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Review

Monocarboxylate transporters: Past, present, and future

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Summary. We review here the 14 members of the Monocarboxylate transporter family (MCTs), their relationship based on sequence homology. The range of substrates transported by different members of this family extends from the standard monocarboxylate metabolites, lactic and pyruvic acids, to aromatic amino acids and thyroid hormones. The family is denoted Solute Carrier Family 16, or SLC16, among 43 SLC families constituting more than 300 members, which are annotated regularly at the website http://www.bioparadigms.org/slc/intro.htm. MCTs classically transport metabolites across plasma membranes with direction controlled by proton and metabolite concentrations independently of energy input, but they may also function in subcellular membranes. Their regulation may be complex, and they are implicated in leukocyte-mediated immunity, hypoxia induced cellular responses, and partitioning of the energy supply in several tissues. We focus here on histologic evidence (involving human tissue where available) and the first four 'classical' members; but we do annotate all 14, and note several candidate or proven genetic diseases that have arisen from MCT mutations. The review progresses through the following sections: (1) MCT1-4: genetics, kinetics, and modulation; (2) Chaperonins and targeting cofactors; (3) Tissue distribution of MCTs; (4) Intercellular lactate/pyruvate shuttles; (5) Transcriptional and translational regulation of MCTs; (6) Properties of other MCTs; and (7) Subcellular localization of MCTs and some future considerations. Along the way we posit questions or suggestions for future research.

Key words: Lactate transporters, Monocarboxylate transporters, Immunohistochemistry, Immunocytochemistry, Intercellular lactate shuttle, Subcellular organelles

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Introduction

The monocarboxylate transporter family includes 14 proteins related to each other by sequence homology. The first four members, MCT1-MCT4, have been experimentally demonstrated to transport aliphatic monocarboxylates, a diverse group of compounds which includes many important metabolites such as lactate, pyruvate and ketone bodies. This transport does not require ATP, and is controlled instead by hydrogen ion and substrate gradients, thus facilitating transport of MCTs either in (influx) or out (efflux) of the cell. Lactate efflux is especially important for cells and tissues which generate large quantities of lactic acid as a result of glycolysis (e.g., red and white blood cells, skeletal muscle, and most tumor cells). Lactate influx is of value to tissues where it is being utilized (via pyruvate) as a respiratory fuel (brain, heart, red skeletal muscle), as a gluconeogenic substrate in liver, or under fasting conditions, as a glycerol-neogenic substrate in white adipose tissue (Beale et al., 2002). Several other members of the family have different substrate specificities: MCT6 has been shown to transport the drug bumetanide (obviously not its primary function), MCT 8 is solely a thyroid hormone transporter, and MCT10, which was first identified as a low-affinity transporter of the aromatic amino acids, also efficiently transports iodothyronines. The detailed structural and functional description of other members of the MCT

family is still incomplete.

MCT1 through MCT4: genetics, kinetics, and modulation

MCT1 is the most well-studied and functionally characterized member of the MCT family, largely due to the fact that it is the only monocarboxylate transporter expressed in human erythrocytes, and it also has the widest tissue distribution. The kinetic parameters of monocarboxylate transport in erythrocytes had been described (Halestrap and Denton, 1974; Deuticke et al., 1978; Dubinsky and Racker, 1978) well before the responsible cDNA was isolated and cloned (Kim Garcia et al., 1994a,b), and they were confirmed after rat MCT1 cDNA was expressed in Xenopus oocytes (Bröer et al., 1998). MCT1 transports a wide variety of monocarboxylates. Three and four carbon atom substrates with C-2 substitutions are preferred, a group which includes such important metabolites as lactate, pyruvate, and the ketone bodies acetoacetate, and ß-hydroxybutyrate. MCT1 lactate transport is stereoselective, with Km for L-lactate being 3-5mM, and for D-lactate 10 times higher (Poole and Halestrap, 1993). A large number of inhibitors of MCT1-mediated transport are known, including generic enzyme inhibitors, such as p-chloromercuribenzoate (PCMB), N-iodo-succinimide, and fluorodinitrobenzene (FDNB), as well as more specific reagents typified by the ß-cyanocinnamates (CC) first described by Halestrap and Denton, (1974), and the recently developed tight-binding pyrollopyrimidine analogs (Murray et al., 2005).

Transporters MCT2 through MCT4 exhibit substrate specificities similar to MCT1, but with distinguishing features. MCT2 has the greatest affinity for both pyruvate (K_m=25 µm) and L-lactate (K_m=1 mM). While sensitive to CC inhibition, MCT2 is not affected by PCMB(S). In contrast, the respective Km values for MCT3 are very similar to those of MCT1, ~1mM for pyruvate and ~5mM for lactate. However, MCT3 is not inhibited by either CC or PCMB(S), and it is localized exclusively on the basolateral membranes of retinal pigment epithelium (RPE) and choroid plexus epithelium (CPE) (Philp et al., 2001). MCT4 exhibits lesser substrate affinities, with Km~30 mM for lactate and for pyruvate ~25 mM (Dimmer et al., 2000) or the much less reasonable ~150 mM (Manning Fox et al., 2000), and it is not as sensitive to the more specific inhibitors.

The differences in kinetic characteristics of these transporters allow for a fine tuning as well as a differential regulation of the transport depending on the requirements of a particular tissue. With regard to L-lactic acid, the ~5-fold K_m increases from MCT2 to MCT1 (or 3) to MCT4 are nearly ideally spaced to cover the full range of concentrations that may occur in human tissues, including the sudden major changes in activity that take place in tissues such as skeletal muscle, brain, retina, leukocytes, and platelets. If MCT 1, 2, and 4 all had the same turnover number and were present in the same membrane at the same concentration, they would provide a relatively smooth increase in transport from the resting lactate level of ~1 mM to the maximum intracellular physiologic level of ~40 mM.

MCT1 was initially cloned from Chinese hamster ovary (CHO) cells (Kim Garcia et al., 1994a); and cDNA sequences from different species, including human, mouse, and rat, were later identified (Halestrap and Price, 1999), all translating into a protein of ~54 kDa. Hydrophobicity analysis of the MCT1 amino acid sequence predicted the presence of 12 transmembrane segments, with both N- and C-termini, plus a large loop connecting transmembrane segments 6 and 7, located in the cytoplasm. This prediction was confirmed experimentally for rat MCT1 by Poole et al. (1996). The presence of 12 transmembrane segments is a common feature of a number of transporters, including the glucose transporters and the ATP-binding-cassette (ABC) protein family (Henderson, 1993).

In the various transporter families, the transmembrane segments are highly conserved and are thought to be directly involved in transport. Indeed, sequence alignment of human MCT1-4 shows that 72 % of the identical amino acids reside in transmembrane segments, which constitute only 50% of the total amino acid content of the protein. Point mutations within these transmembrane domains have been shown to greatly affect both the specificity and transport activity. The spontaneously occurring mutation of phenylalanine 360 to cysteine in domain 10 of MCT1 converted major substrate activity to mevalonic acid (Kim Garcia et al., 1994a), while mutation of arginine 306 to threonine in domain 8 resulted in strongly reduced overall transport activity (Rahman et al., 1999). Mutations in transmembrane segments of MCT8 with striking clinical effects will be described later in this review. We have reported five patients with subnormal red blood cell lactate transport and exertional muscle cramping, who carried mutations in MCT1 cDNA (Merezhinskaya et al., 2000). The 3 patients with the lowest transport rates (< half the normal mean), one of whom was also demonstrated to have a delayed decline of blood lactate after exercise (Fishbein, 1986), were heterozygous for one of two mutations: Lys 204Glu or Gly472Glu. Both mutations were absent in a cohort of 83-90 healthy donors, yielding p values <5.4%. Based on the rat MCT1 transporter topology, the mutations occurred in hydrophilic segments of MCT1: Lys204 in a large cytosolic loop between transmembrane segments 6 and 7 was a conserved amino acid in hamster, rat, mouse, and human, whereas Gly472 in the C-terminal segment was not. The Lys204Glu mutation is the most likely to be significant clinically, and in a recent review (Halestrap and Meredith, 2004) the authors stated, as unpublished data, that they had expressed the mutation in *X. laevis* oocytes but found no distinction from the normal state. However the subject was revisited more recently, and expression of the mutant form in *X. laevis* oocytes was

found to produce a marked decrease in maximum transport rate; and the mutation was found to be quite rare in some Western European populations (K. Kivisto and E. Schaeffeler, personal communications).

Hydrophilic segments of the transporter can also affect its function and stability, as has been demonstrated for rat MCT1 (Galic et al., 2003). Their mutational analysis of conserved amino acids located in the loop between helix 4 and helix 5 showed that the mutation of lysine 142 to glutamine resulted in an increase of K_m for lactate from 5mM to 12mM and a decreased stereoselectivity of the transporter, indicating an involvement of this residue in substrate recognition. In the same loop the mutation of arginine 143 to glutamine had a more pronounced effect: it almost entirely eliminated MCT1 transport activity. An additional role of hydrophilic regions of the transporter stems from their interaction with chaperone proteins, and it will be addressed in the next section.

An indication of the complexity of MCT1 regulation is provided by the recent identification of new mutations involving its non-coding regions, leading to a dominantly inherited hypoglycemic disorder known as exercise-induced hyperinsulinism or EIHI (Otonkoski et al., 2008). Although no mutations were found in the coding region of the gene, one group of patients had a mutation in the 5' untranslated promoter region and the other group had a mutation in the non-coding exon 1, each resulting in increased promoter binding and abnormally high transcript levels. In pancreatic cells, where MCT1 is not normally transcribed, the increased transcription was presumed to lead to pyruvate uptake and metabolism upon exercise, followed by inappropriate insulin secretion via closure of K_{ATP} channels (Koster et al., 2000) despite falling blood glucose. While the patients' fibroblasts also showed elevated levels of MCT1 transcripts, the transporter activities remained normal, indicating further regulatory controls. Thus these mutations may be uniquely specific for the pancreatic ß cell phenotype.

The presence of asparagines in extracellular loops of a transporter would denote possible sites for glycosylation, a phenomenon very common for membrane spanning proteins. However, the predicted size of the loop regions for MCT1 and MCT3 would place the asparagine acceptors too close to the transmembrane domain to accommodate N-linked carbohydrate. Indeed, no glycosylation was found in mouse and hamster MCT1 (Carpenter et al., 1996) and chicken MCT3 (Yoon et al., 1997).

Chaperonins and targeting cofactors

The absence of glycosylation is a rare phenomenon among membrane-spanning proteins, and many such proteins are instead tightly associated with other glycosylated membrane proteins.

Using cross-linking and co-precipitation experiments, MCT1, MCT3 and MCT4 have been shown to interact with a cell surface glycoprotein, CD147 (Kirk et al., 2000; Wilson et al., 2002; Philp et al., 2003a), and MCT2 – with a closely related protein, gp70 (Wilson et al., 2005). CD147 (basigin) has a single transmembrane domain, an immunoglobulin-like extracellular segment, and a short cytoplasmic tail. Results of fluorescence resonance energy transfer (FRET) experiments (Wilson et al., 2002) suggested that two CD147 molecules form a dimer which, in turn, interacts with two molecules of MCT1 within a membrane plane. One of the functions of CD147 appears to be a correct targeting of MCTs to the plasma membrane. MCT1 and MCT4 were correctly localized to the plasma membrane only when co-expressed with CD 147. In cells transfected with only MCT1 or MCT4, but not CD147, the MCTs accumulated in a peri-nuclear compartment, which was not further defined (Kirk et al., 2000). Nakai et al. (2006) compared the tissue distribution of MCT1 and basigin in wild-type and basigin-gene-knockout mice. The initial immuno-histochemical screening revealed that these two proteins are co-localized in most, but not all, wild-type mouse tissues. The elimination of the basigin gene greatly reduced the intensity and distribution of MCT1 in the tissues where both proteins were expressed, with the exception of Leydig cells of the testis and ependymal cells of the brain. The MCT1 localization in tissues where basigin was absent in the wild type, was not affected in the knockout mouse. Curiously, no changes in the tissue levels of MCT1 mRNA or protein were induced by the elimination of basigin gene. This argues that only the localization and not the quantity of the MCT is changed by the absence of basigin, and surprisingly, that MCT1 is not degraded despite its misplacement. In contrast, Philp et al. (2003a) observed a dramatic decrease in MCT1 and MCT3 expression in neural retina upon loss of basigin expression in knockout mice. The findings were reproduced in a human retinal pigment epithelial cell line (ARPE19), wherein MCT4 has replaced MCT3.

Recently, Wilson et al. (2005) demonstrated that CD147 is directly involved in regulating MCT activity. The authors showed that the well-established inhibitor of MCT1 and MCT4 transport activity, p-chloro-mercuribenzene sulfonate (pCMBS), does not affect the transporter directly but rather blocks transport by replacing the disulfide bridge in the Ig-like extracellular C2 domain of CD147. This suggests that a mutation, or even a change in glycosylation, of basigin might exhibit itself by alteration in the activity of MCT 1 or 4. PCMB does not block MCT2- mediated transport because MCT2 complexes with a closely related but different protein, embigin (gp70), which has an unreactive V-domain in place of a C2 domain. The existence of a subset of tissues which correctly express MCT1 in the absence of basigin suggests that other unidentified cofactors may also complex various MCTs to target their proper membrane localization and activity, not only in the plasmalemma, but also in the membranes of

subcellular organelles.

Tissue distribution of MCT

The pattern of MCTs expression among tissues varies within a single animal specie, as studied at both mRNA and protein levels. As noted above, of the 4 physiologically proven lactate/pyruvate transporters, only MCT3 has a uniquely restricted distribution. We studied the human tissue distribution of the other three MCTs at the protein level by Western blot analysis using specific antibodies and validating specificity by both pretreatment of antibody with excess peptide antigen and by showing no cross-reaction among the peptide antigens (Fishbein et al., 2002). MCT 1 was present in 14 tissues examined, with maximum quantified levels in heart, and was the only transporter expressed in red blood cells (MCT2 and MCT4 were undetectable). MCT2 was expressed in 13 tissues, maximally in colon, while MCT4 was expressed at reasonable levels in only 7 tissues, maximally in heart and kidney. For each of the three MCTs, Figure 1 shows the amount of MCT antigen in each tissue relative to that in the maximally expressing tissue taken as 100, and thus provides no information as to the relative amount of the three MCTs in any given tissue. What is really needed, and not yet in sight, is a convincing quantitation of the concentration of all three transporters and their respective turnover numbers in each tissue of interest, in order to gain greater insight into their interacting metabolic roles.

Halestrap's group (Bonen et al., 2000) and collaborators (Bonen et al., 2006) did not find MCT4 in rat heart muscle, and considered it unlikely to be found in highly oxidative tissues like the heart, despite the fact that they had themselves identified MCT4 (then called MCT3) in human heart (Wilson et al., 1998). They therefore ignored our results, and challenged the McClelland and Brooks (2002) report which identified MCT4 in rat heart. In particular, the presumption of a false positive MCT4 band in that report was emphasized by the Bonen et al. (2006) report, which was a survey of many MCTs documented only by visible bands on Western blots using an array of commercial antibodies. There was no densitometric quantitation, no correlation with mRNA, and no peptide antigen blocking to validate specificity.

The hypothesis that MCT4 is restricted to glycolyzing cells and thus must be absent from heart is physiologically unsound, because lactic acid is a preferred cardiac substrate (Laughlin et al., 1993). In addition, the 'ketone bodies', beta-hydroxybutyric acid and acetoacetic acid, are also transported by MCTs, and the heart is an efficient user of these compounds generated by the liver. In any event, at the present time the challenge to the presence of MCT4 in heart has become moot, since the Halestrap group (probably stimulated by the McClelland and Brooks report in 2002 demonstrating a large increase in rat heart MCT4, but not MCT1, upon prolonged hypoxia) have themselves demonstrated that MCT4 is markedly and rapidly induced by hypoxia through the HIF-1α pathway (Ullah et al., 2006). In humans, cardiac hypoxia is a very common occurrence in the hours to days preceding death; and the hypoxia in rats was intentionally produced by McClelland and Brooks. We, in turn, doubt that MCT4 is totally absent from human hearts save under

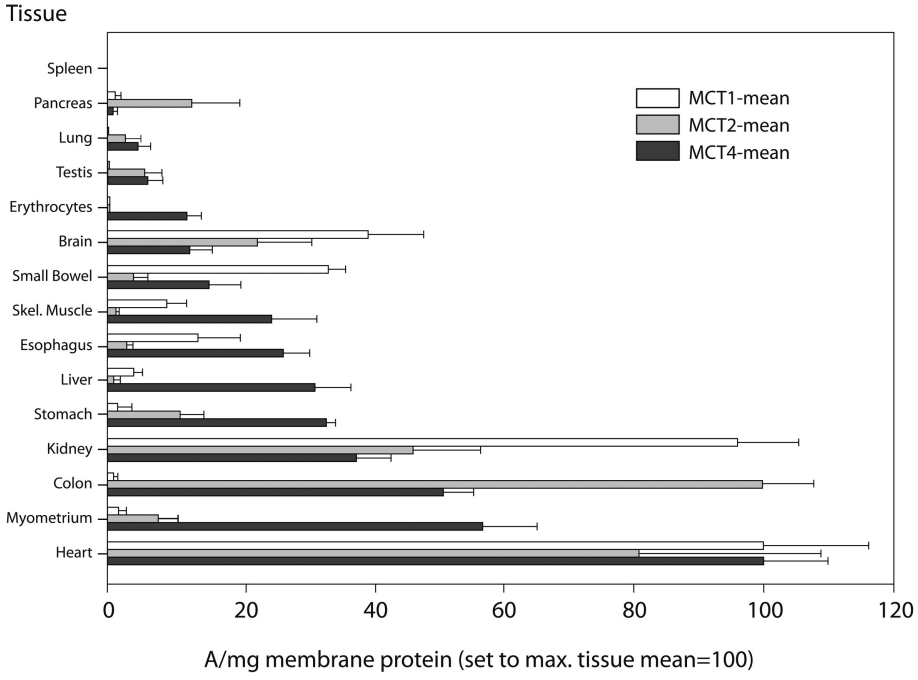


Fig. 1. Relative distribution of MCT1, 2, and 4 in 15 frozen human tissues. For each MCT, the absorbance (A) per mg of protein was averaged for several experiments for each tissue. The mean and standard error were normalized to that of the highest mean tissue level, which was set to 100. (from Fishbein et al. 2002).

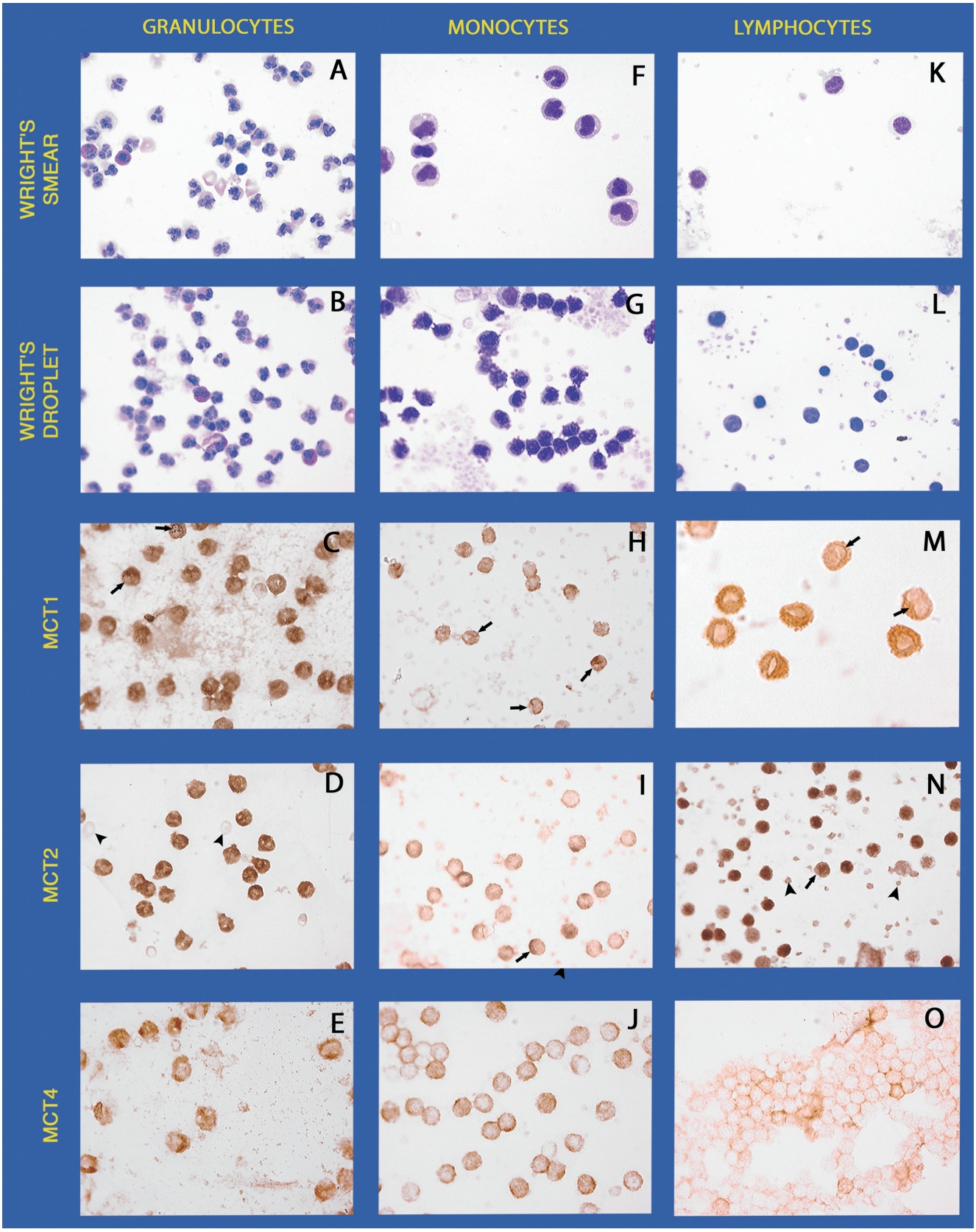


Fig. 2. Wright's and immunohistochemical staining of MCT1, 2, and 4 in separated human granulocytes, monocytes, and lymphocytes. Magnification in panel M is 1.7x that of the other panels. Arrows in C, H, I, M, and N show possible nuclear staining by MCT1 and MCT2 antibodies. Arrowheads in N show platelet plasmalemmal staining by MCT2 antibody, and those in D show unstained erythrocytes as a negative control (from Merezhinskaya et al. 2004) .

hypoxic conditions.

We also found MCT 1, 2, and 4 mRNAs and proteins, again validated for specificity, to be expressed in separated human granulocytes, lymphocytes, and monocytes, as shown in Figure 2, while platelets expressed MCT2 and 4, as shown in Figures 2 and 3; but we did not detect MCT1 (Merezhinskaya et al., 2004, 2006). The challenge of hypoxia, in injured or infected tissues, to the granulocyte and monocyte/macrophage is well known (Schor et al., 2000; Saadi et al., 2002).

These cells of the myeloid series are the first responders of the innate immunity system and must survive in compromising environments. It is now clear that the same HIF-1α transcription factor is essential for their maintenance of glycolytic capacity; in its absence there is a dramatic loss of cell ATP, aggregation, infiltration, and bacterial killing (Cramer et al., 2003). It seems clear to us that the induced high levels of MCT4 in these cells and the consequent export of the high levels of lactic acid generated during aerobic glycolysis must play a significant role in this stage of innate immunity.

An analogous role for MCT1 in the life of the T-lymphocyte has recently been discerned for the case of antibody-mediated immunity. Murray et al., (2005) have shown that lymphocyte activation, provoked by an antigen challenge, was accompanied by increased MCT1 expression induced by the increased rate of aerobic glycolysis. Specific inhibition of MCT1 by nanomolar levels of a new group of potent inhibitors (pyrollopyrimidine analogs) resulted in a significant increase in intracellular lactic acid concentration, decrease in DNA synthesis, and inhibition of the rapid phase of T-cell division necessary for an effective immune response. The arrest of the immune response following the inhibitor treatment makes MCT1 a potential target for a novel immunosuppressive therapy. Indeed, MCT1 inhibition by one of these novel compounds prevented allograft rejection in mouse and rat (Bueno et al., 2007; Ekberg et al., 2007). The pyrollopyrimidine analogs were found to bind to MCT2 as well, albeit with weaker K_i, but not to MCT3 or MCT4. It is not yet known, whether expression of MCT2 or MCT4 (both of which are present in lymphocyte membranes) is similarly involved in the T-lymphocyte activation pathway; but upon cell entry into inflamed tissue we would expect HIF-1α to be activated and thence MCT4 to be upregulated and assist MCT1 in the dissipation of cellular lactic acid.

The tissue distribution of MCT also varies with the specie studied. For example, in the rat MCT1 was the predominant form expressed in liver, while MCT2 took that role in brain. In contrast, the hamster brain did not express MCT2, which was, instead, the major form expressed in liver. In tissues where two or more MCTs are co-expressed, they often have different cellular localizations to satisfy specific conditions. Testicular and epididymal tissues and sperm cells provide a good example. Lactate movement across meiotic and post-meiotic spermatogenic cells is handled by MCT1, MCT2, and MCT4 which are differentially expressed on the membranes of spermatogenic cells during maturation. Both MCT1 and MCT4 were present on both the meiotic pachytene spermatocytes and the post-meiotic round spermatids, but MCT2 was expressed predominantly on the latter (Brauchi et al., 2005). In addition, it was found (Garcia et al., 1995) that during the process of maturation, the MCTs' distribution changes within the sperm cells. For instance, MCT1 was present on sperm heads in the testis and proximal epididymus during the early stages of maturation, but only on the microvillar epithelial surface of the distal

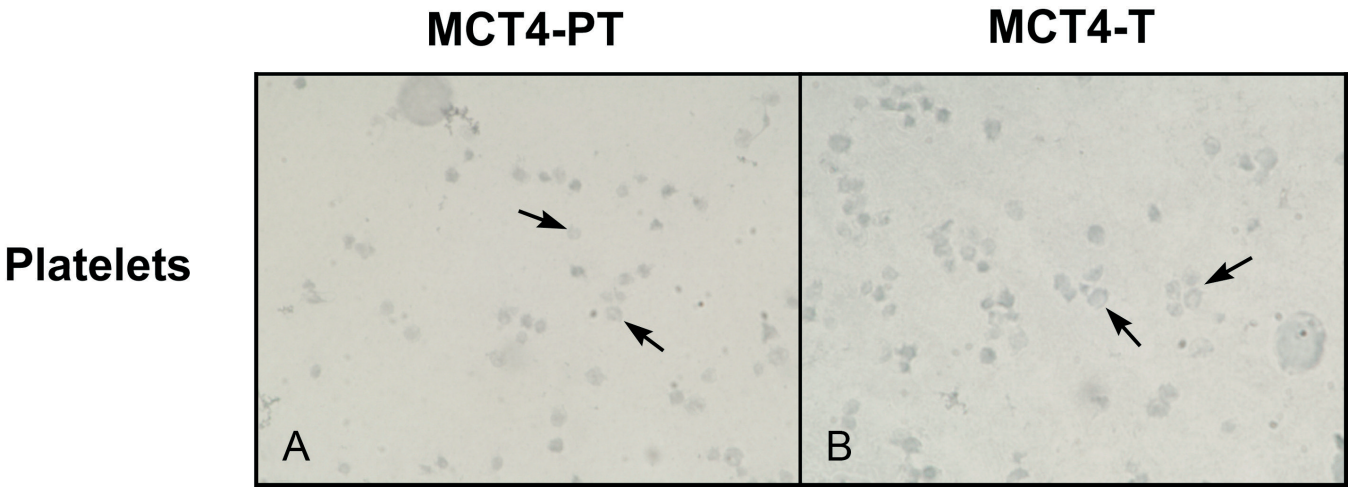


Fig. 3. Immunohistochemical staining of platelets with antibodies to MCT4-terminal peptide (anti-MCT4-T) and to MCT4-preterminal peptide (anti-MCT4-PT) using 3,3'-diaminobenzidine substrate with cobalt enhancement. The arrows show some evident examples of plasmalemmal staining, and a single lymphocyte is present in **A** and **B** to provide a size reference (modified from Merezhinskaya et al., 2006).

epididymus. MCT2, on the other hand, was present exclusively on the tails of sperm through all stages of development and was not expressed in the epithelium. Hopefully, future studies will elucidate the functional rationale for these localizations.

Intercellular lactate/pyruvate shuttles

In at least 4 tissues, research has led to the proposal of intercellular lactate/pyruvate shuttles.

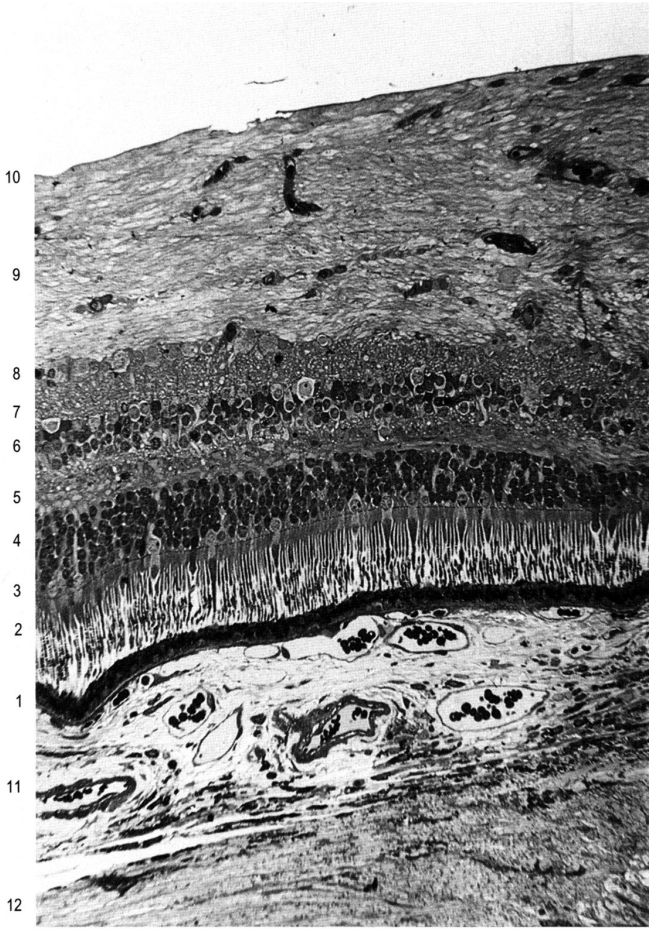
The overall pattern is quite similar in retina, brain, muscle, and testis, where the subdivision of an organ into mainly glycolytic versus mainly oxidative cell types permits the partitioning of the energy supply, with substrate 'feeding' of the latter cell type by the former, for the most efficient energy utilization, after which the residual substrate is finally sent back to liver or muscle for reconversion to glucose or glycogen. We will annotate these four cases, which provide substantial evidence for a novel mechanism contributing to the improved energy efficiency of the tissues.

During visual perception, the retina produces large amounts of lactic acid. The transport of lactate between different compartments then plays an important role in maintaining visual cell function. The retinal pigment epithelium (RPE) mediates the transport of metabolites between the choroidal blood supply and retina. The basolateral surface of RPE is in contact with the capillaries in the choroid, and the apical surface is in contact with neural retina where its microvilli contact (cones) or envelop (rods) the photoreceptor cell outer (i.e., deeper) segments. Figures 4 and 5 show these layers microscopically and schematically, respectively, to clarify the anatomical architecture.

Glucose, transported from choroidal vessels via the RPE to the retinal interstitium, is used there by Müller glial cells for glycolysis. The resulting lactic acid is then transported into the photoreceptor cells where it is re-oxidized to pyruvic acid which is utilized for oxidative phosphorylation. Excess lactic acid is transported back to the choroidal venules via the RPE. Recall that the RPE expresses MCT1 on the apical surface facing neural retina, whereas MCT3 is expressed on the basolateral surface facing the choroid, as shown in Figure 6 (Philp et al., 2003b). Since the lactate concentration in the retinal interstitium (7-13mM) is much higher than in blood plasma (1mM), the coordinated action of MCT1 and MCT3 within RPE appears to provide an energy-efficient source of H+/lactate from glycolyzing cells to the oxidative energy demanding photoreceptor cells via conversion to H+/pyruvate, plus a return shuttle of the residual H+/ lactate from the retinal interstitium back to the blood.

In the retina, MCT1 is highly expressed in Müller cells, photoreceptor inner segments and microvessels forming the inner blood-retinal barrier. MCT2 is detected in Müller cell end-feet and in glial processes surrounding retinal microvessels, and MCT4 is expressed in inner retina. One mystery is why MCT3 is critical, since we noted that the available data indicates Michaelis constants quite similar to those of MCT1. Is it needed to guarantee a localization opposite to MCT1, or has something else been missed? In fact, in the human cell line derived from RPE, ARPE19, MCT4 has replaced MCT3, but with the same localization on the basolateral surface, as shown in Figure 7. Of course the cell line has lost its tissue contiguity, and it is common for such derived and immortalized lines to exhibit differences from the physiological state, yet something has targeted MCT4 to the same site as normally occupied by MCT3.

A similar situation exists in brain where MCT1, MCT2, and MCT4 have been identified. Despite the fact that glucose represents the major source of energy for an adult brain, monocarboxylates, and especially lactate, might provide a divergent source of oxidative energy.



1. Pigment epithelial layer.
2. Rod and cone layer.
3. External limiting membrane.
4. Outer nuclear layer.
5. Outer plexiform layer.
6. Inner nuclear layer.
7. Inner plexiform layer.
8. Ganglion cell layer.
9. Nerve fibre layer.
10. Internal limiting membrane.
11. Choroid.
12. Sclera.

Fig. 4. Microscopic view of retinal and adjacent tissue layers. Orientation here is with the eye looking straight up; vitreous humor at the top and choroid and sclera at the bottom (from Ruskell, 2005, with permission).

This idea is embodied in the astrocyte-neuron lactate shuttle hypothesis. Thus astrocytes (and perhaps other glial cells), which have a high glycolytic activity, release lactate into the extracellular space where neighboring oxidative neurons take it up for oxidation to pyruvate and then through the citric acid cycle to provide their ATP. Blood capillaries in brain are surrounded by astrocytes, with their foot processes in close contact with endothelial plasma membranes. These glial cells express both MCT1 and often MCT4, whereas endothelial cells within blood vessels express MCT1 only, and MCT2 is the major transporter expressed in neurons. Since glial cells outnumber neurons tenfold, the supply of lactate should be plentiful, and it now appears that the astrocytes' heightened activity also dilates the cerebral blood vessels (Takano et al., 2006). The distribution and kinetic characteristics of MCTs in brain are in good agreement with the shuttle hypothesis (Pellerin et al., 1998), and recently, more direct evidence has been provided by two-photon fluorescence imaging via confocal microscopy. In hippocampal slices, this technique could segregate in time and space, both cytoplasmic from mitochondrial changes in NADH, and astrocytes from neurons as well (Kasischke et al., 2004). Upon neuronal stimulation, the data indicate a burst of oxidative phosphorylation depleting the neuronal mitochondrial NADH, with its recovery fueled at least partially by extracellular lactate stimulating increased TCA cycle activity. Following close behind is a burst of glyco(geno)lysis in the nearby astrocytes leading to increased lactate to replenish the extracellular pool.

Now combining this evidence with the transporters of interest, we note that MCT1 located in blood vessels regulates both the influx and efflux of lactate across the blood-brain barrier. In turn MCT1 (and often MCT4) on astrocytes participate in lactate export into the extracellular space, while MCT2, with the highest affinity for lactate, then mediates its uptake into the neighboring neurons, as shown in Figure 8 (Pierre and Pellerin, 2005). Chiry et al. (2006) clearly demonstrated MCT1 in human astrocytes and their end feet adjoining capillary walls by double immunofluorescence labeling, as shown in Figure 9, while the more restricted expression of MCT4 in astrocytes in certain brain regions of the rat are shown in Figure 10 (Pierre and Pellerin, 2005).

Another very recent development in brain microanatomy and function has arisen from the confluence of two separate lines of investigation. Pierre et al. (2007) found that the brains of mice after a prolonged high fat diet, as well as brains of genetically obese or genetically diabetic mice had increased expression of MCT 1, 2, and 4. Almost synchronously, Parton et al. (2007) reported a set of "glucose-excited" POMC (pro-opiomelanocortin) neurons in the brain that respond with increased ATP synthesis that effects the closure of K_{ATP} channels in the cell membrane. Closure of K_{ATP} channels in pancreatic cells induces insulin release and transgenic mice with non-closing channels develop type 2 diabetes (Koster et al., 2000). In obese mice on a high fat diet the POMC cells became defective due to increased activity of a mitochondrial uncoupling protein which impaired glucose stimulated ATP production. Questions then arise as to whether the POMC neurons might signal the pancreas by release of an endorphin, or whether the pancreatic ß-cells of mice

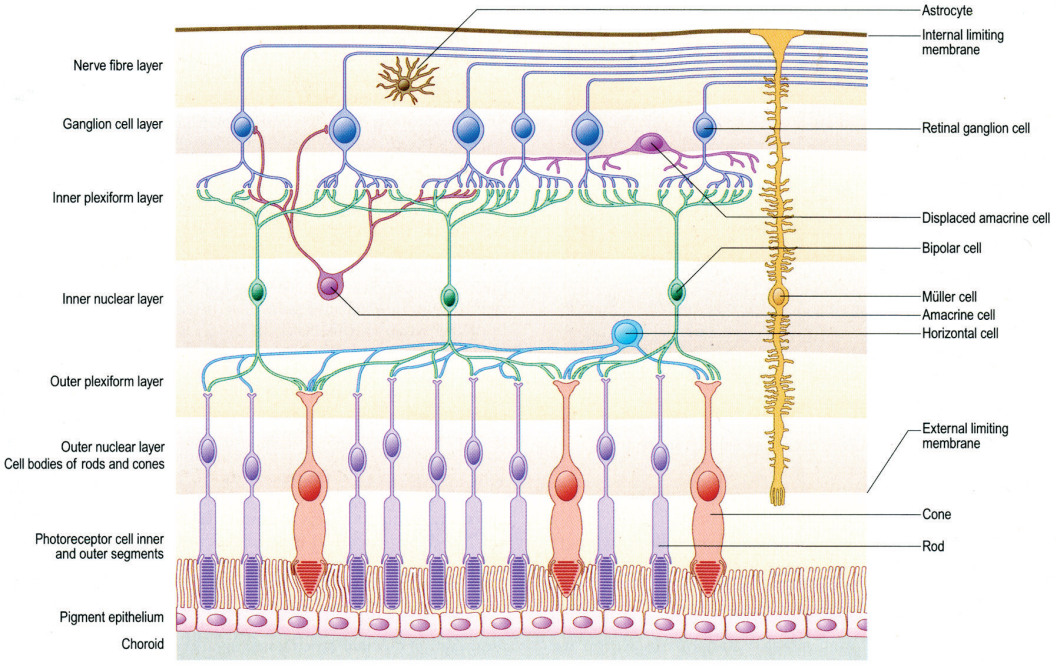


Fig. 5. Schematic representation of the layered arrangement of neuronal cell bodies in the retina and the interconnections of their processes in the intervening pexiform layers. The two principal types of neuroglial cell in the retina are also shown, but the microglia are omitted (from Ruskell, 2005, with permission).

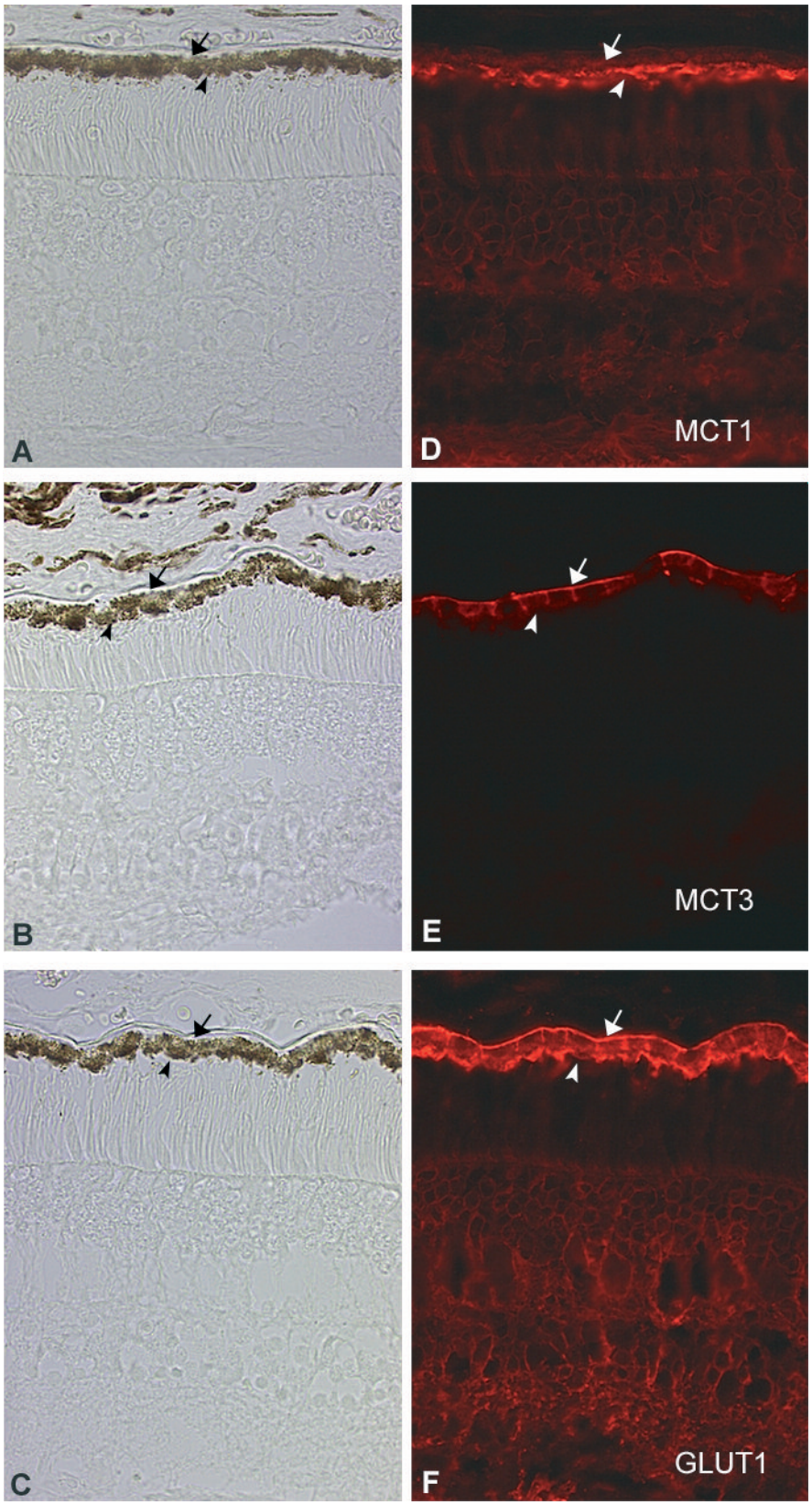


Fig. 6. Light (**A, B, C**) and immunofluorescence (**D, E, F**) micrographs of frozen sections of adult human eye. Orientation here is inverse to that of Fig.4, with choroid at the top. The rod and cone layer can be discerned in **A**, **B**, **C** just below the retinal pigment epithelium (RPE). The arrows touch the basolateral membrane and arrowheads touch the apical membrane of the RPE. MCT1 antibody labeled primarily the apical **(D)**, and MCT3 antibody the basolateral membrane **(E)**, whereas GLUT1 antibody labeled both membrane domains **(F)** (from Philp et al., 2003b, with permission).

on a high fat diet may undergo an analogous futile cycle, in which case upregulation of MCT 1, 2, and 4 would logically follow the ensuing aerobic glycolysis and lactic acid accumulation necessitated by the failure of efficient oxidative phosphorylation.

Skeletal muscle presents a particularly strong case for an intercellular shuttle, because the anatomic, biochemical and physiologic characteristics of the three major fiber types have been so well established; and upon work or exercise it generates the highest amounts of lactic acid in the body. In addition, the large fiber size and random cross-sectional contours permit easy tracking in sequential sections, while its excellent preservation on freezing permits antibody and enzyme studies without the complicating factor of preliminary fixation. In muscle the primary energy source varies with the fiber type. The Fast White (also known as Fast Glycolytic, or type 2b in humans) fibers provide the greatest contractile force, but depend mainly on glycolysis and thus fatigue rapidly from accumulating lactic acid. The Slow Oxidative (Slow Red or type 1 in humans) fibers rely mainly on oxidative phosphorylation for their energy production, and are resistant to fatigue, but have the weakest and slowest force production, while the Fast Oxidative-Glycolytic (Fast Red or Type 2a in humans) fibers have intermediate characteristics.

Most relevantly, the three fiber types are intermingled in the normal muscle, so that any selected fiber is almost never surrounded by fibers of the same type. As a result, the type 2b fiber is in excellent position to export lactic acid that it cannot use and is producing fatigue, to a neighboring 2a or type 1 fiber, which can re-oxidize it. Figure 11 illustrates the distribution of the fiber types determined by enzyme staining in dichroic light and their differential expression of MCTs in sequential frozen sections of an unfixed human muscle by immunofluorescence. MCT 4 is the main isoform expressed in type 2b and some 2a fibers. Indeed, the high Km for lactate (~30mM) makes MCT4 most suitable for handling lactate efflux from fast glycolytic fibers since their intracellular lactate level can reach 30 mM and more upon heavy exercise. MCT1 is predominantly expressed in type 1 (and many type 2a) fibers, which are positioned to take up lactic acid at moderate rates for oxidative phosphorylation. MCT2

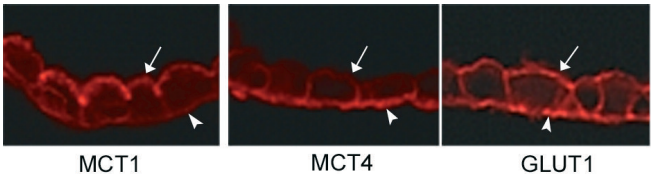


Fig. 7. Immunofluorescence photomicrographs of frozen sections of ARPE-19 cells. MCT1 antibody labeled primarily the apical membrane (here touched by arrows) and MCT4, replacing MCT3 in the cell line, is located by its antibody in the basolateral membrane (here touched by the arrowheads). The glucose transporter, GLUT1, is again found in both membranes (from Philp et al., 2003b, with permission).

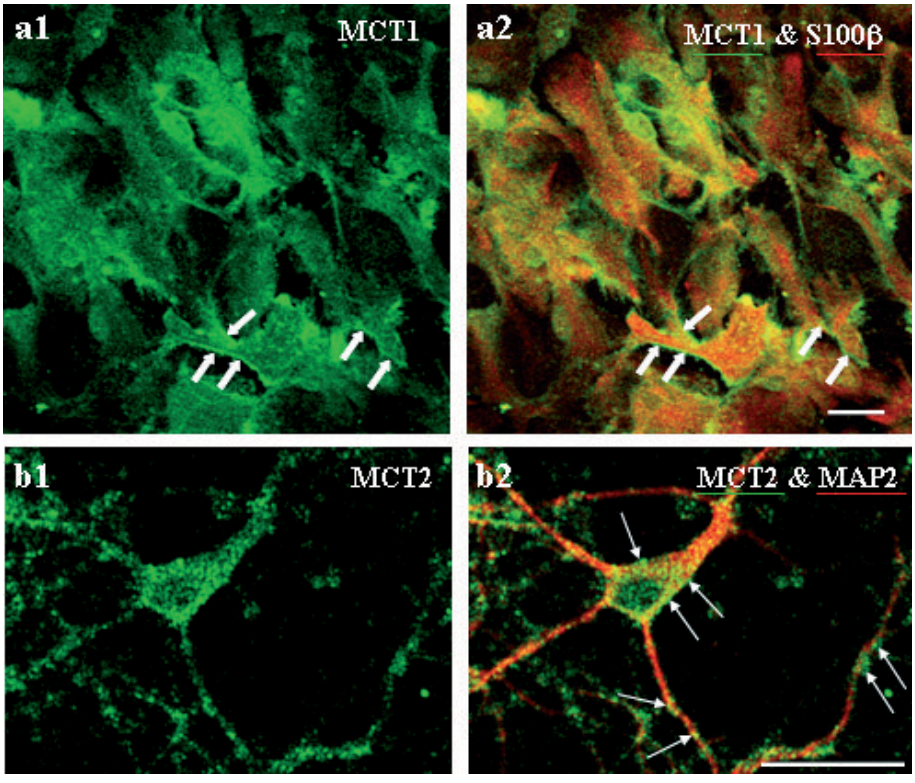


Fig. 8. Double immunofluorescence labeling of cultured astrocytes (a1; a2) and neurons (b1; b2) from mouse cortex, utilizing FITC and Texas Red conjugated second antibodies: MCT1 (green, a1, a2); S100, (red, a2); MCT2 (green, b1, b2), and microtubule-associated protein 2 (MAP2) (red, b2). Arrows indicate MCT1-associated staining of astrocytic cytoplasm and plasma membrane and MCT2-associated staining of the cytoplasm and plasma membrane of neuronal somata and dendrites. Yellow dots and lines indicate co-localization of the MCT with the cell-specific biomarker protein (from Pierre and Pellerin, 2005, with permission).

stained the sarcolemma of but a subset of type 1 fibers, and its frequency varied greatly among different muscles. The metabolic role of these subsets remains unclear at present, but such localizations have also been found in rat plantaris muscle (Hashimoto et al., 2005). Both MCT2 and MCT1 have high affinity for lactate (K_m 1 & 5 mM) which would allow them to efficiently import lactate from the interstitium (which is being fed by the 2b fibers to relieve their acidosis) into neighboring Type 1 and 2a fibers for utilization, rather than simply into the blood capillaries for restorative gluconeogenesis in the liver (the Cori cycle), or into glycogen in resting muscle.

In low-grade neuropathies the characteristic fiber-type dispersion is often lost, because the denervated muscle fibers are re-innervated by neighboring healthy neurons rapidly enough to keep up with the denervation. Thus, no evidence of acute neurogenic atrophy is found in the muscle biopsy to explain the patient's weakness and easy fatigue. Since the re-innervating nerve determines the muscle fiber-type, and is usually different than the original, bouts of this process will typically produce clumps of muscle fibers of the same type, i.e., 'fiber-type grouping', as the only microscopic abnormality. It seems reasonable that the loss of the energetic efficiency of this 'inter-cellular lactic acid shuttle' from 2b to 1 & 2a fibers would be a contributing factor to the patient's symptoms.

Sperm maturation is thought to be controlled, in part, by L-lactate released into the adluminal compartment in the seminiferous tubules by Sertoli cells (Brauchi et al. 2005). Acccording to the proposed 'Sertoli cell-spermatogenic cell interaction model' (Reyes et al., 2002), Sertoli cells secrete fuel substrates (glucose and L-lactate) into the adluminal compartment, and spermatogenic cells can then import and metabolize them, which, in turn, helps to regulate spermatogenesis via changes in intracellular concentrations of adenine nucleotides and Ca^{2+}.

While these examples of lactate/pyruvate shuttles between cell types of the same organ or tissue to improve their energetics do present a new concept, the same cannot be said for interorgan or intracellular shuttles, which have each been well-recognized for many decades.

Finally, we note that full-length MCT1 seems to have been convincingly demonstrated free in human blood plasma (Iizuka et al., 2003), presumably from the degradation of blood cells. The question has been raised as to whether this may constitute a carrier for lactate (or

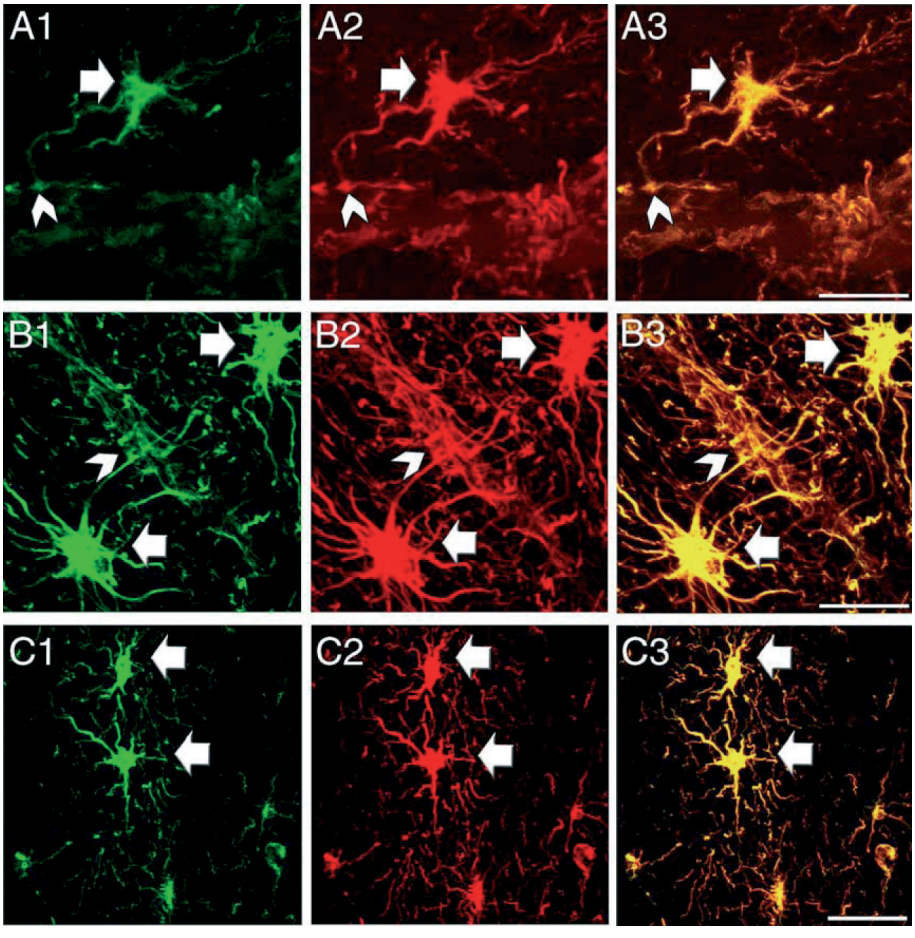


Fig. 9. Double immunofluorescence labeling of human primary auditory cortex via confocal microscopy. MCT1 immunolabeling (green, A1, B1, C1), astrocytic marker Glial Fibrillary Acidic Protein GFAP (red, A2, B2, C2); colocalization of MCT1 and GFAP (yellow, A3, B3, C3). The arrows show astrocytes where both MCT1 and GFAP reside. Arrowheads indicate astrocytic endfeet in contact with blood vessels, labeled with MCT1. A and B contain gray matter astrocytes, C contains white matter astrocytes. Scale bar is 50 µm (from Chiry et al., 2006, with permission)

pyruvate) for distribution to other organs; but the protein level would be far too low for the lactate levels commonly encountered upon work or exercise.

Transcriptional and translational regulation of MCTs

Human MCT genomic sequences were mapped to several different chromosomes (for review see Halestrap and Meredith, 2004), but the detailed analysis of their 5'- and 3'-untranslated regions has not been reported, with the exception of MCT1 and MCT4. The MCT1 promoter does not contain a classical TATA-box motif, but it has potential binding sites for a number of transcriptional factors, some of them known to be associated with the regulatory effects of butyric acid (Cuff and Shirazi-Beechy, 2002). This has a special significance in the colon, since butyrate is a product of microbial fermentation, and its transport across the colonocyte plasma membrane is mediated by MCT1. In a colonic epithelial cell line, butyrate increased the expression of MCT1 (and butyrate uptake) via up-regulation of transcription plus the increase of MCT1 mRNA half-life (Cuff et al., 2002). This is somewhat reminiscent of the mutation-induced failure of the pancreatic ß-cell-specific silencing of MCT 1 that we noted earlier.

As noted earlier, MCT4 expression has recently been shown to be up-regulated by hypoxia via the hypoxia inducible factor, HIF-1α (Ullah et al,. 2006). Deletion analysis of the region upstream from the mouse MCT4 transcription site revealed two hypoxia response elements. The increase in MCT4 expression will facilitate the efflux of the increased amounts of lactic acid formed in hypoxic environments. Neither MCT1 nor MCT2 levels have been found to be regulated by hypoxia, and neither of their gene sequences have hypoxia responsive motifs present in the 5' regions.

Alternative splicing of mRNA represents another

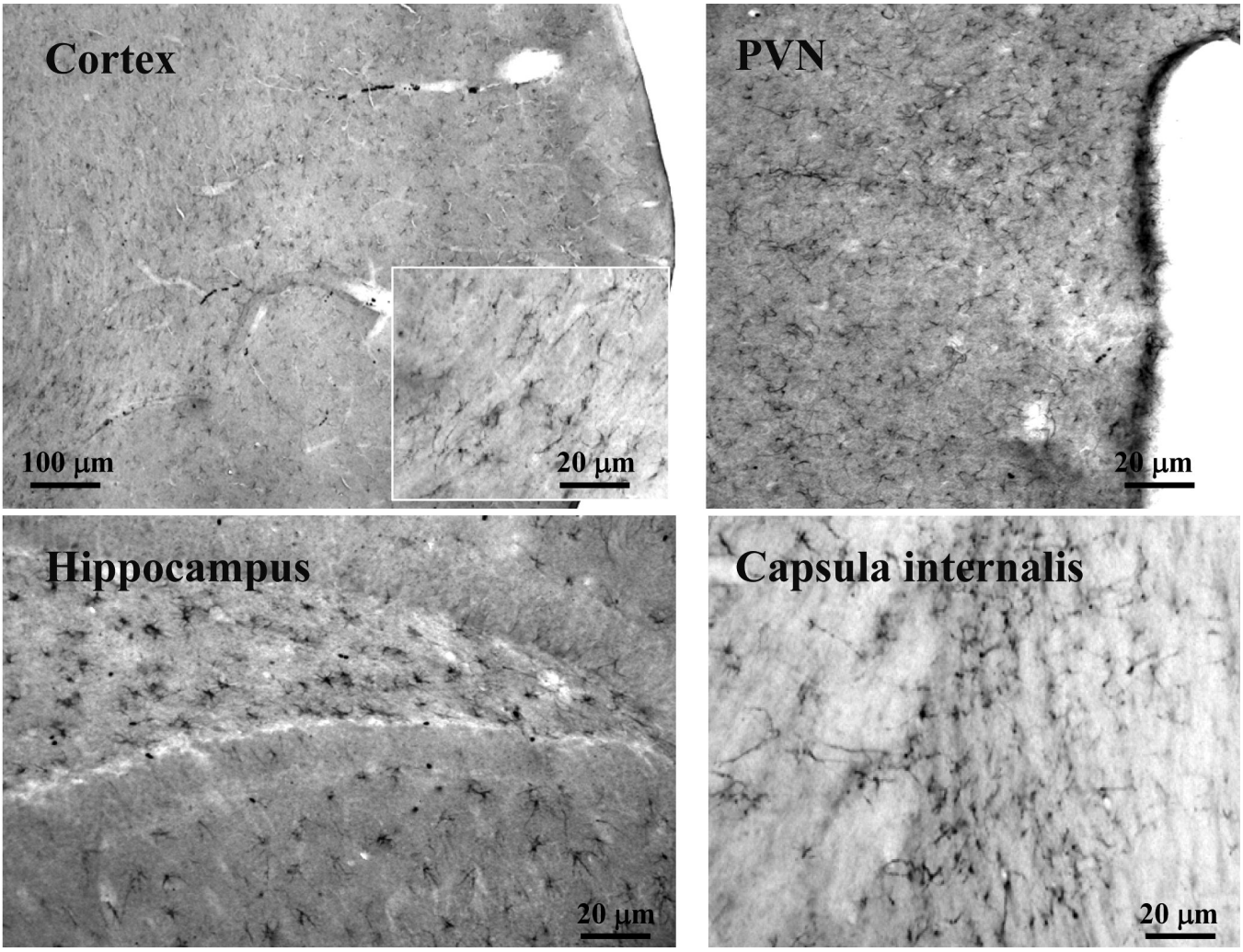


Fig. 10. Expression of MCT4 in astrocytes in various regions of brain, stained by the immuno-peroxidase method. The cortex inset at higher magnification shows the typical stellate forms. PVN: paraventricular nucleus (from Pierre and Pellerin, 2005, with permission).

regulatory mechanism for gene expression. MCT1 mRNA, which has the broadest tissue distribution among all MCTs, displayed no evidence of alternative splicing (Halestrap and Meredith, 2004). In contrast, MCT2 and MCT3 mRNAs display several alternative splicing isoforms. Chicken MCT3 was shown to contain two alternatively spliced non-coding exons, which produce two mRNA transcripts of 2.2 and 2.45 Kb. The shorter transcript is expressed early in embryonic development, while the longer transcript appears later, and increased along with the differentiation of retinal pigment epithelium (Yoon and Philp, 1998). Thus MCT3 expression can be developmentally regulated through the use of different promoters.

Another mode of regulation may entail limited proteolysis. We compared MCT4 identified by the usual C-terminal antibody, with its identification by our pre-C-terminal antibody (Merezhinskaya et al., 2006). Under conditions that excluded the involvement of mRNA splice variants, we found that a truncated version of MCT4, divested of its C-terminal segment, was a major form in many tissues on Western blots. Immunohistochemically, the two antibodies gave identical localizations in most tissues, as demonstrated for skeletal muscle fibers in Figure 12. However, we found two cellular compartments that contained the truncated form, but not the full-length form. One compartment was the capsule of the muscle spindle, as shown in Figure 13, while the other was the lymphocyte cytoplasm, as can be seen in Figure 14. These observations suggest that the truncated MCT4 may be providing a separate specific function, which has yet to be identified.

Properties of other MCTs

Recently, several distant members of the MCT family with less sequence homology to MCT 1-4 were

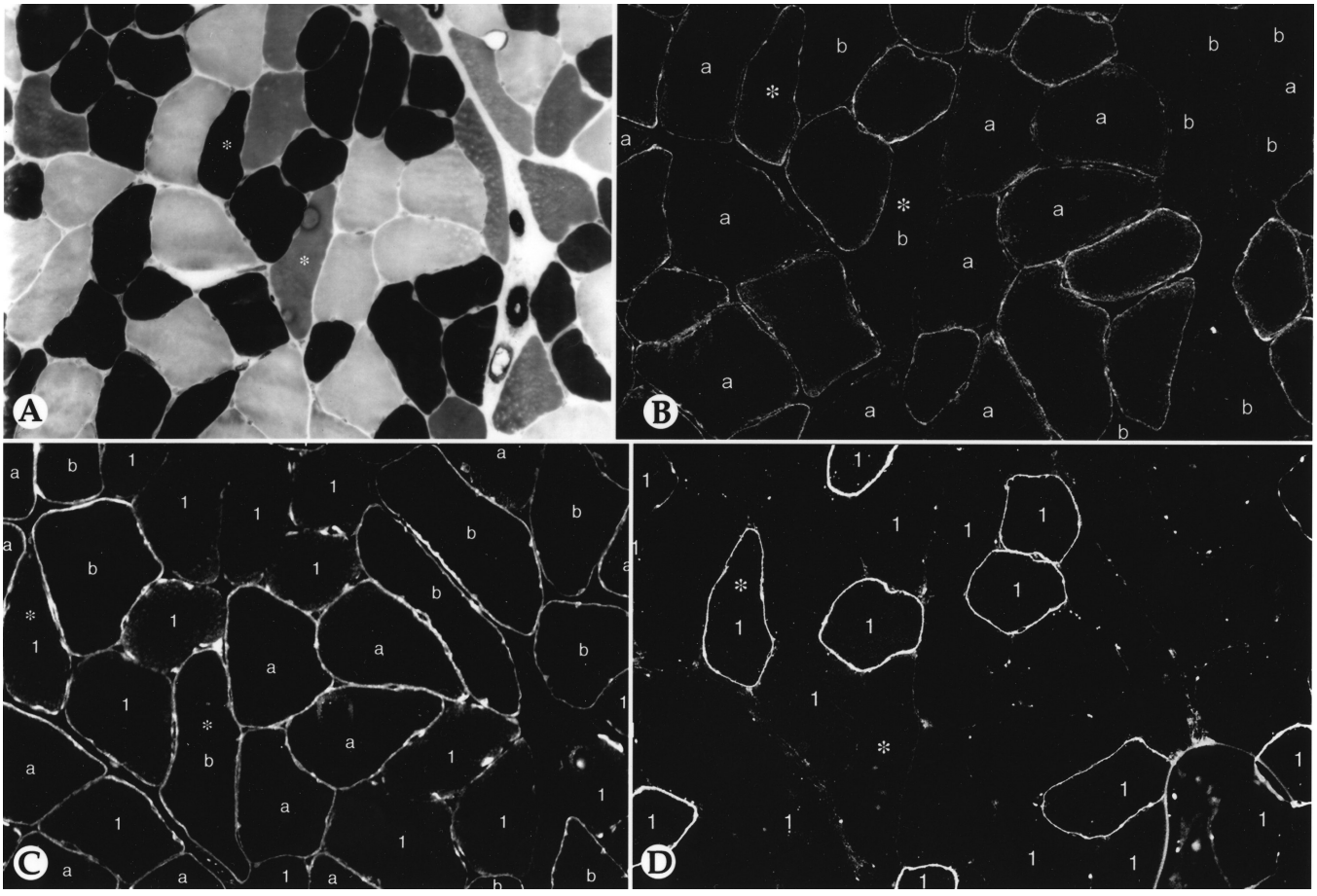


Fig. 11. Immunofluorescent demonstration of the distribution of MCT1 (**B**), MCT4 (**C**), and MCT2 (**D**) in serial sections of unfixed frozen human skeletal muscle, according to fiber types determined in (**A**). The three fiber types were identified using reverse ATPase staining (**A**): Dark –type 1; Light – type 2a (coded here as "a"); Intermediate – type 2b (coded here as "b"). Two fibers useful for orientation are noted with asterisks. In **B**, MCT1 coats the sarcolemma of all type 1 fibers, which are not numbered here, but all 2a and 2b fibers are identified by the small letters. In **C**, all fibers are coded, and MCT4 limns all the type 2 fibers, while type 1 fibers are unstained or have interrupted plaque staining, except where they adjoin capillaries (lower left). In **D**, all type 1 fibers are numbered, and MCT1 is expressed in about half of them, forming a distinct subset, which remains unexplained. No type 2 fibers are stained. A bubble with refractile border distorts the right lower corner (from Fishbein et al. 2002).

functionally characterized. They display different substrate specificities and transport characteristics. TAT1 (or MCT 10) protein cDNA was isolated from rat small intestine by expression cloning in a search for an aromatic amino acid transporter (Kim et al., 2001). The sequence homology search showed 30% identity at the protein level to monocarboxylate transporters 1-4. Expressed in Xenopus oocytes, TAT1 exhibited a low affinity (K_m=5 mM) transport of tryptophan, tyrosine, and phenylalanine and their N-methyl- and N-acetyl-derivatives, but none for lactate or pyruvate. Unlike previously described MCTs, TAT1 transport was not coupled with H^+, but rather supported an electroneutral facilitated diffusion of these amphoteric compounds.

Human MCT8 was cloned during the characterization of a region on the X-chromosome known to contain an inactivation center (Lafreniere et al., 1994). The sequence analysis revealed a presence of Pro (P), Glu (E), Ser (S), and Thr (T) rich domain (PEST) in the N-terminal region of MCT8 which targeted this protein for rapid degradation. Nevertheless, the function of MCT8 remained unknown until very recently, when Visser's group, which was looking for a novel thyroid transporter, noticed that the recently characterized aromatic amino acid transporter TAT1 had a much higher amino acid identity with MCT8 (49%) than with any other MCT (Friesema et al., 2003). Since TAT1 had been reported as being unable to transport either the thyroid prohormone, thyroxine (T4), or the receptor-active form 3,3',5-triiodothyronine (T3), it seemed worth testing whether MCT8 might be the thyroid hormone transporter. Indeed, when a cloned rat MCT8 was expressed in Xenopus oocytes, it caused a 10 fold increase in initial uptake of T3 and T4 (Friesema et al., 2003). The transport was highly specific towards iodothyronines; neither lactate nor Leu, Phe, Tyr, and Trp were transported. Both T3 and T4 transport had K_m values ~ 4 μM, well in the range of physiologic activity.

The importance of MCT8 transport for thyroid hormone action was soon thereafter established, when several mutations in MCT8, including two point mutations in the second (alanine 150 to valine) and ninth (leucine 397 to proline) transmembrane domains, were found to be associated with a reduced transport of thyroid hormone into neurons, resulting in severe X-linked psychomotor retardation (Dimitrescu et al., 2004; Friesema et al, 2004). There followed a number of other reports of this entity, including the recognition that the MCT8 mutations were the genetic cause of one of the earliest described syndromes of X-linked psychomotor retardation (Allan et al., 1944). Most recently, a new MCT8 mutation (proline 537 to leucine) was found to cause the same phenotype, and the authors noted the importance of recognizing neonatal hypotonia as a harbinger to signal early efforts at diagnosis and hormonal therapy, which has so far been unsuccessful (Papadimitriou et al., 2008). A final twist to this story has just been issued by Visser's group, which has now shown by transfection of COS1 cells, that human

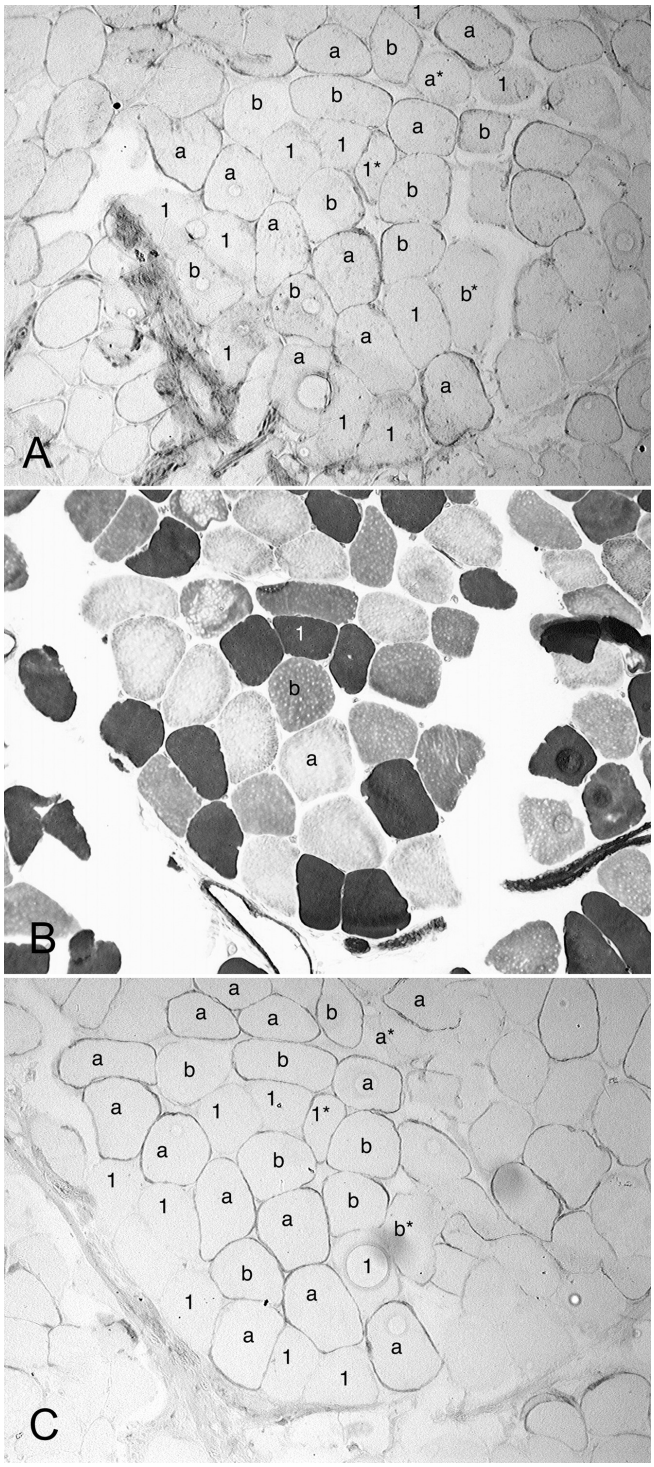


Fig. 12. Immunohistochemical staining of MCT4 in methanol-fixed sections of human skeletal muscle with anti-MCT4-T **(A)** and anti-MCT4-PT **(C)**. Fiber types were identified using reverse ATPase staining of unfixed sections in **B**: Dark – type 1; Light – type 2a (coded here as "a"); Intermediate – type 2b (coded here as "b"). Three fibers with anomalous MCT4 staining are marked with asterisks: staining of a single type 1 fiber, and absence of staining of a single 2a and a single 2b fiber. Like the other fibers, the anomalous three also react the same with both antibodies (from Merezhinskaya et al. 2006).

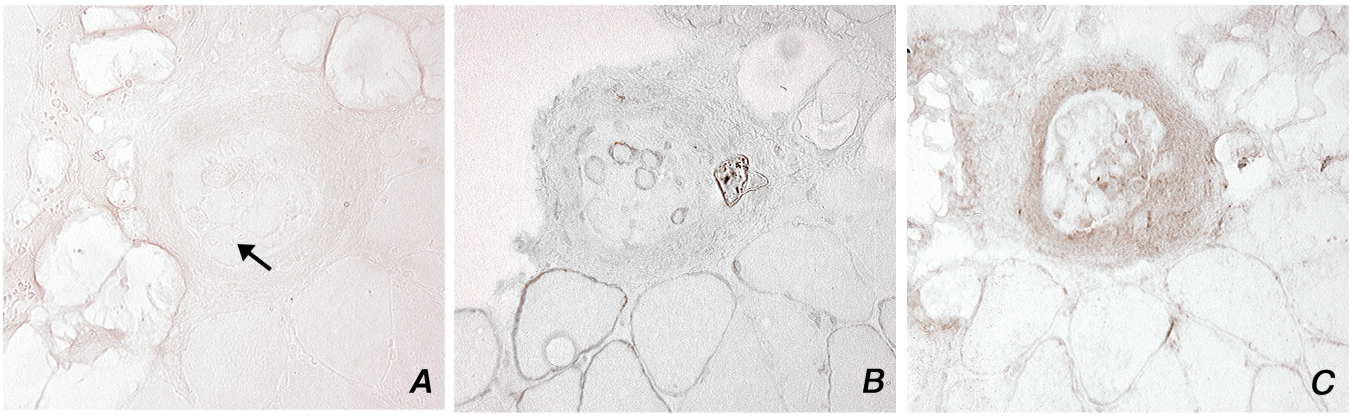


Fig. 13. Immunohistochemical staining of muscle spindles with anti-MCT4-T (**A**) and anti-MCT4-PT (**B**) antibodies. A negative control in the absence of primary antibody is shown in (**A**), with an arrow locating the spindle. Both antibodies stain the small chain fibers, but only anti-MCT4-PT stains the capsule. A crystalline artifact is present in **B** (from Merezhinskaya et al. 2006).

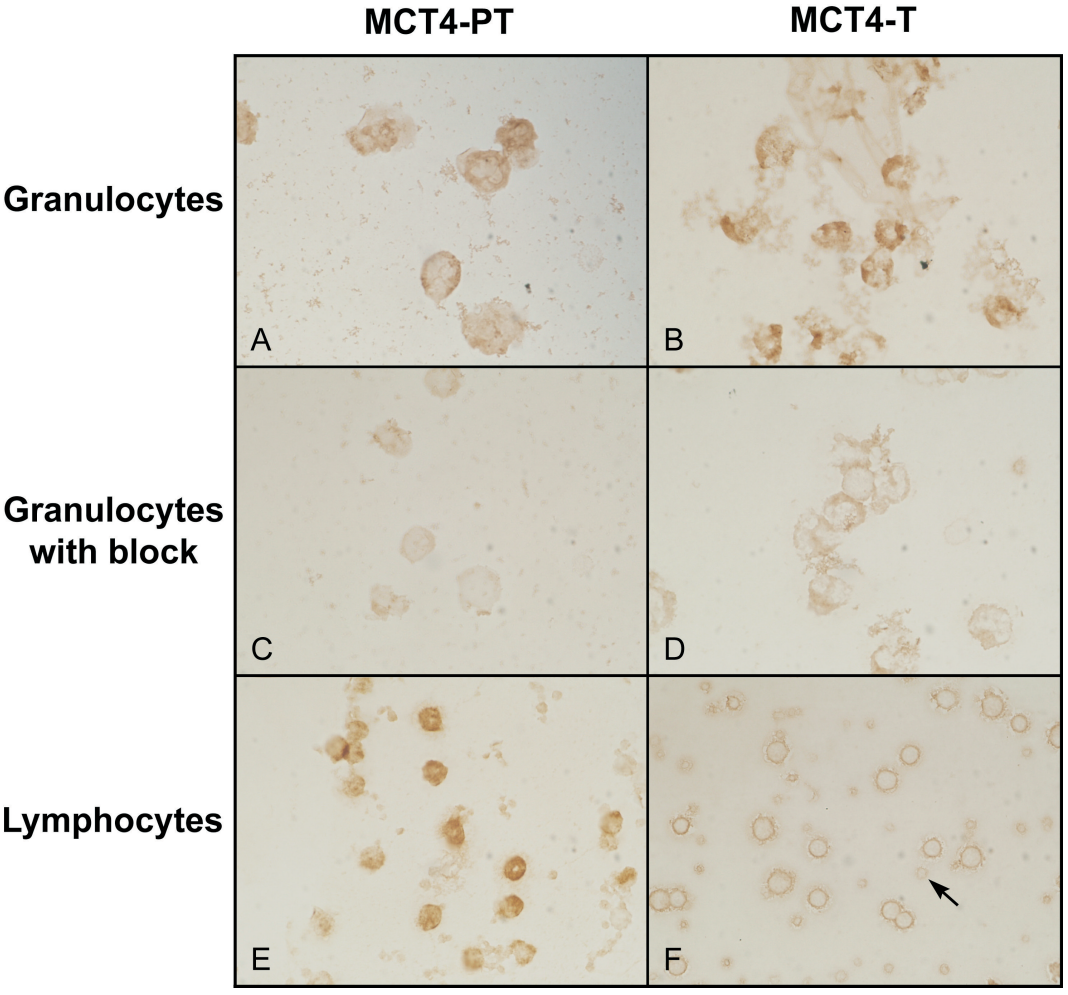


Fig. 14. Immunocytochemical staining of human granulocytes and lymphocytes with anti-MCT4-T (**B,D,F**) and anti-MCT4-PT (**A,C,E**) antibodies. The arrow shows staining of platelet plasmalemma. The blocking effect of pre-treating antibody with the peptide antigen was performed here because granulocyte autolysis was so rapid in the absence of fixation that it could not be demonstrated by Western blotting (modified from Merezhinskaya et al., 2006)

MCT10, aside from its modest ability to transport aromatic amino acids, is as potent a transporter of T4 and T3 as is human MCT8 (Friesema et al., 2008). It is obvious from reported cases that it cannot rescue MCT8 deficiency, but not why, nor what its endocrine functions are. We can expect to see much new information on these items from new studies on available animal models of this disease.

Finally, human MCT6 was expressed in Xenopus oocytes and was shown to transport bumetanide, a sulfonamide diuretic, clearly not the natural substrate, in a pH- and membrane-potential sensitive, but not proton gradient-dependent manner (Murakami et al., 2005). Neither lactic acid nor tryptophane were transported by MCT6. It is, therefore, clear that phylogenetically more distant members of MCT family diverge far enough to acquire substantially different substrate specificity and transport characteristics, extending to the aromatic amino acids.

Subcellular localization of MCTs and some future considerations

In addition to being expressed on the plasma membranes, MCTs have been found on the membranes of intracellular organelles. Early data using high resolution immunogold analysis identified MCT1 on the T-tubule membranes of cardiomyocytes in proximity to mitochondria (Johannsson et al., 1997). In skeletal muscle both MCT1 and MCT4 were found in triads, tubules, sarcoplasmic reticulum and a general pool of intracellular membranes (Bonen et al., 2000).

Using ordinary dichroic light microscopy (Merezhinskaya et al., 2004) we noted nuclear envelope staining by antibodies to MCT1 and MCT2 in each of the three major separated fractions of human leukocytes, as may be seen in Figure 15. In view of the findings of Murray et al. (2005) that blocking MCT1 in T-lymphocytes caused increased intracellular lactic acid, decreased DNA synthesis and failure of rapid phase cell division, it would seem that it is important to keep lactic acid efflux from the nucleus in step with that from the cytoplasm, at least in lymphocytes, and presumably in all immunologic leukocytes. Yet, at present, we are not aware of other studies identifying nuclear envelope MCTs. Isolation and purification of lymphocyte nuclei should permit more decisive studies on whether the MCTs reside within the nuclear envelope, or are instead within apposed endoplasmic (or mitochondrial) reticulum.

Brooks' laboratory decisively demonstrated the presence of MCT1 in cardiac and muscle mitochondria by combining immunohistochemistry with organelle isolation and Western blotting (Brooks et al., 1999a; Butz et al., 2004). The same group subsequently reported evidence that both MCT1 and MCT2 were expressed in the membranes of peroxisomes (McClelland et al., 2003). Recently, Benton et al. (2004) further characterized MCT expression in intermyofibrillar and subsarcolemmal mitochondria. We note that the former (which can interchange only with the cytoplasm of the same fiber) were found to express

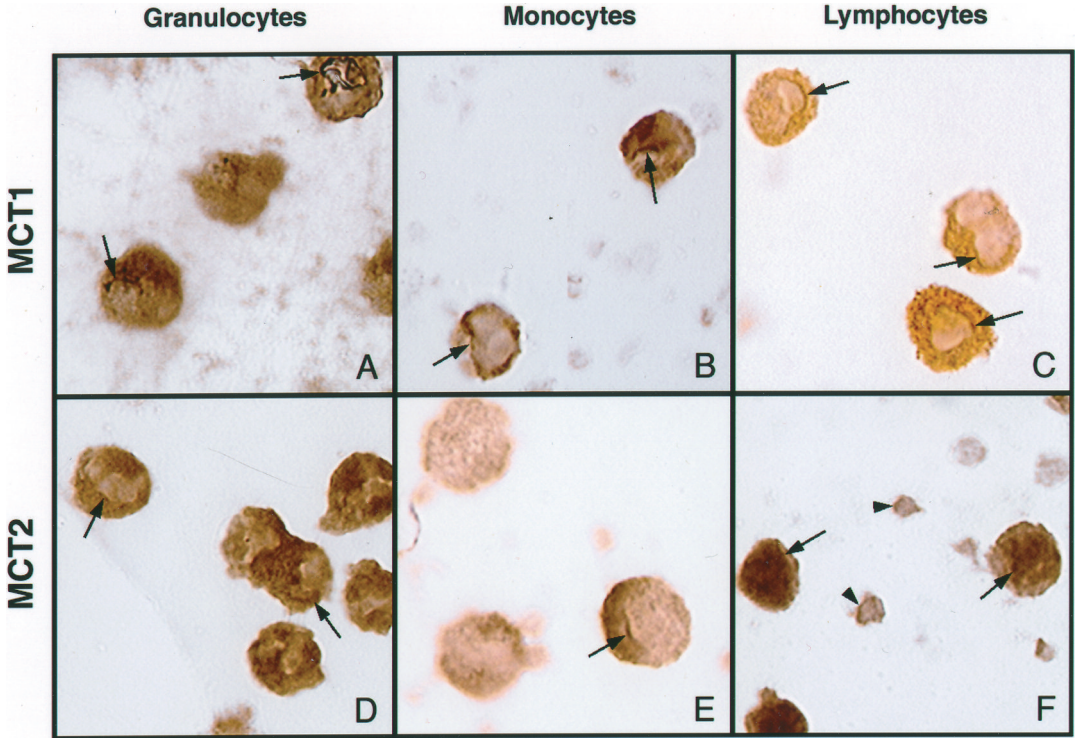


Fig. 15. Immunocytochemical staining of nuclear envelope by MCT1 and MCT2 antibodies in human granulocytes, monocytes, and lymphocytes at high magnification. The arrows point to lines at nuclear folds or perimeter which are darker than the nucleoplasm or cytoplasm suggesting nuclear envelope staining. In F, the arrowheads point to two platelets with plasmalemmal staining (from Merezhinskaya et al. 2004).

only MCT2, whereas the latter (which face neighboring capillaries and muscle fibers, often of a different type) expressed all three transporters. We have explained earlier how the combined expression of MCT1,2,4 would be useful in contracting muscle.

Reports of the presence of MCTs in mitochondria led to the proposal of a 'new intracellular lactate shuttle', summarized by Brooks et al. (1999b). However, mitochondrial shuttles have generally been understood to refer to cyclic reaction sequences which transfer protons from NADH in the cytoplasm to NAD in the matrix for oxidative phosphorylation. The mitochondrion's inner membrane is so specialized that NAD(P)(H+) can not cross it, whereas the outer membrane is quite permeable to most small molecules; so the intermembrane space is more closely equilibrated with the cytoplasm than with the matrix. Over the past few decades physiologic studies have accumulated showing that lactate provides a much larger fraction of the energy source from glycolysis than had been appreciated previously. This would be most conveniently explained by LDH conversion of lactate back to pyruvate when the cytoplasmic levels of pyruvate and/or NADH have fallen low enough, since the modest Gibbs free energy change indicates a readily reversibile reaction. However an alternative view has been offered that the lactate is directly oxidized (Brooks et al., 1999a). This argument would require that LDH be present on the inner face of the inner mitochondrial membrane or in the matrix. This seems a most unfavorable position bioenergetically, as it would short-circuit oxidative phosphorylation by reconverting pyruvate to lactate because of the high NADH/NAD ratio in the matrix (Chance and Williams, 1955; Jobsis and Duffield, 1967; Sahlin and Katz, 1986). If lactate and pyruvate formed a true mitochondrial shuttle (in the original sense), then lactate oxidation should proceed with an ADP/substrate ratio about 20% greater than pyruvate in the absence of other shuttles, which has not been demonstrated (Brooks et al., 1999b). Their observation that lactate provides equivalent state 3 respiratory rates and ADP/O ratios to pyruvate has been contradicted by no less than six other laboratories (Popinigis et al., 1991; Rasmussen et al., 2002; Sahlin et al., 2002; Willis et al., 2003; Ponsot et al., 2005; Yoshida et al., 2007) which found minimal LDH and minimal lactate oxidation in their mitochondrial preparations. However, unlike Brooks, these investigators used Nagarse proteinase (subtilisin), which can certainly degrade mitochondrial proteins (Wilson, 1987) in their preparations. However, strong arguments have been presented against the claim that the protease is removing the LDH (Rasmussen et al., 2002; Sahlin et al., 2002; Yoshida et al., 2007), and the early biochemical consensus, before protease treatment came into wide use, was also negative for LDH and lactate oxidation in mitochondria (Krebs, 1967). At present, we consider the direct-lactate-oxidation proposal (i.e., without reformation of pyruvate) unrealistic, at least in animal tissues.

The most recent report from the Brooks laboratory (Hashimoto et al., 2006) presents a more bioenergetically acceptable proposal. They now locate LDH on the outer face of the inner membrane, and the proton of the NADH formed, upon oxidation of lactate to pyruvate in the intermembrane space, is carried into the matrix by the conventional shuttles. Pyruvate enters via MCT1, which is embedded in the inner membrane along with its chaperone, CD147, and adjacent to cytochrome oxidase, which is proposed to enable the LDH oxidation by coupling it with electron transport reactions. The evidence, obtained in the cultured, immortalized muscle cell line L6 (which is likely not biochemically identical to muscle tissue), is composed of co-precipitation experiments and co-localization of components by confocal microscopy, on isolated mitochondria. The evidence is intriguing, but not decisive, and would benefit from experimental separation of inner and outer membranes and repeat EM, and immuno-/ bio- chemical diagnostics on the pelleted vesicles. Earlier studies on this point (Kline et al., 1986; Brandt et al., 1987) found significant measureable fractions of LDH in the intermembrane (viz., periplasmic) space, but not in the matrix, and also noted the proclivity of the extensive cytosolic LDH to bind to the outer mitochondrial membrane. This naturally arouses concerns that incidental membrane adsorption or porosity during preparation, rather than true integration, may be responsible for intra-mitochondrial LDH. Like adsorption, co-precipitation is often a coincidental process, and does not prove the partners are integral in the natural state.

The concerns noted above extend also to the MCTs. While their presence in mitochondria appears convincing, it is especially difficult to accept that the same chaperone ferrying MCT 1 to the plasmalemma is also embedded in the mitochondrial inner membrane with it. Brooks lab alone has provided evidence for inner membrane localization of MCT1 (Hashimoto et al., 2006). Do we then accept that MCT1 is the long sought after 'mitochondrial pyruvate carrier'? There are a number of discrepancies in the physiology of these two entities that persuade us to decline that decision at this time. While MCT 1-4 transport both pyruvate and lactate, and therefore qualify as exchangers, the mitochondrial pyruvate carrier did not exchange with lactate (Halestrap, 1975), and lactate's entry into mitochondria as measured by proton flux, was not inhibited by the alpha-cyanocinnamates (Halestrap and Denton, 1975). These inhibitors blocked the mitochondrial pyruvate carrier at sub-μM levels (Halestrap, 1975), whereas the human erythrocyte MCT1 plasmalemmal transporter required sub-mM levels (Halestrap, 1976; Fishbein et al., 1988). That contrast has been buttressed by the recent report of new thiazolidine compounds that inhibit mitochondrial pyruvate transport at concentrations >10,000 times lower than are required to inhibit lactate transport by plasmalemmal MCT1 (Hildyard et al, 2005). Partial purifications of the mitochondrial pyruvate carrier yielded MW estimates of 34 kD rather than the ~54 kD

for MCT1-4 (Bolli et al., 1989). Biochemical studies indicate that pyruvate influx may be rate-limiting for its oxidation in heart mitochondria (Shearman and Halestrap, 1984), while in highly active muscle the proton-shuttle systems and/or pyruvate dehydrogenase are likely rate limiting (Spriet et al., 2000). This tempers the usual view that O_2 is the major limiting factor.

Of the 4 MCTs under consideration, only MCT1 bears even a minimal Energy Transfer Signature sequence (Kolarov, 2002) in human, mouse, and hamster, targeting proteins to the mitochondrial inner membrane. However, this sequence is located quite close to the C-terminus, and does not fulfill the more extensive sequences that have been discerned in other inner membrane transporters (Walker, 1992; Palmieri, 2004). None of these differences excludes the possibility of MCT participation, since different host membranes may exert influences on the transport behavior, but it does suggest that a different mitochondrial pyruvate carrier may yet be identified. Indeed, it seemed that this had been accomplished when Hildyard and Halestrap (2003) identified a single candidate protein of 42 kD using cyanocinnamate inhibition of pyruvate uptake in 18 unique knockout mutants in bakers' yeast cultures. However, proof of the projected homologous mammalian counterparts has not yet appeared.

Of course, two different pyruvate transporters might also reside in the same membrane, with different characteristics and function. In this regard we note the report by Valenti et al., (2002), which assumes MCT1 is the 'true' mitochondrial pyruvate carrier and identified a 'new' mitochondrial lactate/pyruvate exchanger in rat heart mitochondria by virtue of its lack of inhibition by 10 μM α-cyano-4-hydroxycinnamate, whereas the same dose completely blocked the entry of pyruvate. This can be interpreted otherwise, however; because, as noted above, MCT1 requires a far higher level of inhibitor to block transport (through plasmalemmal membranes); and MCT1 (or 2 or 4) is certainly equipped to exchange lactate for pyruvate, since both are substrates. We can therefore suspect that the first phenomenon is due to MCT1 (or 2 or 4) carrying out the exchange reaction, aimed perhaps at exporting lactate that has permeated the matrix back to the intermembrane space, or from that space to the cytosol. The second observation then suggests that a 'true' mitochondrial carrier must also exist, more specific for pyruvate and much more sensitive to the inhibitor, but as yet uncharacterized at the molecular level.

Should no mitochondrial pyruvate carrier gene be found, it is worth contemplating alternative splicing or proteolytically truncated forms of MCTs with altered functional characteristics as the source of the mitochondrial carrier. In the case of MCT1, we noted earlier that alternative splicing was absent. On the other hand, the observation that a proteolytic fragment of MCT4 missing the C-terminus, was identified in two cell compartments devoid of the full-length protein (Merezhinskaya et al., 2006) provides impetus for the consideration of a truncated MCT as a potential candidate.

Once we have committed to a highly specific mitochondrial pyruvate carrier, which seems most likely, we need a satisfying extramitochondrial scheme for lactate oxidation, which the physiologists have found to account for a much larger fraction of lactate disposition that had been initially believed. The scheme favored by many (Stainsby and Brooks, 1990; Gladden, 2004; Yoshida et al., 2007) involves slow diffusional gradients in the concentrations of the lactate/pyruvate couple and the NAD/NADH couples creating a quasi-compartmental separation. The glycolytic sequence, presumed to be "remote" from the mitochondrion, generates high levels of pyruvate and NADH (via the glyceraldehydes-3-phosphate dehydrogenase step), thus favoring the LDH conversion of pyruvate to lactate and regeneration of the NAD proton acceptor, while at the mitochondrial transporter and shuttle sites the cytosolic pyruvate and NADH levels are very low, thus favoring their reformation from lactate and NAD. Yoshida et al., (2007) have presented this scheme in detail and noted evidence compatible with it.

It is not a very appealing scenario for this review of one family among 47 families of transporters, totaling more than 300 proteins, all designed to overcome the problems of inefficient diffusion. However, when we consider the evidence of Dzeja et al. (1996, 2002) that creatine kinase and adenylate kinase each transfer high-energy phosphoryl groups from molecule to molecule like a bucket-brigade in order to satisfy the energy requirements of skeletal muscle or cardiac nuclei quickly enough, we are struck by the constraints imposed by slow cytoplasmic diffusion. Like those enzymes, LDH is at what seems like very excessive concentrations in the cytosol, but perhaps not, if its purpose is to be everywhere along the aforementioned gradient to re-equilibrate the substrates it finds at any point.

More appealing would be a true membrane compartment for lactate, namely the intermembrane space, or the intracristal space, which may not be identical (Frey et al., 2002), where pyruvate would be at low levels due to its transported entry to the matrix, while lactate passing through the outer membrane would be blocked from further entry, and the shuttle systems removal of the NADH protons would leave NAD in excess during oxidative phosphorylation. The conditions would be ripe for generation of pyruvate and NADH here if only a tiny fraction of cytoplasmic LDH could reach this compartment in the living cell, and modulate the delivery of substrate to the mitochondrial matrix. The consensus of studies on isolated mitochondria would indicate that this does not happen; but there is a caveat. Advances in electron microscopic technology (Frey et al., 2006) and genetic manipulation (Smirnova et al., 1998; Pitts et al., 1999) have consolidated the conclusion that the mitochondrion in the intact cell is a diffuse reticulum maintained by repeated fissions and fusions, and contains intricate junctional structures at the interface of cristae and inner membrane (Perkins et al., 1997; Renken et al., 2002; Frey et al., 2002) which may

not survive the homogenization and fractionation procedures necessary to isolate the purified spherical mitochondria required to study their many biochemical functions. To a degree, therefore, the mitochondria are the artifacts of their preparative procedure, and may not reveal all the multifarious functions of the intact cellular mitochondrion.

Finally, we return to the main sarcolemmal function of MCT1-4, namely, the prevention of excessive acidosis in heavily glycolysing cells by exporting the resulting lactic acid. Its intra-cellular accumulation is not without some salutary effects, the most well-known being the Bohr effect, describing the increased dissociation of HbO_2 as the blood pH falls below normal. This seems to occur at just the right time to provide extra oxygen to the heart and exercising musculature, which is when the capillary PO_2 has fallen to a critical level (Wasserman, 1999). Less appreciated is the differential binding of lactate to myoglobin at pH=6.5, favoring the deoxygenated form, and thus increasing the dissociation of MbO_2 to give a temporary surge in muscle O_2 availability (Giardina et al., 1996). More recently, studies on isolated contracting soleus muscle (Nielsen et al., 2001) and on single 'skinned' muscle fibers (Pedersen et al., 2004) have found that the accumulation of extracellular K^+ produces a notable loss in contractile force, which is relieved by acidosis (pH ~6.5). This led to a quite provocative 'Perspective' (Allen and Westerblad, 2004) touting lactic acid as a performance enhancing drug. The idea of K^+ as a major fatigue factor is hardly new; the literature from about 1960 on was reviewed by Sjøgaard (Sjøgaard, 1990). The recent studies do provide a new aspect by advancing the argument that (lactic) acid is beneficial. However, they involve extremely high levels of, variously, K^+, $NH4^+$, or acidity, which might be tolerated locally, although lethal systemically. It also seems to us that the conclusion can be reversed: the decline in force due to acidosis would be countered by the increase in extracellular K^+. In addition, Kristensen et al., (2005) have reexamined this question and found that the Nielson and the Pederson results were not replicated in stimulated muscle *in vitro*, or in exercising muscle in vivo. It may be that these two fatigue factors counter each other over some range of work output; but we would strongly discourage administering either potassium salts or lactic acid to athletes, who may be willing to try anything to improve performance.

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