with the neural retina and extends processes into the subretinal space that interdigitate with photoreceptor cell outer segments. Tight junctional complexes at the lateral borders of the RPE impede the movement of even small water-soluble molecules between cells. Substances that are produced and used in large quantities, such as glucose, amino acids, and lactate, are transported into and out of the retina by the RPE (3, 21).

In retina, lactate is produced through aerobic glycolysis and utilized to fuel oxidative phosphorylation (22, 23). Glucose is transported from choroidal vessels to the subretinal space by the RPE. Müller glial cells have a limited number of mitochondria and utilize glucose to produce and release lactate into the subretinal space under aerobic conditions. Lactate is used by photoreceptor cells for oxidative phosphorylation. The Müller glial cells produce lactate, while the RPE regulates lactate levels in the subretinal space by transporting excess lactate from the retina to the choroidal venules (7, 10). A comparable mechanism has been proposed to operate in the brain where glial cell metabolism of glucose provides lactate to neurons for further metabolism (12, 15). This local control of metabolic fuels is referred to as the “lactate shuttle” (12).

In brain, the choroid plexus consists of a branched network of fenestrated capillaries ensheathed by epithelial cells that are connected by tight junctional complexes. The choroid plexus epithelium produces CSF, regulates its ionic composition, and transports micronutrients into the brain (20). Tight junctional complexes of the CPE form a barrier between the CSF and fenestrated capillaries, similar to the organization of the choroid and RPE. In contrast to the subretinal space, under normal conditions, lactate concentrations in CSF are ~1.4 mM, similar to levels found in plasma (19).

Transport of lactate, as well as pyruvate and ketone bodies across the plasma membrane of cells, is mediated by a family of proton-coupled monocarboxylate transporters (MCTs) (16). Eight members of this family have been identified in human tissues and share a 25–70% homology in their primary structure (17). MCTs share structural and functional features but differ in their temporal and spatial distribution. On the basis of their primary structure, the MCTs are predicted to have 12 membrane-spanning domains, and their NH2 and COOH termini are on the cytoplasmic side of the membrane. The membrane-spanning do-

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mains share the greatest sequence identity among the various isoforms, whereas the COOH-terminal regions are not well conserved. MCT1 is expressed in most tissues, whereas MCT2, MCT3, and MCT4 have more restricted distributions (reviewed in Refs. 5 and 6).

MCT3 regulates lactate levels in the subretinal space by transporting lactate across the basolateral membrane of RPE to the choroidal vessels (14). It may play a similar role in maintaining lactate concentrations in the CSF by transporting lactate out of the CPE. In the present study, we isolated the mouse MCT3 cDNA and characterize in detail for the first time the expression pattern of this isoform in a mammalian system.

MATERIALS AND METHODS

Chemicals. All reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise stated.

Tissues. Tissues for RNA, protein, and immunohistochemical analyses were isolated from adult and suckling C3H mice. The animals were euthanized with pentobarbital sodium (150 mg/kg body wt). Eyes were enucleated and the anterior segment was removed with a razor blade. The posterior eyecup was placed in PBS containing 15 mM EDTA and 3% sucrose. RPE or RPE/choroid were peeled off the sclera with fine forceps under a dissecting microscope. Brains were removed from the skull, and the choroid plexus was collected from the lateral and fourth ventricles. Total RNA used for cDNA cloning and Northern blot analysis was prepared from various tissues of C3H mouse using TRIzol reagent (Life Technologies, Grand Island, NY) as described elsewhere (14). Detergent-soluble lysates from RPE and choroid plexus were prepared as previously described (14).

Cloning of mouse MCT3 cDNA. Mouse MCT3 cDNA was cloned from total RNA isolated from RPE by a combination of 5'-rapid amplification of cDNA ends (RACE) and 3'-RACE, the first-strand cDNA was prepared with 3'-capped g of total RPE RNA and an adaptor primer 3AP (provided in -RACE, the first-strand cDNA was prepared with 3'-capped g of total RNA of mouse choroid plexus and a primer SALIM used for cDNA cloning and Northern blot analysis was prepared using the BLAST program provided by the National Center for Biotechnology Information server at the National Institutes of Health.

Southern blot analysis. C3H mouse genomic DNA (20 μg) was digested with BamHI or EcoRI, separated on a 1% agarose gel, and transferred to a Hybond-N nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The membrane was prehybridized for 4 h and hybridized overnight at 45°C to MCT3 digoxigenin-labeled riboprobe at 5 ng/ml of hybridization solution. Blots were washed twice for 5 min in 0.1% SDS/2× SSC (sodium chloride sodium citrate; 1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) at room temperature; once in 0.1% SDS/0.5× SSC, and once in 0.1% SDS/0.1× SSC for 20 min at 45°C. Detection procedure was the same used for Northern analysis.

Northern blot analysis. Five micrograms of total RNA was denatured with 0.5 M glyoxal and 50% dimethyl sulfoxide and separated on a 1% agarose gel in 10 mM sodium phosphate buffer (5 mM Na2HPO4 and 5 mM NaH2PO4, pH 6.5). RNA was transferred and cross-linked to a Hybond-N membrane. The membrane was prehybridized for 4 h and hybridized overnight at 45°C with an MCT3 riboprobe prepared from a 0.6-kb 3'-RACE fragment (1009–1593). Blots were washed twice at room temperature for 5 min each in 0.1% SDS/2× SSC, 20 min in 0.1% SDS/0.5× SSC, and 20 min in 0.1% SDS/0.1× SSC at 65°C and rinsed in maleate buffer (0.1 M maleic acid, pH 7.5, and 150 mM NaCl). The hybridized probe was detected with alkaline phosphatase-conjugated anti-digoxigenin antibody and Lumi-Phos 530 (Roche Molecular Biochemicals, Indianapolis, IN) as previously described (11).

Antibodies. A rabbit polyclonal antiserum to the COOH terminus of MCT3 was generated and characterized by our laboratory (14). Antibody specificity on Western blots and tissue sections was confirmed by incubating with the antibody in the presence of the competing peptide (CAVPFDL-HESIGGHHEARGQKA). Rabbit polyclonal antibody to the COOH terminus of mouse MCT1 was a gift from Dr. Ian Simpson (Pennsylvania State University).
**Immunofluorescence.** Eyes and brains were fixed by immersion in 3.5% formaldehyde in PBS (pH 7.4, 4°C). Tissues were embedded in paraffin, and 6- to 8-μm sections were cut and placed on silanized slides (American Histolabs, Gaithersburg, MD). Sections of adult mouse eye and brain were also purchased from Novagen (Madison, WI). Slides were deparaffinized before use with three 5-min washes in xylene, followed by two 5-min washes in 100% ethanol. Tissues were rehydrated in a graded series of ethanol followed by H2O and PBS and were then blocked for 1 h in PBS with 5% bovine serum albumin.
serum albumin (BSA) and 0.1% Tween 20 (pH 7.4). Samples were incubated for 1 h with primary antibodies diluted in PBS containing 1% BSA and 0.1% Tween 20. Primary antibodies were detected using Cy3-conjugated AffiniPure donkey anti-rabbit IgG (1:250; Jackson ImmunoResearch Laboratories, West Grove, PA) diluted in the same buffer as the primary antibodies. Sections were examined on a Nikon Microphot FX microscope equipped with an Optronics digital camera (Goleta, CA). The images were collected at indicated integrated digital exposure times without adjustment of brightness or contrast. Figures were prepared using Adobe Photoshop 5.5 and Adobe Illustrator 9.0.

**Western blot analysis.** Detergent-soluble lysates were prepared from mouse tissues as previously described (14). Protein concentrations were determined by the bicinchoninic acid assay (Pierce, Rockford, IL) with BSA as a standard. Samples (12.5 μg) were separated on 4–12% Tris-glycine SDS-polyacrylamide gradient gels (Novex, San Diego, CA) and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were incubated for 1 h at room temperature in Tris-buffered saline (TBS) blocking buffer (20 mM Tris, 137 mM NaCl, pH 7.5 with 5% BSA), followed by 1 h of incubation in TBS with primary antibodies diluted 1:1,000. The secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit, was diluted 1:10,000 (Bio-Rad). Enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) was used for detection.

**RESULTS**

**Cloning of mouse MCT3.** MCT3 was initially identified in chicken RPE (13). A human ortholog was subsequently cloned (24). In both chicken and human, MCT3 is preferentially expressed in RPE. In preparation for studies of MCT3 gene deletion, in this report we characterized the molecular structure and determined the tissue distribution of mouse MCT3. Mouse MCT3 cDNA was isolated and sequenced using a combination of 3′- and 5′-RACE and RT-PCR with RPE RNA as detailed in MATERIALS AND METHODS. The resultant cDNA consisted of a 209-bp 5′-untranslated region, 1479 bp of coding sequence, and a 114-bp 3′-untranslated region. An in-frame stop codon (UGA) was present 66 bp upstream from the predicted ATG translation start site. A typical polyadenylation signal sequence, AATAAA, began 16 nucleotides upstream from an observed poly(A) tail. The mouse MCT3 cDNA sequence was deposited in the GenBank database (accession no. AF019111).

**Table 1. Exon/intron boundaries of the mouse MCT3 gene**

<table>
<thead>
<tr>
<th>Exon</th>
<th>Nucleotide Sequence at Splice Site</th>
<th>Exon</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>(119)-caggctecag/tgtgatgtg--(607)--tgccagag/tgdsccagag-(76)</td>
<td>1b</td>
</tr>
<tr>
<td>1b</td>
<td>(76)-tgctagag/tgctagag--(138)--ccagacag/aagcagaag--(225)</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>(225)-ACGGCACGG/tgcatag--(599)--gcagcacag/GCCCGCTGT-(144)</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>(144)-TCAGACAG/tggaggta--(208)--gcagacag/GCCCTGAGG--(768)</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>(768)-CCTCTGCCG/tggtttgg--(394)--tttccctag/GGCCGCCTTG-(467)</td>
<td>5</td>
</tr>
</tbody>
</table>

Nucleotide sequences at the exon/intron borders of the mouse MCT3. Exon sequences are indicated in bold type, and numbers in parentheses indicate the size of either the exon or intron.
Different species were found primarily in the cytoplasmic loop between the sixth and seventh membrane-spanning domains and the COOH-terminal end (Fig. 1).

Genomic structure of mouse MCT3. Mouse MCT3 was shown by Southern blot analysis to be encoded by a single gene (Fig. 2A). A BAC clone containing the MCT3 gene was isolated and sequenced as detailed in MATERIALS AND METHODS and deposited in GenBank under accession no. AF178956. The structure of the gene was determined by comparing the cDNA and genomic sequences. The MCT3 gene spans ~3.75 kb of DNA, comprising six exons and five introns. As shown in Fig. 2B, there are two 5’-noncoding exons (1a and 1b, open boxes) and four coding exons (filled boxes). The structure of the mouse MCT3 gene is virtually identical to the human (24) and chicken (26) MCT3 genes. The intron/exon boundaries in the coding region of the MCT3 gene occur at the same position in mouse, human, and chicken genes, and all conform to the GT/AG rule (Table 1) (11). All coding sequences were spliced within glycine residues, and the amino acid sequences at splice sites are compared among mouse, chicken, and human MCT3. Numbers in the parentheses are number of the amino acids in each exon.

Table 2. Amino acid sequence at splice boundaries

<table>
<thead>
<tr>
<th>Name</th>
<th>Amino Acid Sequence at Splice Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse MCT3</td>
<td>mgagg-(71)-mylytGplssi-(47)-agvltGlgla-(255)-gppsaGrlda-(116)-rgqka</td>
</tr>
<tr>
<td>Chicken MCT3</td>
<td>mgrad-(76)-mylytGpvesi-(47)-agvltGlgmal-(287)-gppsaGrlda-(129)-erdsf</td>
</tr>
<tr>
<td>Human MCT3</td>
<td>mgagg-(71)-mylytGpvesi-(47)-agvltGlgmal-(279)-gppsaGrlda-(1104)-aasv</td>
</tr>
</tbody>
</table>

Amino acid sequences at the splice sites are compared among mouse, chicken, and human MCT3. Numbers in the parentheses are number of the amino acids in each exon.

Tissue distribution of MCT3. Expression of mouse MCT3 mRNA in various tissues was evaluated by Northern blot analysis (Fig. 3A). An antisense RNA probe to the 3’ end of MCT3 hybridized with a single 2-kb transcript in total RNA preparations from RPE. MCT3 transcript was not detected in total RNA prepared from brain, heart, intestine, kidney, liver, muscle, or neural retina. The preferential expression of MCT3 in RPE is also seen in chicken (13).

CPE shares structural and functional features with RPE. To evaluate whether MCT3 was expressed, RNA was prepared from choroid plexus microdissected from mouse brains. It was possible to detect a 2-kb MCT3 transcript in RNA prepared from choroid plexus, whereas it was below detection when whole brain RNA was used. MCT3 mRNA was not detected in cerebellum or cortex (Fig. 3B), whereas MCT1 transcript was detected in cortex, cerebellum, and choroid plexus.

Immunolocalization of MCT3 to the basolateral membrane of RPE. Indirect immunofluorescence was used to examine cellular and subcellular distribution of MCT3 in the eye. Sagittal sections of paraffin-embedded adult mouse eyes were immunostained with anti-MCT3 antibody. As shown in Fig. 4B, MCT3 immunoreactivity was present in the RPE but not in other ocular epithelia. There was no staining in ontologically related tissues such as the ciliary epithelium and neural retina. The bright-field examination of the tissue section is shown in Fig. 4A. Higher magnification of the immunostained tissue (Fig. 4D) and bright-field examination (Fig. 4C) demonstrate that MCT3 labeling was restricted to the basolateral membrane of the RPE.

Immunolocalization of MCT3 to the basolateral membrane of the CPE. The expression of MCT3 in the mouse brain was examined by immunostaining of paraffin sections of mouse brain. As shown in Fig. 5, labeling was found in the basolateral membrane of CPE. MCT3 is not detected in the vascular endothelium or in the ependymal cells, which line the ventricle and are contiguous with the CPE. Similar labeling with the MCT3 antibody was observed in the CPE from...
lateral and fourth ventricles. Immunohistochemical labeling of tissue sections of the eye and the brain suggested that MCT3 was less abundant in the CPE than the RPE. Using integrated digital time, we consistently found that longer exposure times were required to detect a signal in CPE.

The levels of MCT3 protein in the RPE and the CPE were also examined using Western blot analysis. Detergent-soluble lysates prepared from RPE/choroid and choroid plexus were separated on SDS-polyacrylamide gels and electrophoretically transferred to nylon membranes. Duplicated membranes were incubated with anti-MCT3 or anti-actin antibodies. MCT3 protein was more abundant in the RPE than in the CPE, while levels of actin were similar in both samples (Fig. 6A). Differences in the level of protein expression were not reflected in the level of MCT3 mRNA levels. Northern blot analysis of total RNA from RPE and choroid plexus show that the transcript is abundant in both tissues (Fig. 6B).

**Immunolocalization of MCT1 in the RPE and the CPE.** The distribution of MCT1 in the RPE and the CPE was examined using light microscopic immunohistochemistry. Previously, we showed that MCT1 immunoreactivity was abundant in the apical processes of the RPE in rat (14). In the mouse, MCT1 was abundant in the apical processes of the RPE (Fig. 7B, arrow). Immunoreactivity was also detected in the retina in photoreceptor cell inner segments, outer nuclear layer,
inner plexiform layer, and in retinal vessels. In the brain, MCT1 staining was in the ependymal cells lining the ventricle but not in the CPE.

DISCUSSION

Genomic cloning studies reported here demonstrate that mouse MCT3 gene is a single copy gene with a structure nearly identical to the human and chicken MCT3 genes (24, 26). Protein coding region of mouse MCT3 is distributed over four exons separated by three introns. The initial two exon/intron junctions of the MCT3 gene were located in sequences encoding transmembrane domains, with the last exon/intron splice site in a sequence encoding a hydrophilic domain. The locations of splice sites make alternative splicing in coding regions corresponding to membrane domains unlikely. In the chicken, two 5' -noncoding exons are alternatively spliced to exon 2, accounting for the two MCT3 transcripts expressed differentially during development (26). In mouse, exons 1a and 1b are spliced in tandem to exon 2, leading to only one MCT3 transcript. The 5' ends of MCT3 amplified from RPE and CPE were identical.

The genomic structure has been reported for only one other member of the MCT family, MCT8, which was originally called XPCT (4). Mouse MCT8 gene spans 125 kb, separated by five introns. The first intron is 110 kb and is responsible for the large size of the gene. The coding sequence of MCT8 is interrupted by five introns. When MCT3 and MCT8 genes are compared, the fifth exon/intron, in the eleventh transmembrane domain, is conserved (9).

The tissue distribution of MCT3 was examined using Northern blot analysis and immunohistochemical localization. Both MCT3 transcript and protein were expressed preferentially in RPE and CPE, but not in other blood-tissue barriers. Comparable levels of MCT3 transcript were detected in RPE and CPE, but there was disparity in the amount of protein expressed in the two tissues. This suggests that expression of MCT3 in the CPE may be regulated at the level of translation.

The outer retina is metabolically active and converts a large fraction of glucose into lactate, even in the presence of oxygen (1, 23). Lactate concentrations in the subretinal space are high relative to lactate levels in the blood (~1 mM; 13 mM near the outer limiting membrane to 3.8 mM near the surface of the RPE) (1). Physiological studies have shown that lactate is transported from the subretinal space to the choroidal venules by the RPE (2). In the present study, we have shown that the RPE expresses two MCTs (MCT1 and MCT3) that were polarized to distinct membrane domains; MCT1 in the apical membrane, and MCT3 in the basolateral membrane. The coordinated activity of these two transporters could regulate the transepithelial movement of lactate out of the retina. Whereas the RPE cells express two MCT isoforms, the GLUT-1 glucose transporter is expressed on both the apical and basolateral membranes of the RPE (8, 21).

The choroid plexus forms a barrier between the CSF and fenestrated capillaries, similar to the organization of the choroid and RPE. In contrast to the high concentration of lactate in subretinal space, under normal conditions, lactate concentrations in CSF are about 1.4 mM, similar to levels found in plasma (20). MCT1 and MCT3 transcripts were both detected in RNA prepared from choroid plexus. However, MCT1 was not detected in the CPE by immunofluorescence, and MCT3 was expressed, but at lower levels than in the RPE.

In summary, the mouse MCT3 gene has been sequenced and is structurally identical to the chicken and the human MCT3 genes. Expression of the MCT3 transcript and protein is limited to two structurally and functionally similar tissues, the RPE and the CPE. MCT3 was not detected in other blood-tissue barriers such as testis, placenta, and ciliary epithelium, suggesting MCT3 has a specialized role in regulating lactate levels in fluid-bathing neuronal tissues.

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REFERENCES